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IFN-γ-producing Th1 and IL-17–producing Th17 cells are the key participants in various autoimmune diseases, including multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE). Although both of these T cell subsets are known to be regulated by specific transcription factors and cytokines, the role of microRNAs that control these two inflammatory T cell subsets and whether targeting microRNAs can have therapeutic effects are not known. In this study, we show that microRNA-155 (Mir-155) expression is elevated in CD4+ T cells during EAE, and Mir-155−/− mice had a delayed course and reduced severity of disease and less inflammation in the CNS. The attenuation of EAE in Mir-155−/− mice was associated with a decrease in Th1 and Th17 responses in the CNS and peripheral lymphoid organs. The T cell-intrinsic function of Mir-155−/− was demonstrated by the resistance of Mir-155−/− CD4+ T cell-repleted Rag-1−/− mice to EAE. Finally, we found that anti-Mir-155 treatment reduced clinical severity of EAE when given before and after the appearance of clinical symptoms. These findings demonstrate that Mir-155 confers susceptibility to EAE by affecting inflammatory T cell responses and identify Mir-155 as a new target for therapeutic intervention in multiple sclerosis. The Journal of Immunology, 2011, 187: 2213–2221.

Center for Neurologic Diseases, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115

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Address correspondence and reprint requests to Dr. Howard L. Weiner, Brigham and Women’s Hospital, 77 Avenue Louis Pasteur, HIM 730, Boston, MA 02115-5817. E-mail address: hweiner@rics.bwh.harvard.edu

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Abbreviations used in this article: DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; LN, lymph node; LNA, locked nucleotide acid; Mir-155, microRNA-155; miRNA, microRNA; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; ROR, retinoid orphan nuclear receptor; SOCS1, suppressor of cytokine signaling 1; Treg, T regulatory; WT, wild-type.

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Materials and Methods

Mice

C57BL/6 wild-type (WT), miRNA-155−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in a specific pathogen-free condition in the animal facility of Harvard Institutes of Medicine. All mice were 6–8 wk old at the beginning of experiments. All experiments were in accordance with guidelines from the committee on animals at Harvard Medical School.

Induction and evaluation of EAE

Mice were injected s.c. in both flanks with 100 μg myelin oligodendrocyte glycoprotein (MOG)35–55 peptide (MEGVWYRSPSRVVHLYRNGK) dissolved in PBS emulsified in an equal volume CFA-CFA (Difco)
supplemented with 5 ng/ml Mycobacterium tuberculosis H37Ra and injected twice i.p. with 200 µg pertussis toxin (List Biological Laboratories) administered on the day of immunization and 48 h later. Clinical assessment of EAE was performed daily after disease induction, according to the following criteria: 0, no disease; 1, tail paralysis; 2, hindlimb weakness or partial paralysis; 3, complete hindlimb paralysis; 4, forelimb and hindlimb paralysis; 5, moribund state. Mean clinical scores on separate days were calculated by adding scores of individual mice and dividing total number of mice in each group, including mice that did not develop signs of EAE. For histopathological studies, spinal cords were dissected from female mice (n = 5/group), fixed in 10% formalin in PBS, and embedded in a single paraffin block. The 6- to 10-µm-thick sections were stained with H&E and luxol fast blue, and stained sections were evaluated for immune cell infiltration and demyelination.

Generation and isolation of dendritic cells

Dendritic cells (DCs) were derived from bone marrow progenitor cells using a previously described method (16). In brief, the femoral and tibial cells were harvested in DC culture medium (RPMI 1640 medium, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 ng/ml GM-CSF, and 10 ng/ml IL-4) and seeded in 24-well plates at a density of 1 × 10⁶ cells/ml/well. Culture medium was replaced with fresh medium every 3 d. At 6 d, dislodged cells were used as bone marrow-derived DCs. Splenic DCs were isolated using CD11c beads (Miltenyi Biotec).

Analysis of Mir-155 expression

For analysis of Mir-155 expression, real-time RT-PCR analyses were carried out using TaqMan miRNA assays (Applied Biosystems) and relative expression was calculated using the Ct method, as described, and normalized to uniformly expressed snRNA135 (Applied Biosystems). Mir-155 values were expressed relative to the expression of snRNA135. The levels of Mir-155 in CNS-infiltrating DCs during EAE are normalized to DCs isolated from spleen of naive mice.

