Immunopathology and Infectious Diseases

Role of miR-132 in Angiogenesis after Ocular Infection with Herpes Simplex Virus

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MicroRNAs (miRNAs) are small regulatory molecules that control diverse biological processes that include angiogenesis. Herpes simplex virus (HSV) causes a chronic immuno-inflammatory response in the eye that may result in corneal neovascularization during blinding immunopathological lesion stromal keratitis (SK). miR-132 is a highly conserved miRNA that is induced in endothelial cells in response to growth factors, such as vascular endothelial growth factor (VEGF). In this study, we show that miR-132 expression was up-regulated (10- to 20-fold) after ocular infection with HSV, an event that involved the production of both VEGF-A and IL-17. Consequently, blockade of VEGF-A activity using soluble VEGF receptor 1 resulted in significantly lower levels of corneal miR-132 after HSV infection. In addition, low levels of corneal miR-132 were detected in IL-17 receptor knockout mice after HSV infection. *In vivo* silencing of miR-132 by the provision of anti-miR-132 (antagomir-132) nanoparticles to HSV-infected mice led to reduced corneal neovascularization and diminished SK lesions. The anti-angiogenic effect of antagomir-132 was reflected by a reduction in angiogenic Ras activity in corneal CD31-enriched cells (presumably blood vessel endothelial cells) during SK. To our knowledge, this is one of the first reports of miRNA involvement in an infectious ocular disease. Manipulating miRNA expression holds promise as a therapeutic approach to control an ocular lesion that is an important cause of human blindness. *(Am J Pathol 2012, 181: 525–534; http://dx.doi.org/10.1016/j.ajpath.2012.04.014)*

Herpes simplex virus (HSV) causes a chronic immuno-inflammatory response in the eye that is a significant cause of human blindness.[1] The blinding lesion represents a T-cell–orchestrated reaction in the corneal stroma set off by the infection.[2] These stromal keratitis (SK) lesions involve cellular and cytokine events that resemble those seen in some autoimmune diseases, and control measures found effective in autoimmunity often similarly act to control SK. One major feature of SK that is not usually a prominent part of an autoimmune lesion is pathological angiogenesis.[3] Thus, the corneal site where SK occurs is normally an avascular tissue, which is a requisite for normal vision. Once corneal neovascularization (CV) has occurred, inflammatory cells can easily gain access to the eye, vision is impaired, and blood vessels, once formed, are difficult to remove.[2] Hence, a major objective of therapies to control SK and several other ocular lesions is to prevent or control the extent of neovascularization.[4] Several approaches have been evaluated to achieve this objective, but none have proved to be fully effective; alternative strategies are needed. Conceivably, controlling the expression of one or more species of microRNAs (miRNAs) is one such strategy. Thus, several recent studies have indicated that miRNAs are exploitable gene regulators, and many of them may be dysregulated during tissue-damaging inflammatory reactions and during pathological angiogenesis.[5,6] Of particular interest, miR-132 acted as a switch to activate embryonic human vascular endothelial cells to undergo vasculogenesis.[7] The miR-132 was also up-regulated during pathological angiogenesis in a tumor model, and inhibiting miR-132 by antagomir nanoparticles was inhibitory to tumor angiogenesis.[7]

The role for miRNA in tissue damage and angiogenesis caused by an infectious agent is poorly understood. Some recent reports, however, have indicated that miRNA encoded by a virus can play a role in the expression of latency[8] and the induction of tumors[9] and may be involved in human cytomegalovirus immune evasion.[10]

We show herein that targeting miR-132 represents a potentially valuable approach for the control of the CV that occurs during SK. Accordingly, miR-132 expression is up-regulated after infection, and blockade of vascular endothelial growth factor (VEGF)-A activity resulted in significantly lower corneal miR-132 levels. Of particular interest, IL-17 receptor knockout (RKO) mice produced less miR-132 in corneas after infection. In addition, the administration of antagomir-132 nanoparticles led to diminished angiogenic Ras activity that was reflected by significantly reduced angiogenesis and diminished SK lesions. The results of these studies indicate that manipulating miRNAs, as shown herein by targeting miR-132, might provide an additional avenue for the control of an important cause of vision loss.

Materials and Methods

Mice

Female 6- to 8-week-old C57BL/6 mice were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). IL-17RKO mice on a C57BL/6 background were obtained from Amgen (Thousand Oaks, CA). The animals were housed in American Association of Laboratory Animal Care–approved facilities at the University of Tennessee, Knoxville. All investigations followed the guidelines of the institutional animal care and use committee.

Virus

HSV-1 strain RE Tumpey was propagated in Vero cell monolayers (ATCC no CCL81). Virus was grown in Vero cell monolayers (ATCC, Manassas, VA), titrated, and stored in aliquots at −80°C until used.

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 containing 10^4 plaque-forming units of HSV-1 RE Tumpey was applied to one eye. The development of SK lesion severity and angiogenesis in the eyes of mice was examined by slit-lamp biomicroscopy (Kowa Company, Nagoya, Japan). The scoring system used was as follows: 0, normal cornea; 1, mild corneal haze; 2, moderate corneal opacity; 3, severe corneal opacity; 4, opaque cornea and ulcer; and 5, corneal rupture. The severity of angiogenesis was recorded as previously described.[11] According to this system, a grade of four 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 was 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.[12] Briefly, these injections were performed using a 2-cm, 32-gauge needle and syringe (Hamilton, Reno, NV) to penetrate the perivascular region of conjunctiva, and the appropriate amount of soluble VEGF receptor 1 protein/isotype or scrambled sequences/antagomir-132 nanoparticles was administered into the subconjunctival space.

Antagomir Sequences

Anti-miR-132 and scrambled sequences were procured from Ambion (Austin, TX) and used as previously reported.[7]

Nanoparticle Preparation

Optimized histidine-lysine polymers have been applied for small-interfering RNA deliveries *in vitro* and *in vivo*.[13] One HK polymer species, H3K4b, having a lysine backbone with four branches containing multiple repeats of histidine and lysine, was used for packaging small-interfering RNAs against miR-132 or scrambled sequences, 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 polymers–small-interfering RNA nanoparticles were used in mice.

Murine Treatment with Antagomir-132 Nanoparticles

Mice ocularly infected with HSV-1 RE Tumpey were separated into two groups. Antagomir-132 nanoparticle treatment was begun at day 2, with additional doses on alternate days until day 13 after infection. In another group of experiments, the antagomir-132 treatment was started at day 7 or day 10, with additional doses on alternate days until day 13 after infection. The control group received nanoparticles containing scrambled sequences subconjunctivally with the same regimen for respective experiments. These animals were carefully followed for the progression of angiogenesis and SK development. All experiments were repeated two times.

