Ocular infection with herpes simplex virus 1 (HSV-1) can result in a chronic immunoinflammatory stromal keratitis (SK) lesion that is a significant cause of human blindness. A key to controlling SK lesion severity is to identify cellular and molecular events responsible for tissue damage and to manipulate them therapeutically. Potential targets for therapy are miRNAs, but these are minimally explored especially in responses to infection. Here, we demonstrated that Mir155 expression was up-regulated after ocular herpes simplex virus 1 infection, with the increased Mir155 expression occurring mainly in macrophages and CD4⁺ T cells and to a lesser extent in neutrophils. In vivo studies indicated that Mir155 knockout mice were more resistant to herpes SK with marked suppression of T helper cells type 1 and 17 responses both in the ocular lesions and the lymphoid organs. The reduced SK lesion severity was reflected by increased phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 and interferon-γ receptor α-chain levels in activated CD4⁺ T cells in the lymph nodes. Finally, in vivo silencing of miR-155 by the provision of antagonimir-155 nanoparticles to herpes simplex virus 1−infected mice led to diminished SK lesions and corneal vascularization. In conclusion, our results indicate that miR-155 contributes to the pathogenesis of SK and represents a promising target to control SK severity. (Am J Pathol 2015, 185: 1073–1084; http://dx.doi.org/10.1016/j.ajp.2014.12.021)
be a principal cause of tissue damage. This is the situation in ocular lesions of the cornea after HSV-1 infection. Here, we have compared the disease outcome after HSV-1 infection in miRNA-155 knockout (Mir155<sup>−/−</sup>) mice and in mice in which miR-155 was suppressed by antagonir therapy with controls. We show that HSV-1 infection resulted in up-regulation of miR-155, which was mainly produced by the inflammatory cells and T cells in the cornea. Suppression of miR-155 production resulted in milder lesions that were associated with diminished responses of T helper cells type 1 (Th1) and type 17 (Th17) and reduced inflammatory cytokine production. We also demonstrated that miR-155 suppression resulted in increased phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (Ship1) and IFN-γ receptor α-chain (IFN-γR2) levels, molecules known to be required for IFN-γ expression and Th1 differentiation. These results indicate that miR-155 regulates differentiation and effector function of Th1 cells. Thus, our results suggest that miR-155 could be a promising therapeutic target to treat SK.

**Materials and Methods**

**Mice**

Female 6- to 8-week-old C57BL/6 mice were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). Mir155<sup>−/−</sup> mice on C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in facilities at the University of Tennessee (Knoxville, TN) approved by the American Association of Laboratory Animal Care. All investigations followed guidelines of the institutional animal care and use committee.

**Virus**

HSV-1 strain RE Tumpey was propagated in Vero cell monolayers (number CCL81; ATCC, Manassas, VA), titrated, and stored in aliquots at −80°C until used. Ultraviolet (UV) inactivation of the wild-type (WT) HSV virus (1.5 × 10<sup>5</sup> plaque-forming units) was performed for 8 minutes.

**Corneal HSV-1 Infection and Scoring**

Corneal infections of mice were performed under deep anesthesia. The mice were lightly scarified on their corneas with a 27-gauge needle, and a 3-μL drop that contained 10<sup>4</sup> plaque-forming units of HSV-1 RE was applied to one eye. Mock-infected mice were used as controls. These mice were monitored for the development of SK lesions. The SK lesion severity and angiogenesis in the eyes of mice were examined by slit-lamp biomicroscopy (Kowa Company, Nagoya, Japan).

The scoring system was as follows: 0, normal cornea; +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity but iris visible; +4, opaque cornea and corneal ulcer; and +5, corneal rupture and necrotizing keratitis. The severity of angiogenesis was recorded as described previously. 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 score of the four quadrants of the eye were then summed to derive the neovessel index (range, 0 to 16) for each eye at a given time point.

**Subconjunctival Injections**

Subconjunctival injections were performed as previously reported. Briefly, these injections were performed with a 2-cm, 32-gauge needle and syringe (Hamilton, Reno, NV) to penetrate the perivascular region of conjunctiva, and 1-μg
of scrambled sequence (seq)/antagomir-155 nanoparticles was administered into the subconjunctival space.