RNA isolation, cDNA synthesis, and real-time PCR

Total RNA was isolated from cell pellets using RNAeasy mini kit (Qiagen). RNA was stored at −80°C. First-strand cDNA synthesis was performed for each RNA sample from 0.5-1 µg total RNA using TaqMan reverse-transcription reagents. cDNA was amplified using sequence-specific primers (probes used were identified by Applied Biosystems assay identification number: IFN-γ, Mm01181314_m1; T-bet, Mm00450960_m1; IL-17, Mm09999062_m1; ROR-γt, Mm01261019_m1; IL-17F, Mm00521423_m1; Foxp3, Mm00475151_m1; IL-4, Mm00452599_m1; IL-23, Mm00195942_m1; IL-21, Mm00512011_m1; IL-6, Mm00999006_m1; IL-α, Mm09999064_m1; IL-β, Mm01336189_m1; TNF-α, Mm00443258_m1 [Applied Biosystems]) and real-time PCR mix (Applied Biosystems) on ABI7500 cycler. GAPDH gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. All values were expressed relative to the expression of GAPDH.

Anti-mir-155 treatment

An antisense oligonucleotide modified by locked nucleic acid (LNA: Exiqon) was synthesized to inhibit Mir-155. For in vivo anti-mir-155 treatment, 30 µl lipofectamine 2000 (Invitrogen) was mixed with anti-mir-155 (20 µg/mouse) dissolved in 170 µl lipofectamine 2000 (Invitrogen) and injected into the tail vein of mice. Compared with WT mice, Mir-155−/− mice showed delayed onset of neurologic impairment and markedly less severe disease (Fig. 1B). The attenuation of paralytic symptoms in the Mir-155−/− mice was associated with decreased inflammation and demyelination ((Fig. 1C, 1D). Although we found that EAE was associated with demyelination and infiltration of CD4⁺ T cells and CD11c⁺ cells from peripheral lymphoid organs to the CNS in both Mir-155−/− and WT mice, the number of CD4⁺ T cells and CD11c⁺ cells was markedly lower in the CNS of Mir-155−/− mice at the peak of disease (Fig. 1E). Thus, Mir-155 significantly influenced the course of progressive EAE induced by MOG35-55 peptide.

Results

Mir-155−/− mice develop attenuated EAE

To study the role of Mir-155 in EAE development, we first sought to analyze the expression of Mir-155 in mice during the course of EAE. We found that Mir-155 expression was increased in spleen, LN, and CNS during EAE (Supplemental Fig. 1). Because CD4⁺ T cells are the key mediators of pathogenesis in EAE, we investigated whether CD4⁺ T cells express Mir-155 during EAE. Mir-155 expression was analyzed in CD4⁺ T cells (spleen, LN, and CNS) from C57BL/6 mice immunized for EAE with MOG35-55 peptide. We found that Mir-155 expression was markedly increased in CD4⁺ T cells isolated in spleen, LN, and CNS of EAE mice compared with naive mice (Fig. 1A). The potential role of Mir-155 in EAE was next tested using Mir-155−/− mice (Mir-155−/− mice). Compared with WT mice, Mir-155−/− mice showed delayed onset of neurologic impairment and markedly less severe disease (Fig. 1B). The attenuation of paralytic symptoms in the Mir-155−/− mice was associated with decreased inflammation and demyelination ((Fig. 1C, 1D). Although we found that EAE was associated with demyelination and infiltration of CD4⁺ T cells and CD11c⁺ cells from peripheral lymphoid organs to the CNS in both Mir-155−/− and WT mice, the number of CD4⁺ T cells and CD11c⁺ cells was markedly lower in the CNS of Mir-155−/− mice at the peak of disease (Fig. 1E). Thus, Mir-155 significantly influenced the course of progressive EAE induced by MOG 35-55.