Purification of CD31-Enriched Cells

Purification of CD31-enriched cells (presumably endothelial cells) from HSV-infected corneas was performed as previously described.[14] Briefly, the excised corneas were pooled and digested with 60 U/mL Liberase for 35 minutes at 37°C in a humidified atmosphere of 5% CO_2. A single-cell suspension was prepared and stained with anti-CD31–fluorescein isothiocyanate for 30 minutes on ice, and fluorescein isothiocyanate–positive $CD31^+$ cells were sorted using a fluorescence-activated cell sorter. Purity to an extent of 80% to 90% was achieved. These sorted CD31-enriched cells were used for pull-down assays.

Flow Cytometry

Corneal single-cell suspensions were prepared after Liberase digestion of corneas. These corneal cell suspensions were then stained for different cell surface molecules. Briefly, cell suspensions were incubated with

CD45-allophycocyanin (30-F11), CD11b-PerCP (M1/79), Gr1-phosphatidylethanolamine (1A8), CD4-allophyco-cyanin (RM4.5), and CD31–fluorescein isothiocyanate (BD Biosciences) for 30 minutes on ice. Thereafter, cells were washed three times and resuspended in 1% para-formaldehyde. Stained samples were acquired with an FACS Calibur (BD Biosciences), and the data were ana-lyzed using FlowJo software.

qPCR Data

Total mRNA was isolated from corneal cells using TRIzol LS reagent (Invitrogen, Carlsbad, CA). The cDNA pre-pared using 1 μg of RNA was used for subsequent anal-ysis. Quantitative PCR (qPCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems, Fos-ter City, CA) with the iQ5 real-time PCR detection system (Bio Rad, Hercules, CA). The expression levels of the target genes were normalized to β-actin with the ΔC_T method, and relative quantification between control and infected mice was performed using the $2^{-\Delta\Delta C_T}$ formula. The primers used are as follows: Ras–glyceraldehyde-3-phosphate (GAP), 5′-GAGAAGAAGATCCACACGAAGG-3′ (forward) and 5′-CTCCAGGAGTATTATCTGAGGG-3′ (reverse); and β-ac-tin, 5′-CTACCTCATGAAGATCCTGACC-3′ (forward) and 5′-GTCTAGAGCAACATAGCACAGC-3′ (reverse).

TaqMan miRNA qPCR

The miRNAs were extracted from HSV-infected murine corneas using the mirVana miRNA Isolation Kit (Ambion). These extracted miRNAs were converted to cDNAs using the TaqMan MicroRNA Reverse Transcription Kit (Ap-plied Biosystems) and primers for miR-132 and miR-133a. TaqMan MicroRNA Assays (Applied Biosystems) for miR-132 and miR-133a were used to quantify these microRNAs using a real-time PCR detection system (Ap-plied Biosystems). Data were normalized to the internal control small nucleolar RNA 202. miR-133a (which is gen-erally expressed in muscle cells) was used as a negative control for miRNA quantification from corneas.[15]

Neutrophil Depletion with Monoclonal Antibodies

Depletion of neutrophils during SK was performed as previously described.[16,17] Mice ocularly infected with HSV-1 RE Tumpey were separated into two groups. One group of animals was administered 200 μg of anti-Ly6G monoclonal antibody (1A8; BioXcell, West Lebanon, NH) i.p. on alternate days starting from day 7 until day 13 after infection. Experiments were terminated on day 14 p.i., and corneal samples were collected for further analysis. Animals in the control group were given isotype control (IgG2b) Ab (LTF-2; BioXcell) following the same regimen. These experiments were repeated two times.

WB Analysis

The corneal cells were lysed and total protein in the supernatants was quantified using the BCA protein assay kit (Thermo Scientific, Waltman, MA). Samples were denatured in Laemmli buffer and resolved by SDS-PAGE, and proteins were transferred onto a poly-vinylidene difluoride membrane. The membrane was blocked with 5% bovine serum albumin in Tris-buffered saline with Tween 20 at 4°C overnight and subjected to incubation with specific primary and secondary anti-bodies. Protein bands were visualized using chemilu-minescent horseradish peroxidase substrate (Milli-pore, Billerica, MA). After keeping in stripping buffer for 10 minutes, the membrane was reprobed using anti-β-actin antibody. The antibodies used were as follows: mouse Ras (Thermo Scientific), anti-β-actin (C4), anti-Ras-GAP (B4F8), mouse anti-VEGF-A (EE02), goat anti-mouse IgG–horseradish peroxidase, and donkey anti-goat IgG–horseradish peroxidase. (All these antibodies were procured from Santa Cruz Bio-technology).

Ras Activation Assay

Ras activation assays were performed using an active Ras pull-down and detection kit (Thermo Scientific), according to the manufacturer's protocol. Briefly, resin slurry was added to the spin cup in a collection tube, and 100 μg of glutathione *S*-transferase GGA3-PBD fusion protein with 800 μg of total protein (corneal cell lysate) was added to this slurry. This was incubated at 4°C for 1 hour; finally, 50 μL of reducing sample buffer was added and GTP-Ras was pulled down and subsequently analyzed by using Western blot (WB) analysis. These experiments were re-peated two times.

Viral Plaque Assay

Virus titers were measured in the eye swabs taken from HSV-infected mice using plaque assays, as previously described by others.[18]

Statistics

The statistical significance for SK lesion severity and an-giogenesis between two groups was determined using an unpaired two-tailed Student's *t*-test. A one-way anal-ysis of variance, with Bonferroni's post hoc test, was used to calculate the level of significance for some experi-ments. For all statistical analysis, GraphPad Prism soft-ware was used.

Results

miR-132 Is Up-Regulated in Murine Corneas after Ocular Infection with Herpes Simplex Virus

To measure changes in miR-132 levels after ocular HSV infection, tissues were collected at various times and
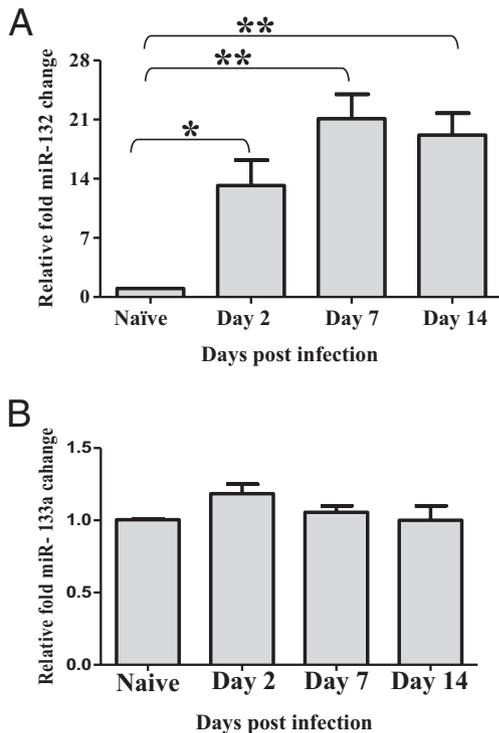
A