**Antagomir Sequences**

Antagomir-155 and scrambled seqs were procured from Ambion (Austin, TX) and were used as previously reported.22

**Nanoparticle Preparation**

Optimized histidine-lysine polymers were applied for siRNA deliveries in vitro and in vivo.23 One HK polymer species, H3K4b, having a lysine backbone with four branches that contain multiple repeats of histidine and lysine, was used for packaging siRNAs against miR-155 or scrambled seqs, with a nanoparticle-to-sequence ratio of 4:1 by mass. The nanoparticles (average size, 150 nm in diameter) were self-assembled, and these histidine-lysine polymer siRNA nanoparticles were used in mice.

**Murine Treatment with Antagomir-155 Nanoparticles**

Mice ocularly infected with HSV-1 RE Tumpey were separated into two groups. Antagomir-155 nanoparticle treatment was begun at day 1, with additional doses on alternate days until day 13 after infection. In another group of experiments, the antagomir-155 treatment was started at day 5, with additional doses every day until day 13 after infection. The control group received nanoparticles that contained scrambled seqs subconjunctively with the same regimen for respective experiments. These mice were carefully followed for the progression of angiogenesis and SK development.

**Administration of IVIGs**

Intravenous immunoglobulins (IVIGs; Gammagard Liquid) was obtained from Baxter (Deerfield, IL). WT and Mir155−/− mice were i.p. injected with IVIG (3.75 mg per mouse) at day 4 after infection. The dose of IVIG was chosen to be 3.75 mg per mouse, based on previous studies.24

**Flow Cytometry**

Single-cell suspensions from cornea, cervical draining lymph nodes (DLNs), and spleen of mice ocularly infected with HSV-1 were collected at day 15 after infection. Aliquots of the above single-cell suspensions were stained for CD4, CD45, CD11b, and Ly6G cell surface markers. In apoptosis studies, single-cell suspensions of DLNs

![Figure 2](image.png)

**Figure 2**  
Mir155−/− mice are resistant to SK. C57BL/6 and Mir155−/− mice ocularly infected with 1× 10⁶ PFU of HSV-1 RE were divided into groups. One group of WT mice received IVIG i.p. at day 4 after infection (WT D4 IVIG), and one group of WT mice received no treatment (WT no IVIG). Disease severity and immune parameters were evaluated at day 15 after infection. A: SK lesion severity and angiogenesis at day 15 after infection are shown. The experiment was repeated three times. B: Representative H&E-stained corneal sections from WT (WT D4 IVIG) and Mir155−/− (Mir155−/− D4 IVIG) mice collected on day 15 after infection. C and D: Mice were sacrificed on day 15 after infection, and corneas were harvested and pooled groupwise for the analysis of various cell types. C: Representative FACS plots for corneal infiltrating total CD4⁺ T cells. Intracellular staining was conducted to quantify Th1 and Th17 cells by stimulating them with PMA/ionomycin. D: Representative plots (left) show percentage of CD4⁺ cells producing IFN-γ or IL-17A after stimulation with PMA/ionomycin in the cornea of infected WT (WT D4 IVIG; black bars) and Mir155−/− (Mir155−/− D4 IVIG; white bars) mice. Plots shown were gated on CD4⁺ T cells. The bar graph (right) represents total numbers of corneal infiltrating CD4⁺ T cells, Th1, and Th17 cells in the corneas of WT (WT D4 IVIG) and Mir155−/− (Mir155−/− D4 IVIG) mice. Data are expressed as means ± SEM. n = 8 to 12 mice per group in three independent experiments (C and D). Statistical significance was analyzed by one-way analysis of variance, with Tukey’s multiple comparison tests (A) and unpaired Student’s t-test (D). **P ≤ 0.01, and ***P ≤ 0.001. D4, day 4; FACS, fluorescence-activated cell sorter; H&E, hematoxylin and eosin; HSV-1, herpes simplex virus 1; IFN-γ, interferon-γ; IVIG, intravenous immunoglobulin; PFU, plaque-forming unit; PMA, phorbol 12-myristate 13-acetate; SK, stromal keratitis; SSC, side scatter; Th1, T helper cell type 1; Th17, T helper cell type 17; WT, wild-type.
were stained for Annexin V by using a kit from BD Biosciences (San Jose, CA). In addition, cells were also costained for CD4, CD44, and viability (Live/Dead fixable dead cell staining kit; Invitrogen, San Diego, CA). To enumerate the functionality of CD4+ T cells, intracellular staining was performed. Briefly, 10^6 cells freshly isolated from DLNs and spleens or the entire corneal samples were left untreated or stimulated with phorbol 12-myristate 13-acetate plus ionomycin along with Golgi plug and incubated for 4 hours at 37°C in 5% CO2. After this period, cell-surface staining was performed, followed by intracellular cytokine staining with a Cytofix/Cytoperm kit (BD Pharmingen, San Diego, CA) to enumerate the number of IFN-γ and IL-17A-producing CD4+ T cells as previously described. Finally, the cells were washed twice and resuspended in 1% paraformaldehyde.