Mir-155 favors T cell-mediated autoimmunity

The candidate contributors to EAE pathology are IFN-γ–secreting Th1 and IL-17–secreting Th17 CD4⁺ T cells. To explore the impact of Mir-155 on the development of pathogenic Th cell subsets, we isolated spleens from WT and Mir-155−/− mice at 10 d after immunization and examined the recall proliferation and cytokine production in response to MOG p35-55 stimulation. We found that splenocytes from WT mice proliferated more robustly and produced higher levels of IFN-γ and IL-17 than splenocytes from Mir-155−/− mice (Fig. 2A–C). Consistent with the clinical scores, ex vivo CD4⁺ T cells isolated from spleen and LN of WT mice expressed higher levels of IFN-γ and IL-17 in comparison with
CD4⁺ T cells from Mir-155⁻/⁻ mice (Fig. 2D, 2E). We then tested ex vivo CD4⁺ T cells isolated from LN and splenocytes from WT and Mir-155⁻/⁻ mice for the presence of IL-17 and IFN-γ by intracellular staining. Consistent with the RNA data, Mir-155⁻/⁻ mice had reduced numbers of Th17 cells in both their LN and spleens compared with WT mice (Fig. 2D, 2E). In addition, we found moderately reduced amounts of Th1 cells in the spleens, but not LN of Mir-155⁻/⁻ mice (Fig. 2D, 2E). Because Th1 and Th17 cells can initiate and perpetuate the CNS inflammatory response in EAE, we next examined the cytokine expression by CNS-infiltrating CD4⁺ T cells during the course of the disease. We found an increased expression of IFN-γ and IL-17 in CD4⁺ T cells isolated from WT mice compared with Mir-155⁻/⁻ mice (Fig. 2F). Both Th1 and Th17 cells have been shown to develop under strict influence by Th2 and Treg cells, respectively (10). Mir-155 has been shown to regulate both Th2 and Foxp3 expressing Treg cells. Reduced numbers of Treg cells and enhanced Th2 differentiation were observed from Mir-155⁻/⁻ mice (13–15). However, Mir-155⁻/⁻ Treg cells are equally suppressive as WT Treg cells on a per cell basis (15). Consistent with previous studies, we also observed reduced Foxp3 expression in CD4⁺ T cells isolated from Mir-155⁻/⁻ mice with EAE in comparison with WT controls (Supplemental Fig. 2A). However, the Th2 cytokine IL-4 expression was not altered between WT and Mir-155⁻/⁻ mice with EAE (Supplemental Fig. 2B). Thus, the observed difference in CNS inflammatory response was not a reflection of skewing toward a Treg phenotype, but rather due to an increased expression of IFN-γ and IL-17 in CNS-infiltrating CD4⁺ T cells of WT mice.

