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Critical Role of MicroRNA-155 in Herpes Simplex Encephalitis

Siddheshvar Bhela,*1 Sachin Mulik,*1,2 Pradeep B. J. Reddy,*3 Raphael L. Richardson,*3 Fernanda Gimenez,*3 Naveen K. Rajasagi,* Tamara Veiga-Parga,* Alexander P. Osmand, † and Barry T. Rouse*

HSV infection of adult humans occasionally results in life-threatening herpes simplex encephalitis (HSE) for reasons that remain to be defined. An animal system that could prove useful to model HSE could be microRNA-155 knockout (miR-155KO) mice. Thus, we observe that mice with a deficiency of miR-155 are highly susceptible to HSE with a majority of animals (75–80%) experiencing development of HSE after ocular infection with HSV-1. The lesions appeared to primarily represent the destructive consequences of viral replication, and animals could be protected from HSE by acyclovir treatment provided 4 d after ocular infection. The miR-155KO animals were also more susceptible to development of zosteriform lesions, a reflection of viral replication and dissemination within the nervous system. One explanation for the heightened susceptibility to HSE and zosteriform lesions could be because miR-155KO animals develop diminished CD8 T cell responses when the numbers, functionality, and homing capacity of effector CD8 T cell responses were compared. Indeed, adoptive transfer of HSV-immune CD8 T cells to infected miR-155KO mice at 24 h postinfection provided protection from HSE. Deficiencies in CD8 T cell numbers and function also explained the observation that miR-155KO animals were less able than control animals to maintain HSV latency. To our knowledge, our observations may be the first to link miR-155 expression with increased susceptibility of the nervous system to virus infection. The Journal of Immunology, 2014, 192: 000–000.

Infections with HSV usually cause lesions at body surfaces such as the skin, mucosal surface, and the eye. Characteristically, after primary infection, HSV establishes a nonreplicating persistent (latent) infection in neuronal tissue, which can break down periodically and cause recurrent lesions at primary lesion sites (1). A rare, yet often tragic manifestation of HSV infection is dissemination to the brain with resultant herpes simplex encephalitis (HSE) (2). In adult humans, HSE is usually caused by HSV-1 and can occur in persons who are seropositive and latently infected with virus (2). In addition, infants can develop encephalitis if seronegative and incur primary infection usually with HSV-2 (2). A rare form of HSE also occurs in children with genetic defects in innate immune defenses (3–5). Once virus enters the brain, the lesions that follow are considered to be

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Abbreviations used in this article: DLN, draining lymph node; HSE, herpes simplex encephalitis; KO, knockout; miR, microRNA; miR-155KO, microRNA-155 knockout; p.i., postinfection; PLN, popliteal lymph node; PNS, peripheral nervous system; rGal-9, recombinant Galectin-9; SK, stromal keratitis; TG, trigeminal ganglia; WT, wild type.

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infection, a lesion that reflects viral dissemination into the nervous system (16). In addition, ganglionic latent infection with HSV-1 reactivated more abundantly from miR-155KO than WT latently infected ganglia upon ex vivo culture. One explanation for the observations was that miR-155KO animals developed diminished virus-specific CD8 T cell responses, particularly those that were functionally effective. Other mechanistic explanations were also discussed.

Materials and Methods

Mice

Female 5–6 wk old C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Breeder pairs of miR-155KO mice on C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME), and additional mice were bred in the Walters Life Sciences animal facility at the University of Tennessee, Knoxville. HSV-specific TCR transgenic mice (gBT-I.3) referred to in the text as gBT mice) were produced in the laboratory of Francis Carbone (University of Melbourne, Melbourne, Australia). The animals were housed in American Association of Laboratory Animal Care–approved facilities at the University of Tennessee, Knoxville. All investigations followed guidelines of the institutional animal care and use committee.

Virus

Three different strains of virus were used. HSV-1 Tumpey (obtained from Dr. Robert Lausch, University of South Alabama), HSV-1 RE (obtained from Dr. Robert Hendricks, University of Pittsburgh), and HSV-1 KOS (obtained from Dr. David Knipe, Harvard University) were used. All strains were propagated and titrated on monolayers of Vero cells (ATCC CCL81) using standard protocols. All virus stocks were aliquoted and stored at −80°C.