For intracellular staining of Ki-67 (BD Biosciences), cells were fixed and permeabilized with the FoxP3 Staining Buffer Set (eBioscience, San Diego, CA) according to the manufacturer’s recommendations. All stained samples were acquired with a FACS LSR (BD Biosciences), and the data were analyzed with the FlowJo software version 8.8.7 (TreeStar Inc., Ashland, OR). For DLN and spleen samples approximately 200,000 events were recorded. For corneal samples, depending on the number of corneas pooled, approximately 300,000 to 1.5 x 10^6 events were recorded.

**Purification of Macrophages, CD4+ T Cells, and Neutrophils**

Briefly, the excised corneas were pooled and digested with 60 U/mL Liberase (Roche Diagnostics, Indianapolis, IN) for 35 minutes at 37°C in a humidified atmosphere of 5% CO2. A single-cell suspension was prepared and stained with different combinations of the following antibodies: anti-CD4 allophycocyanin, anti-CD45 peridinin-chlorophyll protein complex, anti-CD11b phycoerythrin, anti-Ly6G fluorescein isothiocyanate, and anti-CD62L fluorescein isothiocyanate for 30 minutes on ice. Approximately 50,000 to 120,000 CD4+ T cells, macrophages (CD45+/CD11b+, F4/80+), and neutrophils (CD45+ CD11b+, Ly6G+) were sorted using a FACSaria cell sorter (BD Biosciences). The expression of miR-155 was measured on these sorted cells. In separate experiments, lymph nodes from HSV-1-infected WT and Mir155−/− mice were collected at day 15 after infection. A single-cell suspension was prepared and stained with different combinations of the following antibodies: anti-CD4 allophycocyanin, anti-CD44 fluorescein isothiocyanate, and anti-CD62L phosphatidylethanolamine, anti-Ly6G fluorescein isothiocyanate, or F4/80 fluorescein isothiocyanate for 30 minutes on ice. Approximately 50,000 to 120,000 CD4+ T cells, macrophages (CD45+, CD11b+, F4/80+), and neutrophils (CD45+, CD11b+, Ly6G+) were sorted using a FACSAria cell sorter (BD Biosciences). The expression of miR-155 was measured on these sorted cells. Purity to an extent of 80% to 90% was achieved. The expression of miR-155, Ship1, and IFN-γR2 were measured on these sorted cells.
Quantification of mRNA and miRNA Expression Levels by Quantitative Real-Time RT-PCR