**Mir-155 regulates CD4⁺ T cell effector cytokine production**

To determine whether the enhanced T cell autoimmunity in WT mice was a result of intrinsic differences in CD4⁺ T cells, we compared the cytokine profiles of WT and Mir-155⁻/⁻ total CD4⁺ T cells in response to ex vivo stimulation with anti-CD3 and anti-CD28. We found higher production of IFN-γ (Fig. 3A, 3C) and IL-17 (Fig. 3B) by WT CD4⁺ T cells than Mir-155⁻/⁻ CD4⁺ T cells. These results indicate that the enhanced cytokine production of IFN-γ and IL-17 that we observed in WT mice was in part a result of intrinsic differences in CD4⁺ T cells. To further determine the role of Mir-155 on Th1 and Th17 differentiation, WT and Mir-155⁻/⁻ naive CD4⁺ T cells were differentiated into Th1 and Th17 cells. When activated under Th1-polarizing condition, Mir-155⁻/⁻ naive CD4⁺ T cells produced equal amounts of IFN-γ and expressed normal levels of T-bet comparable to CD4⁺ T cells from WT mice (Fig. 3D). We then tested whether there is a defect in IFN-γ production in the memory CD4⁺ T cell compartment that accounted for the reduced IFN-γ levels observed in total CD4⁺ T cells from Mir-155⁻/⁻ mice. For this we sorted memory T cells (CD4⁺CD44highCD62Llow) from WT and Mir-155⁻/⁻ mice and stimulated with anti-CD3 and CD28 Abs. We did not find significant differences in IFN-γ production in memory T cells between WT and Mir-155⁻/⁻ mice (Fig. 3E). However, we did find reduced number of memory CD4⁺ T cells in the spleens of age-matched Mir-155⁻/⁻ mice compared with WT mice (Fig. 3F). Thus, the increased IFN-γ levels that we observed in total CD4⁺ T cells from WT mice are due to more memory CD4⁺ T cells. When naive CD4⁺ T cells are activated by the combination of IL-6 and TGF-β (Th17-polarizing conditions), IL-17 production in
Mir-155−/− T cells was significantly reduced compared with WT-T cells, even when adding IL-23, IL-1β, and TNF-α (Fig. 3G, 3H). In addition, WT-T cells expressed higher levels of Th17-associated molecules such as IL-17F, IL-22, and IL-23R than that of Mir-155−/−-derived Th17 cells (Supplemental Fig. 3A). However, expression of RORγt and IL-21, two other Th17-associated molecules, was not altered between these two groups (Supplemental Fig. 3B), suggesting that Th17 cells are transcriptionally programmed in Mir-155−/− animals even though they do not produce similar levels of effector cytokines. To demonstrate the CD4+ T cell-intrinsic function of Mir-155 in vivo, we transferred WT or Mir-155−/− CD4+ T cells into Rag-1−/− mice and subsequently immunized them with MOG peptide. We found that the T cells both from WT and Mir-155−/− mice are engrafted equally into Rag-1−/− mice and are recovered at normal rates (Fig. 4A). The mice repleted with WT CD4+ T cells exhibited higher IFN-γ and IL-17 production as compared with those reconstituted with Mir-155−/− CD4+ T cells (Fig. 4D). Reduced IL-17 and IFN-γ mRNA expression in the CNS of mice with Mir-155−/− T cells supports the regulatory role of Mir-155 on inflammatory T cell development (Fig. 4E). Moreover, Rag1−/− mice reconstituted with Mir-155−/− CD4+ T cells had reduced numbers of IL-17 single-positive and IL-17+ IFN-γ+ double-positive cells in their brains compared with WT mice (Fig. 4F).

Collectively, these results indicate that Mir-155 deficiency in the CD4+ T cell compartment leads to defective inflammatory T cell development and CNS inflammation during EAE.

*FIGURE 2.* Mir-155−/− mice exhibit lower Th1 and Th17 cytokine production during EAE. Splenocytes from WT and Mir-155−/− mice were harvested 10 d after immunization and were stimulated ex vivo with MOG peptide 35–55. A, In the last 16 h, cells were pulsed with thymidine and assayed for proliferation (cpm). Error bars represent SEM between triplicates. Supernatants from parallel cultures were harvested 72 h after initiation of cultures and assayed by ELISA for IFN-γ (B) and IL-17 (C). Real-time RT-PCR analysis and intracellular staining of IFN-γ and IL-17 in (D) spleen-, (E) LN-, and (F) CNS-derived CD4+ T cells isolated from WT and Mir-155−/− mice with EAE. Data in A–F are representative of three experiments.