Infection of mice

Infections of all mice groups (5–8 wk old) were conducted under deep anesthesia with Avertin (tribromoethanol). For corneal infection, the mice were scarified on their corneas with a 27-gauge needle, and a 3-μl drop containing 10⁶ PFU HSV-1 Tumpey was applied to one eye and was used to monitor the development of encephalitis. In experiments involving HSV reactivation, mice were infected with 10⁷ PFU HSV-RE for corneal infection. The zosteriform infection was used in some of the experiments. The zosteriform infection was performed as described earlier (16). In brief, hair was clipped on each left flank and depilated with Veet hair removal cream after anesthetizing the mice using Avertin i.p. injection. A small area of skin (1 cm²) near the top of the spleen was scarificed with a 27-gauge needle, and 20 μl HSV-1 Tumpey containing 10⁵ PFU virus was applied to hair-depleted area of the skin and massaged. In addition, in some experiments, HSV footpad model was used. Mice were injected s.c. in each hind footpad with 4 × 10⁵ PFU HSV-1 KOS in 30 μl PBS. Mice were scarificated at day 5 postinfection (p.i.), and the popliteal lymph nodes (PLN) were isolated for analysis.

Adaptive transfer of HSV-immune CD8⁺ T cells. To generate HSV-immune CD8⁺ T cells, we scarificated gBT mice on their corneas with a 27-gauge needle, and a 3-μl drop containing 10⁶ PFU HSV-1 Tumpey was applied to one eye. Single-cell suspensions of pooled spleens and PLNs were prepared from immunized mice 7–8 d later, and CD8⁺ T cells were purified using a mouse CD8 T cell isolation kit from Miltenyi Biotec. By flow cytometry analysis, the purified population consisted of 85% CD8⁺ T cells. Ocularly infected miR-155KO animals received an i.v. injection of 20 × 10⁵ purified cells at 24 h p.i.

Immunohistochemistry. Groups of miR-155KO mice and WT mice were ocularly infected with 10⁶ PFU HSV-1 Tumpey, and mice showing signs of encephalitis from each group (day 8 p.i.) were anesthetized with Avertin and transcardially perfused with isotonic sucrose solution; sucrose perfusion was followed by perfusion with a solution of 4% paraformaldehyde. Postfixation of the brain samples was done by immersion of the skull in the same 4% paraformaldehyde fixative for 1 d. After brain extraction from the skull, cryoprotection was done in 10% glycerol on day 1 and 20% glycerol on day 2. Mouse brains were embedded within a single gelatin matrix, freeze cut into 35-μm coronal sections, and collected into 24 series (Neuroscience Associates Knoxville, TN). Each 12th section was then stained as a free-floating section. High-sensitivity immunohistochemistry on multi-brain sections was performed essentially following the protocol described by Osmand et al. (17) and Hoffman et al. (18) This involved treatment with sodium borohydride, blocking with 0.5% Triton X-100, and overnight incubation in a solution of primary Ab at a predetermined optimal concentration, followed by exposure to biotinylated species-specific secondary Ab and enzymatic detection using a 1:500 dilution of reagents A and B from the ABC Elite reagent (Vector Laboratories) and Ni-DAB-glucose oxidase (19). Sections were mounted and coverslipped without the use of counterstains.