B

Figure 1. Expression of miR-132 after HSV infection. miRNA quantification in HSV-infected mice corneas. WT mice are infected with HSV-1 RE in one eye, and six corneas are collected and pooled for miRNA analysis by TaqMan qPCR. The expression levels of miR-132 (**A**) and miR-133a (**B**) at different time points after HSV infection in mice corneas are shown (pooled $n = 6$ mice per group). A one-way analysis of variance, with Bonferroni's post hoc test, is used to calculate the level of significance. $*P \leq 0.05$, $**P \leq 0.01$. Data represent mean $\pm$ SEM. The previously described experiments are repeated three times.

miRNAs were quantified by qPCR. Changes in the levels of miR-132, but not control miR-133a, were evident by day 2 after infection (p.i.) in infected samples, with peak expression levels evident in day 7 and day 14 p.i. samples (Figure 1, A and B). Uninfected scratch controls at the same time points showed no changes in the levels of miR-132 (data not shown). These data demonstrated that miR-132 expression was elevated at multiple time points after ocular HSV infection.

Blockade of VEGF-A Activity Diminishes Corneal miR-132 Levels During SK

The increased expression of miR-132 at time points when virus was cleared from the eyes and CV became evident (usually by day 7 p.i.), which raised the issue as to the triggers responsible for miR-132 up-regulation. Because VEGF-A levels were increased after HSV infection and remained so throughout the disease syndrome,[17] VEGF-A was suspected to be an agonist for miR-132 up-regulation. Moreover, VEGF-A can induce miR-132 expression in the human umbilical vein endothelial cells.[7] To investigate the role for VEGF-A, we measured the effect of VEGF-A blockade on the expression of miR-132 levels. To effect VEGF-A blockade, animals were treated after infection with 5 μg VEGF trap (soluble VEGF receptor 1) or isotype control on alternate days, starting from

day 2 up to day 12 p.i. These conditions previously markedly suppressed VEGF-A protein activity and also significantly inhibited CV.[17] The corneas were collected at day 7 and day 14 p.i., and miR-132 levels were compared in isotype control and VEGF trap-treated animals. When compared with uninfected scratch controls, the miR-132 levels were elevated at day 7 p.i. in isotype-treated animals. However, miR-132 in the recipients of the VEGF trap were, on average, fourfold less than in isotype-treated animals (Figure 2A). The same pattern was evident at day 14 p.i. in mice treated with VEGF trap compared with isotype-treated animals (Figure 2A).

In another set of experiments, neutrophils were depleted[16] during the clinical stage of SK (Figure 2B). Neutrophils are a source of VEGF-A, and their depletion results in lower VEGF-A levels in corneas obtained from HSV-infected mice.[17] Depletion (using 200 μg of anti-Ly6G antibody) was begun at day 7 p.i., with additional doses on alternate days up to day 13. Neutrophil depletion was clearly evident in the spleen and corneas of HSV-infected mice treated with anti-Ly6G antibody (see Supplemental Figure S1, A–C, at *http://ajp.amjpathol.org*). Expression levels of miR-132 were compared in corneal pools from depleted and isotype control mice on day 14 p.i. VEGF-A levels were reduced after depletion of neutrophils in these experiments (Figure 2C). As shown in Figure 2D, miR-132 expression levels were reduced by, on average, threefold in the neutrophil-depleted mice compared with isotype-treated animals. Taken together, the two approaches supported the notion that VEGF-A could be responsible for the up-regulation of miR-132 expression during HSV-induced CV.

Diminished Levels of miR-132 in IL-17RKO Mice after HSV Infection

Our recent observations that mice unable to respond to IL-17A showed reduced corneal VEGF-A levels[19] led us to investigate the possible role of IL-17A on miR-132 expression after HSV infection. To this end, IL-17A RKO mice or wild-type (WT) mice were infected with HSV; miR-132 levels were measured at days 2, 7, and 14 p.i., whereas Ras-GAP levels (miR-132 target) were measured at day 7 p.i. The significant increase in the levels of Ras-GAP was evident in IL-17RKO mice (Figure 2E). As shown in Figure 2, F and G, IL-17RKO mice showed reduced CV and SK lesions compared with WT animals. In addition, when miR-132 levels were measured in the infected corneas at days 7 and 14 p.i., IL-17RKO mice showed a twofold reduction in miR-132 levels compared with WT mice (Figure 2H). The results from these experiments indicated that the cytokine IL-17A was involved in miR-132 up-regulation after HSV infection.

In Vivo *Silencing of miR-132 by Antagomir-132 Nanoparticles Reduces Angiogenic Ras 1 in Corneas*