Mir-155 positively regulates Th1- and Th17-polarizing cytokine expression in DCs

Polarization of T cells to Th1, Th2, or Th17 phenotypes is a critical feature of cell-mediated immunity, and is influenced by production of cytokines by DC. For example, DC-secreted IL-12 has been shown to favor Th1 development, whereas DC-secreted IL-10 or IL-27 has been shown to induce IL-10-producing Tr1 cells (17, 18). Likewise, DC-secreted IL-1β and IL-6 appear to synergize with IL-23 in the induction of IL-17 by CD4+ T cells (19). To
further understand the nature of defective Th1 and Th17 immune responses in vivo, we explored the possibility of an intrinsic requirement for Mir-155 in DC function. Therefore, we tested whether Mir-155 modulates the expression of Th1- and Th17-polarizing cytokine by DCs. For this, we stimulated bone marrow-derived DCs from WT and Mir-155−/− mice with the TLR ligand LPS and analyzed cytokine expression. In agreement with previous study in macrophages (20), LPS stimulation induced Mir-155 expression in DCs (Fig. 5A). Although LPS stimulation induced the expression of Th1-polarizing cytokine IL-12 and Th17-polarizing cytokines IL-1β, IL-6, IL-23, and TNF-α both from WT and Mir-155−/− DCs, significant differences were observed between DCs from Mir-155−/−...
mice compared with WT mice. In Mir-155−/− DCs, decreased levels of both Th1- and Th17-polarizing cytokines were observed in response to LPS stimulation (Fig. 5B, 5C), although we did not observe a difference in Th2-polarizing cytokine. Furthermore, Mir-155 deficiency did not alter DC development in vitro, as we do not see any difference in DC numbers from WT and Mir-155−/− mice-derived BM-DC culture (Supplemental Fig. 3C). Similarly, reduced expression of both Th1- and Th17-polarizing cytokines was observed from macrophages in response to LPS stimulation (Supplemental Fig. 3D, 3E). Indeed, in a DC–T cell coculture system, Mir-155 deficiency in DCs resulted in significantly less IFN-γ and IL-17 in CNS-derived CD4+ T cells isolated from Rag-1−/− mice reconstituted with T cells from WT and Mir-155−/− mice.

Anti–Mir-155 treatment resulted in inhibition of EAE and reduction of CNS inflammation

We then investigated whether systemic administration of anti–Mir-155 in vivo affects the course of EAE. Therapeutic efficacy of LNA-modified oligonucleotide (LNA–anti-miR) technology has recently been reported (21, 22). Administration of LNA-modified anti–Mir-155 during the preclinical stage of EAE (beginning on day 5 after immunization) significantly ameliorated clinical disease (Fig. 6A). In addition, we found that peripheral administration of anti–Mir-155 during EAE affected both CD4+ T cells and APCs, including DCs and macrophages (Supplemental Fig. 4). To investigate whether anti–Mir-155 treatment affected Ag-specific
recall responses, we isolated spleen and LN of MOG-immunized mice treated with anti–Mir-155 or scrambled control and stimulated them in vitro with MOG peptide. We found that splenocytes from anti–Mir-155–treated mice had a diminished MOG-specific proliferative response, reduced IFN-γ and IL-17 secretion (Fig. 6B, 6C). In addition, consistent with our data and with the dramatic inhibitory effect on the clinical EAE symptoms by anti–Mir-155, we found that anti–Mir-155 treatment prevented upregulation of IL-17 and IFN-γ transcripts in CD4+ T cells isolated from the CNS of the anti–Mir-155–treated mice (Fig. 6D). Moreover, DCs isolated from LN of anti–Mir-155–treated mice had lower levels of TNF-α expression (Fig. 6E). Thus, anti–Mir-155 suppresses EAE in association with a decrease in Th1 and Th17 responses in the CNS and peripheral lymphoid organs. The increased IL-17 and IFN-γ expression in WT mice suggested that Mir-155 favored the emergence of Th17 cells, which would sustain inflammation and exacerbate clinical signs of EAE. Thus, the reduction of IFN-γ and IL-17 is most likely

**Discussion**

IFN-γ and IL-17 are produced by Th1 and Th17 cells, respectively. They are key proinflammatory mediators of cellular immunity that underlie crucial pathological events during development of EAE. Although genetic studies showed that neither IFN-γ nor IL-17 is essential for the initiation of the disease, multiple studies are in agreement that high levels of IFN-γ and/or IL-17 correlate with severity of the disease. In MS patients, elevated levels of IL-17 and IFN-γ correlated with exacerbation of the disease. The Th1 lineage and chemokine receptor-mediated events can initiate EAE by facilitating entry of Th17 cells into the CNS (23, 24). Furthermore, the ratio of Th1 cells to Th17 cells can affect the CNS lesions and disease severity (8). Our study provides evidence that Mir-155 is an important positive regulator of CNS autoimmune inflammation. We found that mice deficient in Mir-155 exhibit a delayed course and reduced severity of disease and less inflammation in the CNS. The attenuation of EAE in Mir-155−/− mice was associated with a decrease in IL-12 and Th1-polarizing cytokine expression in DCs isolated from spleen (F) and LN (G) of WT and Mir-155−/− mice with EAE. Data are representative of three experiments.
a major underlying mechanism for the amelioration of EAE in Mir-155−/− mice. Finally, we found that in vivo silencing of Mir-155 using LNA-modified anti–Mir-155 reduced clinical severity of EAE associated with a decrease in Th1 and Th17 cells. In addition, it was reported by O’Connell et al. (25) that Mir-155−/− mice develop reduced footpad inflammation during delayed-type hypersensitivity. Moreover, a recent study by Blueml et al. (26) suggest that Mir-155−/− mice are resistant to collagen-induced arthritis; this was associated with decreased Th17 cell numbers. Thus, our results together with others suggest that Mir-155 regulates both Th1 and Th17 responses that can markedly affect T cell-mediated autoimmunity.