FIGURE 1. miR-155KO mice are highly susceptible to encephalitis after HSV-1 infection and have elevated viral titers in the brain but no difference in the cornea. Groups of WT and miR-155KO animals were ocularly infected with 1 × 10⁶ HSV-1 Tumpey. (A) Survival of age-matched WT and miR-155KO was established over 9 d. (B) Brains were harvested from WT and miR-155KO mice at day 9 p.i. Brains were homogenized and centrifuged, and supernatants were tested for virus titers. (C) The presence of virus in the cornea was measured at day 6 p.i. by swabbing the HSV-infected eye with a sterile swab and assaying for the virus by plaque assay. The level of significance was determined by a Student t test (unpaired). Error bars represent means ± SEM (n = 5–8 mice/group). Experiments were repeated at least three times. *** p < 0.001.
Abs and reagents. Allophycocyanin-conjugated anti-mouse CD8α (53-6.7), FITC-conjugated anti-mouse TNF-α, allophycocyanin-conjugated anti-mouse IFN-γ, FITC-conjugated anti-mouse CD49d, FITC-conjugated anti-mouse CD44, and Golgi transport inhibitor (brefeldin A) were purchased from BD Biosciences. Allophycocyanin-conjugated and PE-conjugated H-2Kb/gB498–505 (S5IFEARL) tetramers were provided by the National Institutes of Health Tetramer Core Facility (Emory University, Atlanta, GA). Recombinant mouse Gal-9 was provided by GalPharma, Japan. CD8 T cell isolation kit was obtained from Miltenyi Biotec. Primary Abs and reagents for immunohistochemistry were purchased from BD Bioscience and DAKO, respectively. The secondary Abs Donkey Anti-Rat IgG (H+L) and Donkey Anti Rabbit IgG (H+L) were purchased from Jackson ImmunoResearch.

Preparation of trigeminal ganglia single-cell suspensions. At 14 d after HSV-1 RE ocular infection, mice were anesthetized and euthanized by exsanguinations (20). Trigeminal ganglia (TG) were excised and subjected to collagenase treatment (Sigma-Aldrich, St. Louis, MO) at a concentration of 3 mg/ml for 90 min at 37˚C. After incubation, the TGs were dispersed into single cells by trituration. Each single-cell suspension was then plated in 48-well tissue culture plates. The cells were cultured in DMEM with 10% FCS and 10 U/ml recombinant murine IL-2 (R&D Systems) as described previously (20).

Ex vivo reactivation experiments. Each TG sample isolated from miR-155KO mice was divided into two aliquots. One aliquot was left unmanipulated, whereas the other aliquot received 1 × 10^6 CD8 T cells isolated at day 8 p.i. from lymph nodes of HSV-1–infected WT mice. Similarly, each WT TG was divided into two aliquots and one aliquot was left unmanipulated, whereas the other aliquot received 1 μM recombinant Galectin-9 (Gal-9), a procedure shown in a previous report to block CD8 T cell infiltration (21). TG cultures were incubated in DMEM in a 5% CO₂, humidified incubator at 37˚C for a 10-d period, and culture supernatant samples were collected at 24-h intervals and assayed for infectious virus by plaque titrations on Vero cells. Gal-9 (1 μM) and IL-2 (10 U/ml) concentrations were constantly maintained throughout the culture period.

Flow cytometry
Single-cell suspensions isolated from draining cervical lymph nodes, and TG samples of mice ocularly infected with HSV-1 were collected at different time points. Foot infection also was used in separate experiments; PLNs were isolated and made into single-cell suspensions after HSV-1 footpad infection. Aliquots of the earlier single-cell suspensions were stained for CD8 and Kb-Gal tetramer cell-surface markers. To enumerate the functionality of CD8 T cell, we performed intracellular staining with freshly isolated draining lymph node (DLN), PLN, or TG suspensions from WT and miR-155KO mice. The cells were cultured in U-bottom 96-well plates and left untreated or stimulated with gB498–505 (S5IFEARL) peptides (1 μg/ml) and incubated for 6 h at 37˚C in 5% CO₂. Brefeldin A (5 μg/ml) was added for the duration of the culture period to facilitate intracellular cytokine accumulation. After this period, cell-surface staining was performed, followed by intracellular cytokine staining using a Cytofix/Cytoperm kit (BD Pharmingen) to enumerate the number of IFN-γ- and TNF-α–producing CD8 T cells as previously described (22). Finally, the cells were washed three times and resuspended in 1% paraformaldehyde. The stained samples were acquired with a FACSCalibur (BD Biosciences), and the data were analyzed using the FlowJo software.

Viral plaque assay
Virus titers were measured in the brain, TG, and skin of HSV-infected mice as described previously by others (9, 21, 23). In addition, mouse corneas were swabbed with sterile swabs (Fisher HealthCare, USA) at 6 d after ocular infection. Virus titers in all samples were measured using standard plaque assay as described previously (24).