Total mRNA and miRNA was isolated from corneal and lymph node cells by using mirVana miRNA Isolation Kit (Ambion). For RNA, cDNA was made with 500 ng of RNA by using oligo (dT) primer and ImProm-II Reverse Transcription System (Promega, Madison, WI). For miRNA, cDNA was made with 5 ng of miRNA by using the TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA) and primers for miR-155 and small nucleolar RNA 202. TaqMan miRNA Assays (miR-155 and small nucleolar RNA 202) and TaqMan gene expression assays [IL-6, IL-1β, IL-17A, IFN-γ, chemokine (C-C motif) ligand 2 (CCL-2), chemokine (C-C motif) ligand 1 (CXCL-1), Ship1, and IFN-γRα] were purchased from Applied Biosystems and were used to quantify miRNAs and mRNAs by using a 7500 Fast Real-Time PCR System (Applied Biosystems). The expression levels of the target genes were normalized to β-actin for mRNA and small nucleolar RNA 202 for miRNA with the ΔΔCT method, and relative quantification between control and infected mice was performed with the formula $2^{-\Delta\Delta CT} \times 1000$.

Statistical Analysis

Statistical significance for SK lesion severity and angiogenesis between the two groups was determined with unpaired two-tailed Student’s t-test. One-way analysis of variance with Tukey’s multiple comparison tests was used to calculate the level of significance for some experiments, where noted in the results. $P \leq 0.05$ was considered as significant, and results are expressed as means ± SEM. For all statistical analysis, Prism software version 6.0e (GraphPad Inc., San Diego, CA) was used.

Results

miR-155 Is Up-Regulated in Murine Corneas after Ocular Infection with HSV

To investigate the potential role of miR-155 in mediating tissue damage during SK, mice were ocularly infected with HSV-1, and miRNA levels were quantified by RT-qPCR in pools of corneal extracts at various times after infection. The results showed that miR-155 expression was up-regulated by day 2 after infection (approximately 10-fold compared with uninfected corneas) and increased with progression of diseases at day 7 and day 14 after infection (approximately 20-fold compared with uninfected corneas) (Figure 1A). To demonstrate if replicating virus was required to up-regulate miR-155 in eyes, we infected mice with live WT/UV-inactivated HSV, and miR-155 expression was analyzed. Our results show that UV-inactivated virus up-regulated the miR-155 equivalent to live WT virus, indicating that replicating virus is not required for miR-155 induction in eyes (Supplemental Figure S1). To further determine the cellular source of SK-induced miR-155, neutrophils, macrophages,
and CD4+ T cells were purified from WT HSV-1-infected corneas at day 15 after infection, and miRNA extracts were tested by RT-qPCR. CD4+ T cells taken from the lymph node of Mir155−/− mice served as a negative control for these experiments. Data showed miR-155 expression in neutrophils, macrophages, and CD4+ T cells with the highest expression being observed in CD4+ T cells and macrophages (Figure 1B). Collectively, these data indicated that miR-155 expression was elevated at multiple time points after ocular HSV-1 infection and that macrophages and CD4+ T cells were the main source of the miR-155 during the clinical phase of the lesions.

Mir155−/− Mice Show Defective CD4+ T-Cell Responses and Reduced SK Lesion Severity

To further study the role of miR-155 in SK pathogenesis, the outcome of HSV-1 infection was compared in WT mice and mice lacking miR-155 because of gene KO. Because HSV-1–infected Mir155−/− mice usually develop lethal herpetic encephalitis,26 infected mice were given a source of anti-HSV antibody (human IVIG) 4 days after infection, which protects them against encephalitis, but does not interfere in the WT mice with the extent of SK lesion severity. Mice in all groups were infected, but levels of SK disease severity and the extent of angiogenesis were significantly diminished in the Mir155−/− mice (Figure 2A). The administration of IVIG had no effect on the severity of lesions in the WT mice (Figure 2A). Further documentation of reduced severity in Mir155−/− mice could be shown by histologic evaluation (Figure 2B) and by quantifying the extent of the inflammatory cellular events in subpools of corneas taken from WT and Mir155−/− mice at day 15 after infection. The frequencies and numbers of total CD4+ T cells, Th1 cells, and Th17 cells in the pooled corneal samples were decreased fivefold to sixfold in the Mir155−/− mice compared