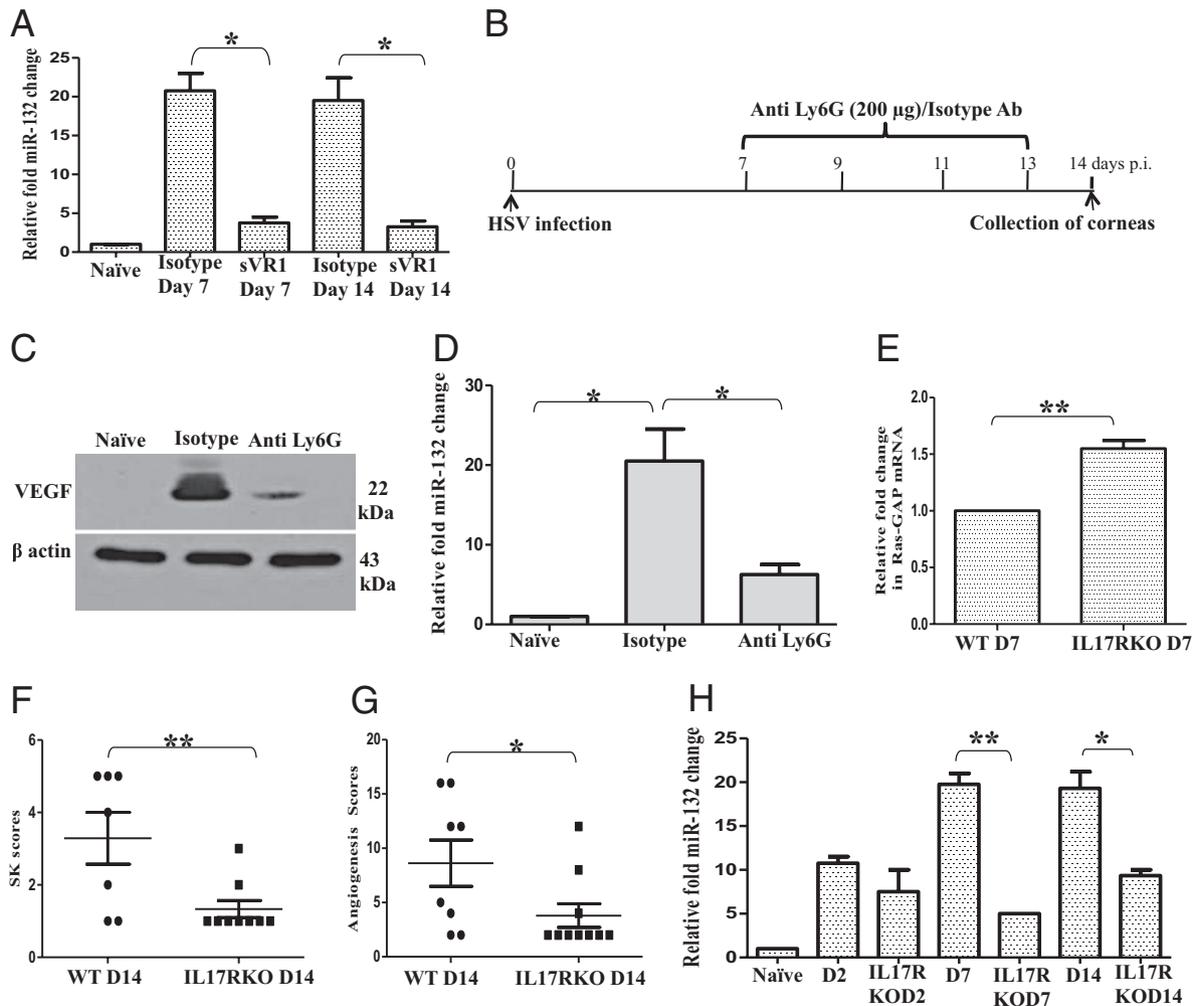Experiments were performed using nanoparticles containing an antagomir for miR-132 or scrambled se-

Figure 2. IL-17A and VEGF-A up-regulates miR-132 in corneas. WT mice are infected with HSV-1 RE in one eye, and six corneas are collected and pooled for miR-132 analysis by TaqMan qPCR. The 5-μg soluble VEGFR1/isotype treatment is begun on day 2 p.i., with additional doses on alternate days until day 6, and miR-132 quantification is performed at day 7 p.i. (pooled n = 6 mice per group). In another set of experiments, 5-μg soluble VEGFR1 (sVR1)/isotype treatment is continued up to day 12 p.i., and corneas are analyzed for miR-132 expression at day 14 p.i. (**A**) (pooled n = 6 mice per group). A one-way analysis of variance, with Bonferroni's post hoc test, is used to calculate the level of significance. Data represent mean ± SEM. Neutrophil depletion is performed using anti-Ly6G antibody, as shown (**B**). The HSV-infected mice are treated with 200 μg of anti-Ly6G/isotype antibody, as shown, and corneas are analyzed for VEGF-A levels by using WB analysis and for miR-132 expression by using qPCR at day 14 p.i. (**C** and **D**, respectively) (pooled n = 6 mice per group). The level of significance is determined by the Student's *t*-test (unpaired). WT and IL-17RKO mice are infected with HSV-1 RE in one eye, and six corneas are collected and pooled for Ras-GAP mRNA analysis at day 7 (D7) p.i. (**E**) and miR-132 analysis by TaqMan qPCR at day 14 (D14) p.i. SK lesions (**F**), angiogenesis scores (**G**), and miR-132 expression (**H**) in HSV-infected IL-17RKO mice and WT mice at days 2, 7, and 14 p.i. are shown (pooled n = 6 mice per group). Data represent mean ± SEM. All experiments are repeated two times. *$P \leq 0.05$, **$P \leq 0.01$.

quences to determine the effect of antagomir treatment on miR-132 levels in postinfection corneas. A dose of 2.5 μg of antagomir-132 provided significant miR-132 knockdown when measured at day 7 p.i. (Figure 3, A and B). Because miR-132 targets Ras-GAP mRNA,[7] levels of both Ras-GAP mRNA and protein were measured. These levels were elevated in mice treated with antagomir-132 nanoparticles compared with scrambled sequence recipients (Figure 3, C and D).

Additional experiments were performed to measure expression of active Ras 1 after *in vivo* knockdown in infected animals, because miR-132 promoted VEGF signaling by influencing active Ras levels in human umbilical vein endothelial cells.[7] Mice infected with HSV were given 2.5 μg of antagomir-132, or scrambled sequences, subconjunctivally, starting from day 2 p.i., with additional

doses on alternate days until day 10 p.i. Active Ras 1 was pulled down from 6 corneas collected from each group at day 11 p.i. (Figure 4A). As shown in Figure 4, B and C, total Ras 1 levels were similar in all groups, but an increase in GTP-Ras 1 was evident in animals treated with scrambled sequences compared with uninfected controls. In contrast, a reduction in GTP-Ras 1 was observed in the antagomir-132 treatment group. In addition, active Ras 1 pulled down from the CD31-enriched cells (presumably blood vessel endothelial cells) sorted from scrambled sequences or antagomir-132–treated mice corneas at day 11 p.i. revealed a reduction in active Ras 1 in antagomir-132–treated mice (see Supplemental Figure S2, D and E, at *http://ajp.amjpathol.org*). Curiously, there was no difference in VEGF-A levels between these groups (Figure 4, D and E). Collectively, these results