Statistics
Mortality data were analyzed by log-rank testing (taking into account both time of death and final mortality). The statistical significance between two groups was determined using unpaired two-tailed Student t test. One-way ANOVA with Bonferroni’s post hoc test was used to calculate the level of significance for some experiments; **p ≤ 0.001, *p ≤ 0.01, and *p ≤ 0.05 were considered significant, and results are expressed as mean ± SEM. For all statistical analysis, GraphPad Prism software was used.

Results

Differential susceptibility of miR-155KO and WT mice to ocular infection with HSV
Upon ocular infection with HSV, mice experience development of a T cell–orchestrated immunoinflammatory lesion in the cornea (stromal keratitis [SK]), and susceptible strains may succumb to encephalitis (25, 26). The latter outcome has also been advocated to represent an immunoinflammatory reaction to virus replication (8, 9). Because miR-155KO animals express higher resistance than WT animals to the induction of some immunoinflammatory diseases (12, 13), we anticipated that miR-155KO animals would be more refractory than WT animals to both SK and HSE. We did observe significantly heightened resistance to SK (these data will be documented in a separate manuscript), but unexpectedly, miR-155KO animals were markedly more susceptible to HSE than were the WT animals. Thus, under infectious conditions with a strain of HSV-1 virus that failed to cause detectable illness or symptoms of encephalitis in WT animals, 75–80% (in three separate experiments) of miR-155KO animals experienced encephalitis, and most had to be terminated by 9 d p.i. (Fig. 1A). By 6 d p.i., affected animals became lethargic, lost weight, and showed ruffled fur, hunched appearance, and signs of incoordination. To cause encephalitis with the same virus strain in WT required a virus dose that was 1000 times greater, and then <20% experienced development of encephalitis. Brains were collected from encephalitic miR-155KO animals, both to investigate pathological changes...
and to quantify levels of virus present. High virus levels of HSV were detectable in brain homogenates in all, showing signs of encephalitis by day 9 p.i., although none had detectable virus in ocular swabs at day 6 p.i. (Fig. 1B, 1C). Virus could not be detected in the brains at day 9 p.i. or in the ocular tissue at day 6 p.i. in the WT animals when infected at the low virus dose that caused encephalitis in the miR-155KO animals (Fig. 1C).

Brain sections from miR-155KO and WT animals examined 8 d.p.i. and showing signs of encephalitis revealed differences in the nature of pathological changes. Thus, the density of CD8 T cell infiltration in the posterior temporal lobe was notably more abundant in the WT animals than in the miR-155KO animals (Fig. 2A). There was also marked differences in the extent of astrocytosis indicative of inflammatory reactions to infection with the response more abundant in WT animals (Fig. 2B).

The earlier observations are consistent with the viewpoint that the CNS damage in the miR-155KO animals was likely the consequence of the direct effects of virus infection rather than an immunopathological response to infection. Further support for this notion also came from experiments that showed that ocularly infected miR-155KO animals could be protected from development of encephalitis if treated with acyclovir starting at 4 d p.i. (Fig. 3A, 3B). Moreover, animals killed 5 d after treatment expressed minimal levels of virus in brain extracts compared with untreated animals (Fig. 3C). In separate experiments, we could recover infectious virus from the brains of both miR-155KO and WT mice 1 d before acyclovir treatment. However, higher viral titers were evident at day 4 p.i. in the miR-155KO animals (Fig. 3D).

Our results are consistent with the notion that miR-155KO animals succumb to encephalitis with lesions in the brains likely the direct consequence of viral infection rather than representing the result of an inflammation reaction to infection, as some advocate accounts for encephalitis in WT mice (9).