Figure 5 Preventive administration of antagomir-155 diminishes SK lesion severity and cellular infiltration. C57BL/6 mice were ocularly infected with 1×10⁶ PFU of HSV-1 RE. A: Antagomir-155/scrambled seq (5′-AUUCAUGACGUACUGACCU-3′) nanoparticle treatment was given subconjunctively. Disease severity and immune parameters were analyzed at day 14 p.i. B: SK lesion severity and angiogenesis at day 14. C: Representative H&E-stained corneal sections of scrambled seq–treated and antagomir-155–treated mice were collected on day 14 p.i. Mice were sacrificed on day 14 p.i., and corneas were harvested and pooled groupwise for analysis of various cell types. D: The frequency and total cell number of CD4+ T cells and neutrophils (CD45+ CD11b+ Ly6G+) (plot was gated on CD45+ cells) infiltrated in the corneas of control (scrambled seq) and antagomir-155–treated mice. Data are expressed as means ± SEM. n = 8 to 12 mice per group of two independent experiments. Statistical significance was analyzed by the Student’s t-test (unpaired). ***P ≤ 0.001. H&E, hematoxylin and eosin; HSV-1, herpes simplex virus 1; PFU, plaque-forming unit; p.i., after infection; seq, sequence; SK, stromal keratitis; SSC, side scatter.
miR-155 Promotes CD4\(^+\) T-Cell Proliferation after HSV-1 Infection

Prior studies have shown that miR-155 targets several pathways involved in survival and proliferation of effector T cells.\(^{16,27}\) We determined whether proliferation or apoptosis could account for the reduced number of effector T cells in miR-155−/− mice. WT and \(\text{Mir155}^{+/+}\) mice were ocularly infected with HSV-1 and were treated with IVIG at day 4 after infection as described in Materials and Methods. Mice were sacrificed on day 14 after infection, and proliferation and apoptosis were detected by Ki-67 and Annexin V staining, respectively. Our results reveal that \(\text{Mir155}^{+/+}\) CD4\(^+\) T cells show significantly reduced proliferative response both in the DLNs and cornea (Figure 4, A and B). However, no differences were found in Annexin V\(^+\) CD4\(^+\) CD44\(^hi\) cells in the DLNs of WT and \(\text{Mir155}^{-/-}\) mice (Figure 4C). Thus, our data suggest that reduced numbers of Th1 and Th17 responses seen in \(\text{Mir155}^{-/-}\) mice could be due to defective proliferation of CD4\(^+\) T cells both in the DLNs and the cornea.

Provision of Antagomir-155 Nanoparticles Diminishes SK Lesion Severity

To evaluate the effect of locally inhibiting miR-155 expression on the extent of SK, mice were subconjunctively given antagomir-155, or control scrambled seq nanoparticles, with treatment starting on either day 1 (preclinical phase) or day 5 (early clinical phase) after infection (Figure 5A and Supplemental Figure S2A, respectively). Treatment begun on day 1 resulted in significantly reduced SK scores and angiogenesis in the antagonim-155−treated mice (Figure 5B). Furthermore, histopathologic analysis on day 14 after infection showed that corneas from control scramble seq−treated mice were inflamed and contained a massive infiltrate of inflammatory cells compared with antagonim-155−treated mice (Figure 5C). An examination of subpools of collagen-digested corneas at the termination of experiments on day 14 revealed that the number and frequencies of inflammatory cells (CD4\(^+\) T cells and neutrophils) were significantly diminished in the antagonim-155−treated group compared with the control scramble seq−treated group (Figure 5D).

In the experiments in which treatment was begun in the early clinical phase on day 5 after infection when replicating virus is mostly cleared from the HSV-1−infected corneas, SK and angiogenesis scores were also significantly diminished in the antagonim-155−treated mice (Supplemental Figure S2B). In addition, histopathologic analysis showed decreased cellular infiltration and inflammation in the cornea in the antagonim-155−treated mice.
Antagomir-155 Nanoparticle Treatment Reduces Proinflammatory Cytokine and Chemokine Levels in the Corneas of HSV-1–Infected Mice