A

Antagomir-132 (0.1-2.5 µg)/scrambled sequences

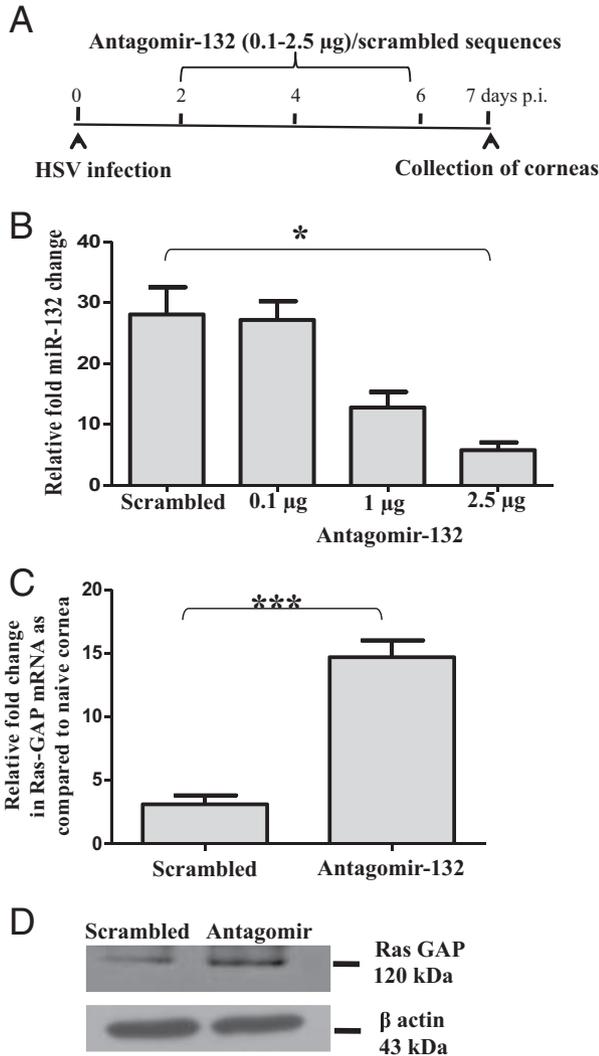HSV infection   Collection of corneas

B

C

D

Figure 3. Knockdown of miR-132 by antagomir-132 nanoparticles in eyes. **A:** The different doses of antagomir-132/scrambled sequences nanoparticle treatment are given to HSV-infected mice, as shown. The 2.5-µg antagomir-132 treatment regimen results in peak miR-132 knockdown in the corneas (**B**) (pooled $n = 6$ mice per group). A one-way analysis of variance, with Bonferroni's post hoc test, is used to calculate the level of significance. *$P \leq$ 0.05. These experiments are repeated two times. WT mice are infected with HSV-1 RE in one eye, and six corneas are collected and pooled for analysis by qPCR or WB. The 2.5-µg antagomir-132/scrambled sequences are injected subconjunctivally in HSV-infected mice, and the quantification of Ras-GAP mRNA (**C**) from corneas isolated from different groups is performed (pooled $n = 6$ mice per group). The level of significance is determined by Student's t-test (unpaired). ***$P \leq$ 0.001. These experiments are repeated two times. Data represent mean ± SEM. Reducing WB for Ras-GAP protein after treatment of HSV-infected mice with 2.5 µg of antagomir-132/scrambled sequences (**D**) (pooled $n = 6$ mice per group). The representative WB image of two independent experiments is shown.

indicated that the provision of antagomir-132 to HSV-1–infected mice reduced the angiogenic Ras 1 activity but did not affect VEGF-A levels in the corneas.

Provision of Antagomir-132 Nanoparticles Diminishes Corneal Neovascularization

To evaluate the effect of inhibiting miR-132 expression on the extent of angiogenesis induced after infection, animals were given an optimal dose subconjunctivally of

antagomir-132, or control nanoparticles, starting on either treatment day 2 or day 7. As shown in Figure 5, A–C, treatment begun on day 2 resulted in significantly reduced CV in the antagomir-treated animals and SK severity was also significantly reduced. A visible reduction in angiogenesis was evident in the eyes of mice treated with antagomir-132 (see Supplemental Figure S2, A–C, at *http://ajp.amjpathol.org*). An examination of collagen-digested corneas at the termination of experiments on day 14 revealed that the number of inflammatory cells was significantly diminished in the antagomir-132–treated group (Figure 5, D–I). We also measured the ocular viral titers on day 5 p.i. after administration of antagomir-132 or scrambled sequences, and a slight increase in viral levels was evident in antagomir-132–treated animals (see Supplemental Figure S3, A and B, at *http://ajp.amjpathol.org*).

In the experiments in which treatment was begun on day 7 p.i., significantly diminished angiogenesis was also evident in the antagomir-132–treated animals (Figure 6, A–C). At the end of experiments on day 14, subpools of corneal collagen digests were analyzed by flow cytometry to enumerate the numbers of the CD31$^+$ cells (a marker for vascular endothelial cells) and Gr1$^+$CD11b$^+$ cells (Figure 6, D–G). Both cell populations were significantly reduced in number in the recipients of antagomir-132 compared with those that received the scramble
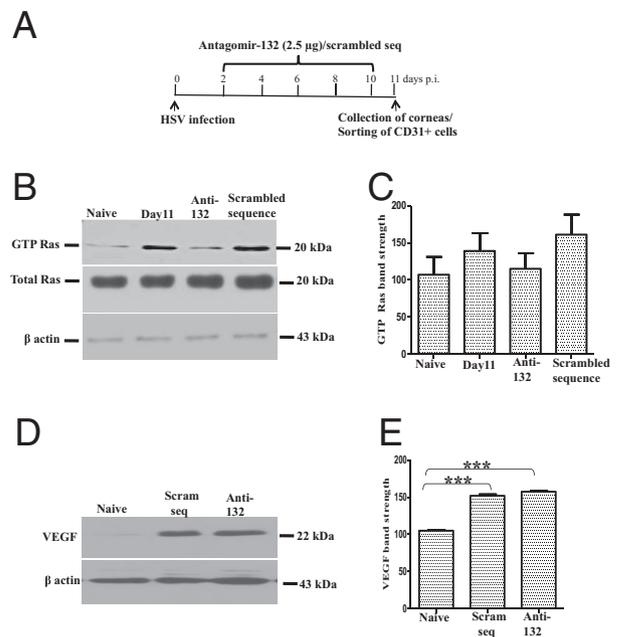
A