miR-155 is required for optimal CD8 T cell responses

To investigate whether miR-155 influences the nature of HSV-1–specific CD8 T cell responses, we infected miR-155KO and WT mice intradermally in the hind footpads with HSV-1 strain KOS, and effector CD8 T cell responses were measured in the draining PLNs at day 5 p.i. when responses are at their peak (27, 28). The results show that the total numbers of HSV gB tetramer-specific CD8 T cells per lymph node were significantly reduced (~3-fold) in miR-155KO mice compared with WT control animals (Fig. 4A). We also investigated the homing capacity of CD8 T cells in the miR-155KO animals. Analyzing expression of the homing molecules VLA-4 and CD44, we found ~1.5- to 3-fold reduced expression in the infected miR-155KO animals compared with the WT animals (Fig. 4B, 4C). In addition, no differences were evident in the expression of the homing molecule LFA-1 between the infected WT and miR-155KO animals (data not shown). When cell numbers were compared using the intracellular cytokine staining (ICS) assay to detect virus-specific IFN-γ–producing cells, differences between miR-155KO and WT responses were of even greater magnitude (average of 5-fold; Fig. 4D). As an additional measure of functional responses, numbers of CD8 T cells that produced both IFN-γ and TNF-α or a single cytokine alone were compared in the two groups. This approach revealed that dual cytokine-producing CD8 T cells were reduced ~10-fold in miR-155KO compared with WT (Fig. 4E), a result we take to indicate that the CD8 virus-specific response in miR-155KO mice was functionally impaired. In additional experiments, this trend was also seen in the DLNs at day 9 after ocular infection with the HSV-1 strain that caused encephalitis in miR-155KO mice (data not shown).

In separate experiments, WT and miR-155KO mice were infected with another strain of HSV-1 (HSV-1 RE) that did not cause HSE in miR-155KO mice. In such experiments, TGs were collected 14 d.p.i. and processed either for viral reactivation experiments (described in a subsequent section) or to recover T cells to measure virus-specific CD8 T cell responses by using both tetra-
mer and the ICS assay to quantify cytokine producers. The total numbers of gB tetramer-specific CD8 T cells were ∼2-fold higher in WT compared with miR-155KO mice (Fig. 5A). The number of total CD8 T cells that produced IFN-γ in the WT group was ∼4-fold higher compared with miR-155KO animals. In addition, the dual cytokine (IFN-γ and TNF-α)-producing cells were ∼4.5-fold more frequent in WT mice as compared with miR-155KO mice (Fig. 5B, 5C).

Taken together, the earlier data demonstrate that the absence of miR-155 results in diminished CD8 T cell response, which is particularly evident when using assays that measure numbers of functional CD8 T cells.

HSV-immune CD8+ T cells from gBT mice protect miR-155KO animals from lethal herpetic encephalitis

To determine whether the reduced number and function of CD8 T cells is one of the reasons for HSE, we carried out adoptive transfer experiments. Infected miR-155KO mice were given HSV-immune CD8+ T cell transfers from gBT mice at 24 h p.i. and recipients were monitored clinically over the next 9 d. Eighty percent of the miR-155KO mice succumbed to death by day 9 p.i.; however, 100% of the miR-155KO mice that received HSV-immune CD8 T cells at 24 h.p.i. survived (Fig. 6A). Animals were subsequently sacrificed at day 9 p.i., and brains were collected to quantify levels of virus present. High virus levels were detectable in the brain homogenates in all miR-155KO animals showing signs of encephalitis by day 9 p.i., although none had detectable virus in the group of animals that received CD8 T cell adoptive transfers (Fig. 6B).

Virus reactivation differences between latently infected miR-155KO and WT mice

In additional experiments, WT and miR-155KO mice were infected with a strain of HSV-1 (HSV 1RE) that did not cause HSE in miR-155KO mice. In such experiments, TGs were collected 14 d.p.i., and aliquots were exposed to different treatments. The culture supernatants were tested daily to detect infectious virus over a 10-d period. Unmanipulated cultures revealed differences in the viral reactivation pattern between miR-155KO and WT TG. Whereas ∼15% of WT cultures showed reactivation, ∼90% of the miR-155KO cultures reactivated (Fig. 7). Infectious virus was detectable in the miR-155KO culture supernatants by day 2 after culture,
but not until day 3 in the WT cultures that reactivated. Although the majority of WT cultures did not reactivate, all were judged to be latently infected because the addition of 1 mM rGal-9 (a procedure shown previously to cause ex vivo reactivation [21]) caused virus reactivation in all cultures (Fig. 7).