To assess the effect of antagomir-155 treatment on proinflammatory cytokine and chemokine production in SK, mRNA was prepared from corneal extracts on day 14 after infection following treatment that was started on day 1 after infection. Samples from mice treated with antagomir-155 showed a sevenfold to eightfold reduction of proinflammatory cytokines IL-1β and IL-6 and a threefold to fourfold reduction of IFN-γ and IL-17 compared with scrambled seq control-treated mice (Figure 6A). In addition, levels of chemokines involved in neutrophil CXCL-1 and monocyte CCL-2 migration were significantly decreased in antagomir-155–treated mice (Figure 6B). These data indicate that the provision of antagomir-155 to HSV-1–infected mice resulted in marked reduction of proinflammatory cytokines and chemokines.

miR-155 Targets Ship1 and IFN-γRα, Which Are Down-Regulated in Activated CD4+ T Cells

Observations in nonviral systems had indicated that miR-155 might influence several immune events.7–10 Because CD4+ T cells of mainly the Th1 phenotype primarily orchestrate SK, we chose to evaluate the Ship1 and IFN-γRα response because these are involved in regulating IFN-γ expression and Th1 differentiation, respectively.2,17,18 Experiments were performed to measure the effects of miR-155 on levels of Ship1 and IFN-γRα in naive and activated CD4+ T cells. Accordingly, WT and Mir155+/− mice were ocularly infected with HSV-1. At day 15 after infection, naive CD4+ T cells (CD4+ CD62Lhi CD44lo) and activated CD4+ T cells (CD4+ CD62Llo CD44hi) were quantified from the lymph nodes and levels of miR-155, Ship1, and IFN-γRα were quantified. As expected, there was a minimal level of miR-155 in naive CD4+ T cells, but activated CD4+ T cells showed an approximate 20-fold up-regulation (Figure 7A). The mRNA levels of Ship1 and IFN-γRα were elevated in the activated CD4+ T cells of Mir155+/− mice compared with the WT mice, but no differences were observed in naive CD4+ T cells among WT and Mir155+/− mice (Figure 7, B and C). Collectively, these results indicated that Mir155 was up-regulated in activated CD4+ T cells, which correlated with low Ship1 and IFN-γRα mRNA levels in those T cells.

Discussion

In this report, we describe the influence of miR-155 on the expression of ocular lesions caused by infection with HSV-1. The expression of miR-155 was shown to
Outcome (SK) in HSV-1 increased proinflammatory reactions, along with their heightened effector function. This IFN-γ induced Th1 and Th17 cells, the major orchestrators of SK. miR-155 degrades Rginterferon-γ receptor, Ship1, and conceivably other targets that cause increased numbers of these cells, along with their heightened effector function. This increased proinflammatory response leads to severe SK. Right panel: Outcome (SK) in HSV-1–infected Mir155−/− mice. HSV-1 infection of Mir155−/− mice leads to higher levels of IFN-γRx and Ship1, resulting in reduced numbers and functionality of Th1 and Th17 cells, which results in attenuation of SK lesions. HSV-1, herpes simplex virus 1; IFN-γRx, interferon-γ receptor α; Ship1, phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1; SK, stromal keratitis; Th1, T helper cell type 1; Th17, T helper cell type 17; WT, wild-type.

Figure 8 Illustration of miR-155–induced regulation of inflammatory response during HSV-1 infection. Left panel: Outcome (SK) in HSV-1–infected WT (Mir155+/+) mice. HSV-1 infection leads to ocular infiltration of Th1 and Th17 cells, the major orchestrators of SK. miR-155 degrades IFN-γRx, Ship1, and conceivably other targets that cause increased numbers of these cells, along with their heightened effector function. This increased proinflammatory response leads to severe SK. Right panel: Outcome (SK) in HSV-1–infected Mir155−/− mice. HSV-1 infection of Mir155−/− mice leads to higher levels of IFN-γRx and Ship1, resulting in reduced numbers and functionality of Th1 and Th17 cells, which results in attenuation of SK lesions. HSV-1, herpes simplex virus 1; IFN-γRx, interferon-γ receptor α; Ship1, phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1; SK, stromal keratitis; Th1, T helper cell type 1; Th17, T helper cell type 17; WT, wild-type.