Antagomir-132 (2.5 µg)/scrambled seq

HSV infection   Collection of corneas/
Sorting of CD31+ cells

B

C

D

E

Figure 4. Reduction in angiogenic Ras activity after antagomir-132 treatment. WT mice are infected with HSV-1 RE in one eye, and six corneas are collected and pooled for analysis by GTPase activation assays. **A:** WT mice infected with HSV are treated with antagomir-132/scrambled sequences nanoparticle, as shown. The corneas collected at day 11 p.i. after antagomir-132/scrambled sequences nanoparticle treatment are subjected to Ras (**B** and **C**) pull down, followed by reducing WB analysis for Ras of the respective groups (pooled $n = 6$ mice per group). The representative WB image is shown. The corneas collected at day 11 p.i. after antagomir-132/scrambled (Scram) sequences nanoparticle treatments are subjected to WB for the detection of VEGF (**D** and **E**). The representative WB image is shown. These experiments are repeated two times, and densitometry analysis of the band strength is shown. A one-way analysis of variance, with Bonferroni's post hoc test, is used to calculate the level of significance. ***$P \leq$ 0.001. Data represent mean ± SEM.
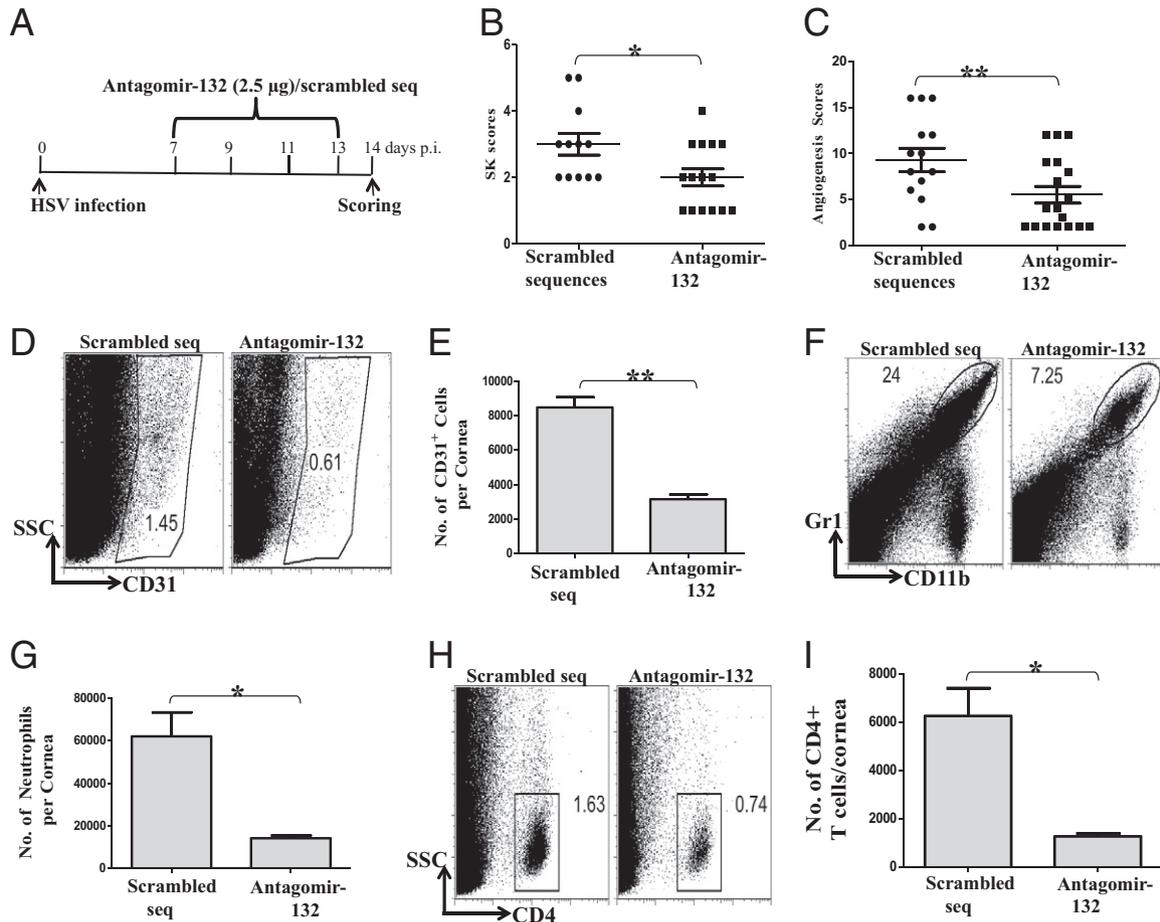
Figure 6. Provision of antagomir-132 nanoparticles therapeutically diminishes angiogenesis and SK. WT mice are infected with HSV-1 RE in one eye, and six corneas are collected and pooled for analysis by flow cytometry. The therapeutic 2.5-μg antagomir-132 nanoparticle treatment (**A**) results in reduction in SK (**B**) and angiogenesis (**C**) scores in HSV-infected animals. The frequency and total cell number per cornea for endothelial cells (CD31$^+$ cells) (**D** and **E**, respectively), Gr1$^+$CD11b$^+$ cells (**F** and **G**, respectively), and CD4$^+$ T cells (**H** and **I**, respectively) show a significant reduction in total cell number per cornea after therapeutic antagomir-132 nanoparticle treatment ($n = 12$ to 18 mice per group). The level of significance is determined by Student's t-test (unpaired). *$P \leq 0.05$, **$P \leq 0.01$. Data represent mean ± SEM. These experiments are repeated two times. SSC, side scatter; seq, sequence.

tion no longer present in the eye. However, inhibition of CV was not complete after antagomir-132 treatment, and it is possible that using additional approaches, along with antagomirs, might be more successful. One critical event influenced by miR-132 was the response of VEGFR2-expressing vascular endothelial cells to VEGF-A, as was recently described by Anand et al.[7] miR-132 targeted Ras-GAP, an endogenous inhibitor of Ras, which, when activated, is responsible for causing vascular endothelial cells to proliferate. In agreement with these observations, we could show in our study that the administration of miR-132 antagomirs diminished Ras activity in the CD31-enriched cells (presumably blood vessel endothelial cells). Our results indicate that modulating miRNA represents an effective approach to control CV, which reflects in reducing the severity of SK lesions caused by HSV infection. The results are summarized in Figure 7.

miRNAs are becoming well-known as regulators of immunity and inflammation, and manipulating their expression holds promise as a therapeutic maneuver.[20] Few studies have focused on the relevance of miRNA in the control of pathological angiogenesis. However, it is becoming evident that levels of several miRNA species are

changed during tumor angiogenesis, which have been the systems mainly investigated. The miRNAs with changed expression include miR-132, miR-20a, miR-21, and miR-106a.[5] Recently, Anand et al[7] drew attention to a likely major role for miR-132 during angiogenesis because miRNA screens of embryonic vascular endothelial cell (human umbilical vein endothelial cell) responses to VEGF-A stimulation revealed that miR-132 expression was the most elevated.[7] Moreover, they could show that normal expression of miR-132 was necessary for vascular development and that preventing its up-regulation was beneficial in a tumor angiogenesis system.

Apart from the effects of miR-132 on angiogenesis, the miRNA may also influence aspects of neural function, which has occurred in some other systems.[21–23] Because the pathogenesis of HSV often involves stress-related effects on a latent infection in the nervous system, it is conceivable that regulation by miR-132 is involved in this process. This issue merits further investigation.