With the miR-155KO cultures, CD8 T cells isolated from the lymph nodes of WT HSV-infected mice were added to culture aliquots to determine the effect on virus reactivation. This procedure prevented virus reactivation in all cultures (Fig. 7). Accordingly, our results demonstrate that viral reactivation from latency occurs far more readily with cultures from miR-155KO animals than WT, and this observation might be attributed, at least in part, to differences in CD8 T cell function.

**Differential susceptibility of miR-155KO and WT mice to intradermal infection with HSV**

Animals infected in the scarified skin with HSV experienced development of so-called zosteriform skin lesions, which as first demonstrated by Simmons and Nash (16), reflect the consequence of viral entrance into sensory nerve endings followed by viral replication in the dorsal root ganglia and subsequent spread to the dermatome. When groups of WT and miR-155KO were infected intradermally with identical viral dosage of HSV, the outcome was significantly different in the development of zosteriform lesions. Thus, a greater proportion of miR-155KO mice developed lesions compared with WT mice. By day 6 p.i., 100% of the miR-155KO mice had developed lesions compared with only 25% in the WT mice. In addition, miR-155KO mice exhibited lesions that were far larger than in those in WT that developed lesions (Fig. 8A). In addition, whereas by day 7 p.i. the majority of the miR-155KO mice developed hind-limb paralysis, all of the WT mice remained free from any neurologic signs (Fig. 8B). In some experiments, test mice were terminated at day 6 p.i., and virus levels were assayed in the skin encompassing the inoculation site, as well as in the brain. In such experiments, it was only possible to detect virus in the brains and skin isolated from miR-155KO animals (Fig. 8C, 8D). Thus, our results demonstrate a marked increase in susceptibility of miR-155KO to HSV infection in a model that reflects spread within the nervous system.

**Discussion**

HSV infection usually causes lesions at body surface sites, but occasionally the virus spreads to the brain, inducing life-threatening encephalitis (2). We show in this report that mice unable to pro-
duce miR-155 may develop HSE post ocular infection with the lesion primarily the direct consequence of virus replication in the CNS. Affected animals could be protected from HSE by acyclovir treatment commenced 4 d p.i., and pathological features in the CNS were consistent with direct viral destructive effects. miR-155KO animals were also more susceptible to development of zosteriform lesions, a reflection of viral replication and dissemination within the nervous system. One explanation for the heightened susceptibility to HSE and zosteriform lesions could be because miR-155KO animals develop diminished CD8 T cell responses especially when the numbers of functional effector CD8 T cell responses were compared. Indeed, adoptive transfer of HSV-immune CD8+ T cells into infected miR-155KO mice provided protection from HSE. Deficiencies in CD8 T cell numbers, function, and homing capacity may also explain the observation that miR-155KO animals were less able than WT animals to maintain latency upon ex vivo culture. To our knowledge, our observations may be the first to link miR-155 expression with susceptibility of the nervous system to virus infection.

HSE is a rare manifestation of HSV infection and can be a devastating disease, especially if not treated promptly (2). Most cases in adult humans are caused by HSV-1, and these usually occur in latently infected persons whose previous clinical consequences of infection were either not observed or were only mild surface lesions. Little is understood regarding the triggers that cause reactivated virus to traffic to the brain or the pathogenic mechanisms involved at causing the brain damage. Occasional cases of human HSE can occur in children with genetic defects in TLR3-dependent IFN responses (3–5), but in the great majority of HSE cases, genetic defects in immune function have not been demonstrated (2). Moreover, even profound immunosuppression, as can occur during AIDS or immunosuppressive therapy, rarely results in HSE. In HSE in humans, encephalitis appears to be largely the consequence of virus replicating in and destroying cells, an idea supported by the success that can be achieved using antiviral drug therapy (2). However, others advocate that an inflammatory reaction to the brain infection can also contribute or perhaps be mainly responsible for the encephalitis (9). Enthusiasm for the later idea has mainly come from experimental studies in mice where innate immune signaling-dependent activation of polymorphonuclear leukocytes and macrophages, and the production of inflammatory mediators in response to HSV were shown necessary for the development of fulminate lesions of encephalitis (7, 8). Other studies indicate that encephalitis in susceptible mouse strains may represent an immunopathological response because it fails to respond to antiviral therapy but is controllable by procedures that diminish inflammatory cells (9). More than likely, the pathogenesis of HSE involves multiple mechanisms with studies in mice not accurately reflecting the pathogenesis of the natural