Our study focused on miR-155 because this molecule was known to be involved in immune and inflammatory reactions, yet its role during viral infections had received minimal investigation. Two reports showed that miR-155 influenced the magnitude of CD8+ T-cell responses to a virus infection and another showed that diminished miR-155 responses led to more severe virus-induced lesions in nervous tissue with mice more susceptible to encephalitis. This latter outcome was at least in part the consequence of diminished CD8+ T-cell responses known to participate in protection of the central nervous system against HSV infection. The present report evaluates the effect of miR-155 expression on the inflammatory consequences of a virus infection, which in the model investigated occurs because of an inflammatory process orchestrated by CD4+ T cells. Our results clearly showed that, when miR-155 expression was suppressed, either because of gene KO or the administration of miR-155–specific antagonirs, lesions of SK were markedly reduced. In fact, our results reflect observations made in some mouse models of autoimmune inflammation in which lesions were less evident in situations when miR-155 expression was limited. In addition, some human autoinflammatory lesions appear to be influenced by the magnitude of the miR-155 response. In the viral immunoinflammatory model, the main effect of miR-155 suppression was a significantly reduced CD4+ Th1 T-cell reaction along with diminished non–antigen-specific inflammatory cell infiltration into the cornea, with the latter cells mainly responsible for tissue damage. These effects were apparently the consequence of miR-155 affecting the levels of Ship1 and IFN-γRx in activated CD4+ T cells, both of which were significantly elevated when miR-155 expression was inhibited. Ship1 is a phosphatase that negatively regulates cytokine signaling via repression of the PI3K pathway and its deletion from CD4+ T cells results in elevated IFN-γ expression. Thus, in our system because activated CD4+ T cells in Mir155−/− mice showed higher levels of Ship1, it is likely that these cells would have lower IFN-γ expression as shown in other models. In addition, lower IFN-γ expression could also be explained by defective differentiation of Th1 cells in Mir155−/− mice. Regulation of IFN-γ signaling through the IFN-γR is necessary for the differentiation of Th1 cells. miR-155, by targeting IFN-γRx mRNA, might down-regulate the IFNγ receptor in activated CD4+ T cells that differentiate into Th1 cells. We also observed much higher levels of IFN-γRx in the activated CD4+ T cells, suggesting defective Th1 differentiation in the Mir155−/− mice. The observed phenotype of reduction in Th1 and Th17 cell numbers could also be explained by impaired proliferation and/or survival of responding T cells. Our data support reduced
proliferation of \( \text{Mir}155^{-/-} \) CD4\(^{+}\) T cells rather than reduced survival on the basis of the use of markers to indicate such effects.

As shown in some other systems, miR-155 could also degrade additional crucial targets such as suppressor of cytokine signaling 1 (SOCS1) that negatively regulates T-cell function.\(^{35}\) T-cell–specific deletion of SOCS1 results in multiorgan inflammatory lesions, which are correlated with high levels of IFN-γ.\(^{35}\) We measured and compared the expression of SOCS1 in activated CD4\(^{+}\) T cells from WT and \( \text{Mir}155^{-/-} \) mice but observed no significant differences between the two (data not shown), suggesting that Ship1 and IFN-γRα were the main targets of miR-155 in our study. miR-155 was also shown in \( \text{in vitro} \) cultures of CD4\(^{+}\) T cells to influence IL-10 production with a higher fraction of \( \text{Mir}155^{-/-} \) CD4\(^{+}\) T cells producing IL-10.\(^{36}\) However, in the SK situation we failed to observe enhanced IL-10 production in the corneas of either \( \text{Mir}155^{-/-} \) or antagonim-155–treated mice (data not shown). Nevertheless, we observed significantly reduced numbers of Th17 cells in HSV-infected \( \text{Mir}155^{-/-} \) mice. This is consistent with the previous reports that show the role of miR-155 in Th17 cell differentiation.\(^{37,38}\) Targets other than Ship1 and IFN-γRα could be subject to modulation by miR-155, and this issue is under further investigation.