In addition, rapid responses to some viruses, which include HSV, may induce miR-132,[24] although such responses are likely to represent innate reactions to the virus, rather than being responses to virus gene expres-
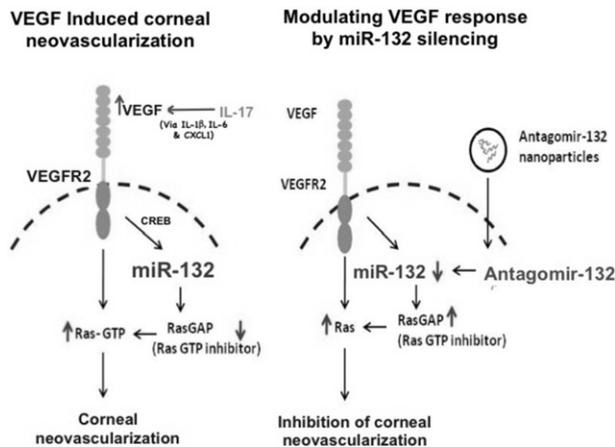
Figure 7. Illustration of antagomir-132–mediated inhibition of CV. **Left panel:** Outcome (CV) in HSV-infected untreated mice. HSV infection leads to up-regulation of IL-17 in corneas. IL-17 (along with IL-6 and virus-infected epithelial cells) increases VEGF (probably via increasing IL-1b, IL-6, and Cxcl1) levels in the eyes. VEGF, thus acting through VEGFR2 receptors on the blood vessel endothelial cells, up-regulates miR-132 expression via cAMP response element–binding protein (CREB) transcription factor. miR-132 removes Ras-GAP (intrinsic inhibitor of Ras), leading to activation of Ras and CV. **Right panel:** Modulation of CV by miR-132 silencing. The administration of antagomir-132 nanoparticles leads to deposition of antagomir-132 cargo in blood vessel endothelial cells, resulting in silencing of miR-132. This leads to higher levels of Ras-GAP, which, thereby, inhibits angiogenic Ras activity, resulting in inhibition of CV.

sion. In line with this lipopolysaccharide, a Toll-like receptor (TLR)-4 ligand may also cause the rapid expression of miR-132 in macrophages *in vitro*.[22] In our studies, we noted early up-regulation (by 48 hours pi) of miR-132, which might represent a response to the well-characterized TLR ligand activity of HSV.[25,26] Preliminary *in vitro* experiments support this interpretation (unpublished data) and our past observations that TLR-2 and TLR-9 knockout mice have diminished CV compared with WT animals, which is in line with the same interpretation.[18]

There are reasons, however, to consider that the direct binding effects by virus to cells may not be the major stimulus for miR-132 production, particularly by vascular endothelial cells. Accordingly, in the mouse model, virus replication is usually confined to the corneal epithelium, but neovascularization occurs in the underlying stroma.[2] Because miRNAs exert regulatory effects only in cells that produce them, any effect of infection is expected to be mediated indirectly via soluble molecules, such as cytokines and cell breakdown products, generated during the infection. Our data, along with the published report of others,[7] indicate that one agonist responsible for causing miR-132 production in vascular endothelial cells was VEGF-A. Evidence for this conclusion came from *in vivo* observations that procedures that caused diminished VEGF-A production or activity resulted in reduced levels of miR-132. For example, lowering VEGF-A levels by a VEGF trap approach, or reducing the presence of some cell types, such as neutrophils that produce VEGF-A, resulted in diminished miR-132 levels. Perhaps more convincing in favor of VEGF-A as responsible for miR-132 production, we showed that animals treated with nanoparticles containing miR-132 antagomirs had reduced levels of downstream signaling products of

VEGF-A stimulation, such as active Ras in the CD31-enriched cells (presumably blood vessel endothelial cells) than controls (Figure 7).

An unsolved issue, however, is to explain the connection between the virus infection and the VEGF-A production. Some have advocated that virus-infected cells themselves produce VEGF-A,[27] but perhaps the more common circumstance is VEGF-A production by several uninfected cell types in response to one or more agonists generated as a consequence of the infection. These agonists include proinflammatory cytokines, such as IL-6, that can cause inflammatory cell types to produce VEGF-A in our previous study.[28] An additional cytokine that could participate in causing VEGF-A production is IL-17A. This cytokine is produced in the cornea after HSV infection, mainly by innate immune cells in initial stages and, later on, by CD4 Th17 T cells.[29] In support of IL-17A as involved in miR-132 production, we showed that levels of miR-132 produced in WT mice after infection were higher than those in animals unable to respond to IL-17A because they lacked the IL-17A receptor. Accordingly, IL-17RKO animals displayed more miR-132 target, Ras-GAP, in corneas after HSV infection.

Our observation that miR-132 appears to be involved in orchestrating CV in an infectious disease situation is a novel finding. It leads to the question about the therapeutic potential of targeting miRNAs as a means to control clinical diseases. Progress on this topic has already been accomplished in some autoimmune and neoplastic diseases.[30] Many studies focus on the miR-155 that is involved in regulating inflammatory pathways.[31] miR-155 can be up-regulated by exposure to several TLR ligands and is overexpressed during some autoimmune diseases, as well as a diverse array of immune cell cancers.[20] Of particular interest, knockout mice exist that lack miR-155. Such mice are resistant to experimental autoimmune encephalomyelitis and collagen-induced arthritis and generate T-cell responses that emphasize the type 2 helper T-cell pattern.[32] In preliminary studies, we have shown that miR-155 may also be involved in the pathogenesis of SK (unpublished results). In disease situations that involve changes in one or more species of miRNAs, the issue will be to determine whether modulating their expression represents a more viable approach to control inflammatory and neoplastic diseases than other procedures. We are attempting to answer this question in the SK system by comparing the miRNA antagomirs in the nanoparticles approach with other control procedures. So far, there are no results to report. Whatever occurs, there is at least one theoretical advantage of targeting miR-132 to control angiogenesis. Thus, antiangiogenic therapies that target single pathways, such as VEGFR signaling, often develop resistance by up-regulating alternative angiogenic growth factors.[33] This problem can be avoided using miR-132 because it removes an endogenous regulator (Ras-GAP) involved in controlling responses to several angiogenic factors. On the other hand, because miRNAs have multiple mRNA targets, a potential downside of the approach could be a crossfire effect that turns off some useful gene expression, alongside that of the gene being selected for silenc-

ing. The results of future studies will reveal when situations are most appropriate for the use of miRNA for therapy.