**FIGURE 6.** Survival in miR-155KO mice after receiving HSV immune CD8 T cells. miR-155KO animals were ocularly infected with 1 x 10^4 HSV-1 Tumpey and were divided in two groups. One group (n = 8) received adoptive transfer of HSV immune CD8+ T cells via tail vein at 24 h p.i. (A), whereas the other group served as a control. Survival of age-matched miR-155KO mice and miR-155KO animals that received CD8 T cell adoptive transfer was established over 9 d. (B) Brains were harvested from miR-155KO animals and miR-155KO mice that received CD8 T cell adoptive transfer mice at day 9 p.i.Brains were homogenized and centrifuged, and supernatants were tested for virus levels. *p ≤ 0.05, **p ≤ 0.005.

**FIGURE 7.** Comparison of ex vivo HSV-1 reactivation between miR-155KO and WT mice. Individual TGs excised on day 14 p.i. from HSV-1 (RE)-infected WT and miR-155KO mice were dispersed into single-cell suspensions, and the cultures were established in a 48-well plate. Each TG (n = 6) sample from miR1-55KO mice was divided into two aliquots. One aliquot was left unmanipulated and the other aliquot received 1 x 10^5 CD8 T cells isolated at day 8 p.i. from lymph nodes of HSV-1-infected WT mice. Similarly, each WT TG was divided into two aliquots, and one aliquot was left unmanipulated, whereas the other aliquot received 1 mM rGal-9. TG cultures were incubated in DMEM medium for a 10-d period, and samples of culture supernatant were collected at 24-h intervals and assayed for infectious virus by plaque titrations on Vero cells. Bar graph represents the percentage of virus reactivation from various experimental combinations. The experiment has been repeated three times.
human disease. We advocate, however, that the miR-155KO mice could represent a more appropriate model than other mouse systems to understand the pathogenesis of human HSE and to evaluate novel therapies. Accordingly, the encephalitis in miR-155KO animals appeared to represent primarily the consequences of viral replication events. Thus, the disease was readily controllable with antiviral therapy even when this was begun 4 d p.i., a time point when HSV was readily detectable in the brains of miR-155KO animals and presumably could be inducing an inflammatory response. Immunohistochemical analysis of brain lesions of miR-155KO animals revealed lesser T cell inflammatory infiltrates in affected areas along with less reactive astrocytosis as compared with WT animals with encephalitis. We interpret this to mean that the nature of lesions in miR-155KO animals is less immunoinflammatory than those in the WT animals.

We suspect that one reason miR-155KO animals readily developed HSE was because of their reduced virus-specific T cell responses to infection. Another might relate to the role of miR-155 could play in susceptibility of neural tissue to HSV infection. According to our previous reports showing that CD8-deficient animals failed to control HSV in the brain and developed encephalitis (30). This argument was also supported by the adoptive transfer experiments where HSV immune CD8 T cells adoptively transferred to miR-155KO mice were shown to be fully protective. However, further experiments are needed to clarify whether the apparent defect in miR-155KO CD8 T cells is a problem with priming, effector cytokine production, homing defects, or additional events such as the numbers of cells that can access the nervous system. Furthermore, although we favor the idea that differences in CD8 T cell activity accounted for the difference in outcome in miR-155KO and WT mice, other explanations merit exploration such as differences in NK cell homeostasis or levels of IFN induced, which have both been implicated as providing protection in herpetic encephalitis (7, 38–40).