What is inducing Mir-155?

Mir-155 locus is located within a region known as B cell integration cluster, which was originally thought to be a proto-oncogene associated with lymphoma. Mir-155 was first implicated in the oncogenesis of hematopoietic malignancies based on the finding that B cell integration cluster/Mir-155 expression is upregulated in lymphomas of B cell origin and chronic lymphocytic leukemia (27, 28). Consistent with this observation, transgenic expression of Mir-155 in B cells causes acute lymphoblastic leukemia (29). Although the hazards of unbalanced Mir-155 expression are clearly demonstrated by the diversion of lymphoid and myeloid cells to an oncogenic fate, recent findings suggest that Mir-155 was induced upon activation of myeloid and lymphoid cell types in the mouse. Various TLR ligands have been shown to induce Mir-155 expression in monocyte and macrophage cell lines (20). This induction was dependent on the signaling pathways initiated by TLR activation, implicating Mir-155 as a downstream player in innate immune function. In addition, Mir-155 is induced in macrophages and DCs after exposure to a variety of inflammatory cytokines such as IFN-β, IFN-γ, and TNF-α (20). In addition to innate immune cells, T lymphocytes also display similar induction of Mir-155 in response to activating stimuli (30).

What are the relevant Mir-155 targets?

Many direct targets of Mir-155 in DCs and T cells have been identified. Mir-155 has been shown to limit Th2 cell development through repression of c-Maf (14). It was established that Mir-155 is required for the differentiation and proliferation, but not the immunosuppressive action of CD4+ Treg cells (15). Further studies indicate that Mir-155 downregulates the protein suppressor of cytokine signaling 1 (SOCS1), which promotes competitive fitness and increased proliferation through the IL-2 and STAT5 signaling pathways (13). The essential role of Mir-155 elucidated by targeting SOCS1 expression is
not only confined to Treg cells, but also macrophages and DCs. Recently, it has been shown that IL-12 production by DCs is regulated by Mir-155-mediated targeting of SOCS1 (31). Consistent with previous studies, we found activated DCs from Mir-155−/− mice secreted less IL-12 than those from WT mice. Overexpression of Mir-155 in myeloid cells leads to myeloproliferative disorder through suppression of SHIP1 expression (29). Both SHIP1 and SOCS1 have been shown to negatively regulate cytokine expression in DCs (32, 33). It is well known that the functional phenotype of CD4+ T lymphocytes is dictated by the cytokine secretion by DCs. In addition to the Th1-polarizing cytokine IL-12, we found reduced expression of Th17-polarizing cytokines in DCs from Mir-155−/− mice with EAE. Altered expression of Th1 and Th17-polarizing cytokine expression in DCs that influence the differentiation of Th1 and Th17 responses could be an additional mechanism by which Mir-15−/− mice are resistant to EAE. Mir-155 has been shown to target DC-specific intracellular adhesion molecule-3 grabbing non-integrin and reduces its pathogen-binding ability on maturation after directly targeting the intracellular adhesion molecule-3 grabbing non-integrin (DC-SIGN). Mir-155 suppresses an infiltration of an IL-22 osteopontin axis in dendritic cells and its modulation by IFN-γamma limits IL-17-mediated autoimmune inflammation. Proc. Natl. Acad. Sci. USA 107: 11495–11500.