Acknowledgments

References

1. Pepose JS: Herpes simplex keratitis: role of viral infection versus immune response. Surv Ophthalmol 1991, 35:345–352
2. Biswas PS, Rouse BT: Early events in HSV keratitis: setting the stage for a blinding disease. Microbes Infect 2005, 7:799–810
3. Zheng M, Schwarz MA, Lee SJ, Kumaraguru U, Rouse BT: Control of stromal keratitis by inhibition of neovascularization. Am J Pathol 2001, 159:1021–1029
4. Kim B, Tang QQ, Biswas PS, Xu J, Schiffelers RM, Xie FY, Ansari AM, Scaria PV, Woodle MC, Lu P, Rouse BT: Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor pathway genes: therapeutic strategy for herpetic stromal keratitis. Am J Pathol 2004, 165:2177–2185
5. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM: A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 2006, 103:2257–2261
6. Zhou QB, Gallagher R, Ufret-Vincenty R, Li XY, Olson EN, Wang SS: Regulation of angiogenesis and choroidal neovascularization by members of microRNA-23 similar to 27 similar to 24 clusters. Proc Natl Acad Sci U S A 2011, 108:8287–8292
7. Anand S, Majeti BK, Acevedo LM, Murphy EA, Mukthavaram R, Scheppke L, Huang M, Shields DJ, Lindquist JN, Lapinski PE, King PD, Weis SM, Cheresh DA: MicroRNA-132-mediated loss of p120RasGAP activates the endothelium to facilitate pathological angiogenesis. Nat Med 2010, 16:909–914
8. Umbach JL, Kramer MF, Jurak I, Karnowski HW, Coen DM, Cullen BR: MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. Nature 2008, 454:780–783
9. Gottwein E, Mukherjee N, Sachse C, Frenzel C, Majoros WH, Chi JT, Braich R, Manoharan M, Soutschek J, Ohler U, Cullen BR: A viral microRNA functions as an orthologue of cellular miR-155. Nature 2007, 450:1096–1099
10. Nachmani D, Lankry D, Wolf DG, Mandelboim O: The human cytomegalovirus microRNA miR-UL112 acts synergistically with a cellular microRNA to escape immune elimination. Nat Immunol 2010, 11:806–813
11. Rajasagi NK, Reddy PBJ, Suryawanshi A, Mulik S, Gjorstrup P, Rouse BT: Controlling herpes simplex virus-induced ocular inflammatory lesions with the lipid-derived mediator resolvin E1. J Immunol 2011, 186:1735–1746
12. Dana MR, Zhu SN, Yamada J: Topical modulation of interleukin-1 activity in corneal neovascularization. Cornea 1998, 17:403–409
13. Leng Q, Scaria P, Lu P, Woodle MC, Mixson AJ: Systemic delivery of HK Raf-1 siRNA polyplexes inhibits MDA-MB-435 xenografts. Cancer Gene Ther 2008, 15:485–495
14. Mulik S, Sharma S, Suryawanshi A, Veiga-Parga T, Reddy PBJ, Rajasagi NK, Rouse BT: Activation of endothelial roundabout receptor 4 reduces the severity of virus-induced keratitis. J Immunol 2011, 186:7195–7204
15. Koutsoulidou A, Mastroyiannopoulos NP, Furling D, Uney JB, Phylactou LA: Expression of miR-1, miR-133a, miR-133b and miR-206 increases during development of human skeletal muscle. BMC Dev Biol 2011, 11:34
16. Zhang X, Majlessi L, Deriaud E, Leclerc C, Lo-Man R: Coactivation of Syk kinase and MyD88 adaptor protein pathways by bacteria promotes regulatory properties of neutrophils. Immunity 2009, 31:761–771
17. Suryawanshi A, Mulik S, Sharma S, Reddy PBJ, Sehrawat S, Rouse BT: Ocular neovascularization caused by herpes simplex virus type 1 infection results from breakdown of binding between vascular endothelial growth factor A and its soluble receptor. J Immunol 2011, 186:3653–3665
18. Sarangi PP, Kim B, Kurt-Jones E, Rouse BT: Innate recognition network driving herpes simplex virus-induced corneal immunopathology: role of the toll pathway in early inflammatory events in stromal keratitis. J Virol 2007, 81:11128–11138
19. Suryawanshi A, Veiga-Parga T, Reddy PB, Rajasagi NK, Rouse BT: 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. J Immunol 2012, 188:3434–3446
20. O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D: Physiological and pathological roles for microRNAs in the immune system. Nat Rev Immunol 2010, 10:111–122
21. Luikart BW, Bensen AL, Washburn EK, Perederiy JV, Su KG, Li Y, Kernie SG, Parada LF, Westbrook GL: miR-132 mediates the integration of newborn neurons into the adult dentate gyrus. PLoS One 2011, 6:e19077
22. Shaked I, Meerson A, Wolf Y, Avni R, Greenberg D, Gilboa-Geffen A, Soreq H: MicroRNA-132 potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase. Immunity 2009, 31:965–973
23. Nudelman AS, DiRocco DP, Lambert TJ, Garelick MG, Le J, Nathanson NM, Storm DR: Neuronal activity rapidly induces transcription of the CREB-regulated microRNA-132, in vivo. Hippocampus 2010, 20:492–498
24. Lagos D, Pollara G, Henderson S, Gratrix F, Fabani M, Milne RS, Gotch F, Boshoff C: miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator. Nat Cell Biol 2010, 12:513–519
25. Zheng M, Klinman DM, Gierynska M, Rouse BT: DNA containing CpG motifs induces angiogenesis. Proc Natl Acad Sci U S A 2002, 99:8944–8949
26. Zhang SY, Jouanguy E, Ugolini S, Smahi A, Elain G, Romero P, Segal D, Sancho-Shimizu V, Lorenzo L, Puel A, Picard C, Chapgier A, Plancoulaine S, Titeux M, Cognet C, von Bernuth H, Ku CL, Casrouge A, Zhang XX, Barreiro L, Leonard J, Hamilton C, Lebon P, Heron B, Vallee L, Quintana-Murci L, Hovnanian A, Rozenberg F, Vivier E, Geissmann F, Tardieu M, Abel L, Casanova JL: TLR3 deficiency in patients with herpes simplex encephalitis. Science 2007, 317:1522–1527
27. Wuest TR, Carr DJJ: VEGF-A expression by HSV-1-infected cells drives corneal lymphangiogenesis. J Exp Med 2010, 207:101–115
28. Biswas PS, Banerjee K, Kinchington PR, Rouse BT: Involvement of IL-6 in the paracrine production of VEGF in ocular HSV-1 infection. Exp Eye Res 2006, 82:46–54
29. Suryawanshi A, Veiga-Parga T, Rajasagi NK, Reddy PBJ, Sehrawat S, Sharma S, Rouse BT: Role of IL-17 and Th17 cells in herpes simplex virus-induced corneal immunopathology. J Immunol 2011, 187:1919–1930
30. O'Neill LA, Sheedy FJ, McCoy CE: MicroRNAs: the fine-tuners of Toll-like receptor signalling. Nat Rev Immunol 2011, 11:163–175
31. O'Connell RM, Kahn D, Gibson WSJ, Round JL, Scholz RL, Chaudhuri AA, Kahn ME, Rao DS, Baltimore D: MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. Immunity 2010, 33:607–619
32. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, van Dongen S, Grocock RJ, Das PP, Miska EA, Vetrie D, Okkenhaug K, Enright AJ, Dougan G, Turner M, Bradley A: Requirement of bic/microRNA-155 for normal immune function. Science 2007, 316:608–611
33. Bergers G, Hanahan D: Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer 2008, 8:592–603