A diminished protective CD8 T cell response to reactivation. In support of this, we could show that miR-155KO CD8 T cells showed diminished levels of VLA-4 and CD44, both shown in other systems to influence brain homing of T cells (36, 37). We suspect that the diminished protective CD8 T cell response permitted virus to traffic effectively to the brain and PNS, and that once there, fewer protective CD8 T cells were around to abort infection. This is consistent with the previous reports showing that CD8-deficient animals failed to control HSV in the brain and developed encephalitis (30). This argument was also supported by the adoptive transfer experiments where HSV immune CD8 T cells adoptively transferred to miR-155KO mice were shown to be fully protective. However, further experiments are needed to clarify whether the apparent defect in miR-155KO CD8 T cells is a problem with priming, effector cytokine production, homing defects, or additional events such as the numbers of cells that can access the nervous system. Furthermore, although we favor the idea that differences in CD8 T cell activity accounted for the difference in outcome in miR-155KO and WT mice, other explanations merit exploration such as differences in NK cell homeostasis or levels of IFN induced, which have both been implicated as providing protection in herpetic encephalitis (7, 38–40).

A diminished protective CD8 T cell response in miR-155KO animals was also demonstrated using two models that reflect the activity of CD8 T cells. First, in a foot pad infection model, we could show that miR-155KO animals generated lesser numbers of HSV-specific CD8 T cells than WT animals in DLNs, which was especially evident when IFN-γ-producing cell responses were compared. CD8 T cells are required to contain HSV replication in ganglia, and they orchestrate this response largely by IFN-γ production, homing defects, or additional events such as the numbers of cells that can access the nervous system. Furthermore, although we favor the idea that differences in CD8 T cell activity accounted for the difference in outcome in miR-155KO and WT mice, other explanations merit exploration such as differences in NK cell homeostasis or levels of IFN induced, which have both been implicated as providing protection in herpetic encephalitis (7, 38–40).

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In addition to encephalitis, we also observed that miR-155KO mice were more susceptible than the WT animals to development of zosteriform lesions, an event that requires dissemination of virus within the nervous system (16). Accordingly, with doses of virus that produced barely noticeable lesions in WT, almost all miR-155KO animals developed overt lesions, and many had to be killed because of hind-limb paralysis. The miR-155KO animals failed to control HSV, and virus was easily detectable in the brains of miR-155KO animals but could not be demonstrated in the brains of WT animals.

Currently, it is not clear how miR-155 influences the magnitude and functionality of CD8 T cell responses, but there are several possibilities. First, it might result from the fact that miR-155KO mice also generate impaired Th cell responses (12, 13), and optimum CD8 T cell responses are known to require signals from CD4 Th cells (43, 44). It is also conceivable that miR-155 plays a direct role during CD8 T cell differentiation. Thus, some have observed that in the absence of miR-155, type 1 IFN-driven proliferative responses of CD8 T cell are defective (33, 34), whereas others suggest that CD8 T cells survive less well and show defective responses to PI3K/AKT signaling (34). It has also been suggested that in the absence of miR-155, SOCS1 is upregulated, which expresses suppressive effects on T cell function (32). Further studies are clearly needed to clarify how miR-155 expression influences the CD8 T cell response.

Our results also raise the issue whether miR-155 expression somehow influences the dissemination of HSV to and replication within the nervous system. Thus, miRNAs could influence expression of proteins involved in axon transport, but this point has not been investigated to our knowledge. Alternatively, miRNAs could influence the infectivity and replication efficiency in target cells within the nervous system. It is known, for example, that miR-155 regulates microglia immune responses by targeting SOCS-1 and promoting cytokine and NO production (45, 46). Therefore, it is conceivable that the glial cells in miR-155KO mice could be defective in cytokine and NO production, a possibility we are currently investigating. We are also investigating whether different cell types taken from miR-155KO and WT mice show differential susceptibility to HSV replication events.

In conclusion, our report makes the novel observation that miR-155 expression influences the dissemination of HSV to and replication within the nervous system. Thus, miRNAs could influence expression of proteins involved in axon transport, but this point has not been investigated to our knowledge. Alternatively, miRNAs could influence the infectivity and replication efficiency in target cells within the nervous system. It is known, for example, that miR-155 regulates microglia immune responses by targeting SOCS-1 and promoting cytokine and NO production (45, 46). Therefore, it is conceivable that the glial cells in miR-155KO mice could be defective in cytokine and NO production, a possibility we are currently investigating. We are also investigating whether different cell types taken from miR-155KO and WT mice show differential susceptibility to HSV replication events.

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Disclosures

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