It was interesting to observe that, soon after HSV ocular infection, levels of miR-155 were increased in corneal tissue. The stimulus for this early expression would likely not be the direct result of viral infection of epithelial cells but would more likely represent the innate immune-stimulating activity of the virus. This was supported by our data showing that UV-inactivated virus could trigger miR-155 up-regulation at day 2 after infection. Thus, HSV-1 is known to express at least three Toll-like receptor ligand activities,\(^{39–41}\) and others have documented that Toll-like receptor stimulation can be a potent agonist for miR-155 production.\(^{10}\) Some herpes viruses do themselves express several miRNAs, but homologs of miR-155 are not one of them. In the case of HSV-1, the miRNAs expressed by the virus are mainly involved in decisions between productive and latent cycles of replication.\(^{42}\) In the clinical phase of SK, virus is no longer present in the cornea, and at this time we could show that the main producers of miR-155 were proinflammatory T cells along with nonspecific inflammatory cells, especially macrophages. Macrophages form a minor cell population that infiltrate corneas during SK. Some reports suggest that macrophages have antiviral activity, which helps in clearing the virus from the cornea,\(^{43}\) whereas other studies show that macrophages are involved in the development of immunologically driven SK.\(^{5}\)

The stimulus for miR-155 production was not identified, but it was most likely to include either Toll-like receptor ligand activity of the virus or cytokines such as IFN-β, IFN-γ, and tumor necrosis factor α that are present during SK and which are shown in other systems to act as miR-155 agonists.\(^{10}\) In addition, miR-155 also signals innate cells, such as neutrophils and macrophages, to produce proinflammatory cytokines and chemokines.\(^{9,10,44}\) In cystic fibrosis patients, for example, increased miR-155 expression in neutrophils led to increased neutrophil recruitment via IL-8 activity.\(^{9}\) It was also reported that miR-155–mediated degradation of B-cell lymphoma 6 protein in macrophages leads to attenuation of NF-kB signaling, resulting in diminished production of proinflammatory cytokines and chemokines.\(^{44}\) Conversely, miR-155 induced repression of SOCS1 in macrophages enhanced type I IFN signaling.\(^{45}\) In a collagen-induced arthritis model, \( \text{Mir}155^{-/-} \) animals exhibited low levels of inflammatory mediators in joints.\(^{11}\) \( \text{Mir}155^{-/-} \) null mice were also seen to be resistant to bacterial keratitis, an effect mediated by miR-155 activity on macrophages.\(^{36}\) In agreement with these observations, we could show in our study that diminished miR-155 activity led to a dampened proinflammatory milieu in the eyes. Therefore, the SK resistance phenotype observed in \( \text{Mir}155^{-/-} \) mice is likely the consequence of multiple events such as poor neutrophil and macrophage activity, dampened proinflammatory milieu, and attenuated Th1 and Th17 cell responses. This activity of miR-155 could make it a viable therapeutic target to treat HSV-induced ocular lesions.

One major rationale for research on miRNAs is the prospect that manipulating their levels could represent a viable new approach to therapy. In fact, accumulating evidence indicates that the miRNA environment changes in the course of an inflammatory response with the miRNA species present in the proinflammatory phase differing from those prominent during the resolution phase of an inflammatory response.\(^{47,48}\) It is still not clear if miRNAs are primarily responsible for this switch of events, but evidence is accumulating to support the concept.\(^{48}\) In such a scenario, miR-155 may act as a facilitator of the resolution phase of an inflammatory reaction. Consistently, we showed that the use of an antagonim to miR-155 at the early clinical phase diminished the severity of lesions. It seems likely that in a disease situation manipulating the expression of multiple miRNA species that are involved in different aspect of pathogenesis might be the most fruitful approach to therapy. Consistently, in our preliminary results we have observed that manipulating miR-155 along with miR-132, a miRNA involved in angiogenesis,\(^{22}\) may provide more therapeutic value than using either miRNA alone. Current ongoing research is evaluating the value of manipulating the levels of multiple miRNA in an attempt to achieve a more effective resolution of ocular inflammatory reactions.

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Supplemental Data

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