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Original article

Micro RNA-155 participates in re-activation of encephalitogenic T cells

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ABSTRACT

MicroRNAs (miR) are small non-coding RNAs involved in the immune response regulation. miR-155 has been attributed a major pro-inflammatory role in the pathogenesis of multiple sclerosis and its animal model experimental autoimmune encephalomyelitis (EAE). Here, a role of miR-155 in re-activation of encephalitogenic $CD4^+$ T cells was investigated. Dark Agouti rats were immunized with myelin basic protein (MBP) emulsified in complete Freund's adjuvant. $CD4^+$ T cells were purified from draining lymph node cells (DLNC) obtained in the inductive phase and from spinal cord immune cells (SCIC) isolated at the peak of EAE. $CD4^+$ T cells obtained from SCIC (*i.e., in vivo* re-activated cells) had markedly higher expression of miR-155 in comparison to those purified from DLNC (not re-activated). Likewise, *in vitro* re-activation of DLNC with MBP led to increase in miR-155 expression. Further, DLNC and DLNC $CD4^+$ T cells were transfected with an inhibitor of miR-155 during *in vitro* re-activation. As a result, expression of important $CD4^+$ T cell effector cytokines IFN-γ and IL-17, but not of regulatory cytokines IL-10 and TGF-β, was reduced. These results imply that miR-155 supports re-activation of encephalitogenic $CD4^+$ T cells. Our results contribute to a view that miR-155 might be a valuable target in multiple sclerosis therapy.

1. Introduction

Multiple sclerosis is a chronic, inflammatory, demyelinating and neurodegenerative disease of the central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE) is a model of multiple sclerosis that has been intensively contributing to our understanding of the auto-immune response in the disease pathogenesis [1]. It is widely accepted that immune reactivity against the CNS antigens starts at the periphery where auto-reactive T helper (Th) cells, belonging to Th1 and/or Th17 population are activated [2,3]. While it is well known that these encephalitogenic $CD4^+$ T lymphocytes are activated in lymph nodes draining the site of immunization (DLN) in EAE, the site of their initial activation in multiple sclerosis is still elusive. Nevertheless, it is widely accepted that, both in multiple sclerosis and in EAE, these cells migrate into the CNS where they have to be re-activated by local antigen-presenting cells in order to initiate inflammation and autoimmune attack upon the CNS tissue [2,3]. Importantly this reactivation has to be performed in the interaction of encephalitogenic T cells and professional antigen-presenting cells, as $CD4^+$ T cells are dependent on recognizing their cognate antigens in a complex with MHC class II [2]. The phenomenon of re-activation is not yet completely understood, yet it is considered as "an absolute requirement" for pathogenesis of inflammatory diseases of the CNS [2,3].

MicroRNAs (miR) are small non-coding RNAs that are important post-transcriptional regulators of gene expression. They play a profound role in regulation of the immune response, while their aberrant expression is involved in onset and progress of immune-related disorders, including autoimmune diseases [4]. Accordingly, a number of miR have been found up-regulated or down-regulated in various immune cells in multiple sclerosis [5]. Among these, miR-155 seems to be particularly important for multiple sclerosis pathogenesis. This miR was found elevated in peripheral blood mononuclear cells of multiple sclerosis patients and a haplotype of three single nucleotide polymorphisms corresponding to mir-155 processing was associated with the disease status[6]. Also, miR-155 expression was up-regulated in active brain lesions in multiple sclerosis where it was associated with impaired expression of CD47 in astrocytes and with consequent dysregulation of macrophage inflammatory activity [7]. Moreover, miR-155 was shown of major importance for inflammatory activity of microglia [8]. Finally, it was demonstrated essential for differentiation of Th1 and Th17 cells [9]. Concordantly, deficiency

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of miR-155 protected mice from EAE through inhibition of dendritic and Th17 cells [10].

Here, expression of miR-155 in the process of re-activation of encephalitogenic cells was investigated. Also, effects of miR-155 inhibition on cytokine production in encephalitogenic T cells were determined. miR-155 expression correlated with the re-activation, while the inhibition of its activity reduced generation of typical Th1 and Th17 cytokines, *i.e.,* IFN-γ and IL-17, respectively.

2. Materials and methods

2.1. Experimental animals, EAE induction and evaluation

Female Dark Agouti rats were used in this study. The rats were maintained in the animal facility of the Institute for Biological Research "Siniša Stanković." Animal manipulation and experimental procedures were approved by the local Ethics Committee (Approval N°03-10/13 and 03-04/15). Active EAE was induced by immunization of rats with myelin basic protein (MBP 100 μg/rat, purified from guinea pig brains, kind gift from Professor Alexander Fluegel, University of Goettingen, Germany) emulsified with complete Freund's adjuvant (CFA, Difco, Detroit, MI). The rats were injected intradermally with 100 μL of the emulsion. Clinical signs of EAE were scored as follows: 0 = no clinical signs; 1 = flaccid tail; 2 = hind-limb paresis; 3 = hind-limb paralysis; 4 = moribund state or death.

2.2. Isolation of cells and cell cultures

Lymph node cells (LNC) were obtained from popliteal, inguinal and para-aortal lymph nodes of un-immunized rats. Draining lymph node cells (DLNC) were isolated from popliteal lymph nodes on day 7 after the immunization (inductive phase of disease). Spinal cord immune cells (SCIC) were obtained at the peak of disease (13–15 days post immunization, clinical sign 2–4). from spinal cords of the rats perfused with sterile phosphate buffered saline. Spinal cords were homogenized and SCIC were obtained from 30%/70% Percoll (Sigma–Aldrich, St. Louis, MO) gradient. $CD4^+$ T cells were purified from DLNC and SCIC using biotin conjugated antibody specific for CD4 (eBioscience, San Diego, CA) and IMagSAv particles plus (BD Biosciences, San Diego, CA). The cells were grown in RPMI-1640 medium (PAA Laboratories, Pasching, Austria) supplemented with 2% rat serum(RS) for antigen-specific response or with 5% fetal calf serum (FCS, PAA laboratories) for other cultivations. MBP (10 μg/ml), anti-CD3 antibody and anti-CD28 antibody (both from BD Biosciences, 1 mg/ml, each), antagomiR-155 and irrelevant oligonucleotide (Anti-mmu-miR-155-5p and negative control, both from Qiagen, Venlo, Netherlands) were used for *in vitro* treatments.

2.3. Transfection of DLNC and $CD4^+$ T cells

For transfection of the cells with anti-mmu-miR-155-5p a lipofectamine-based method was applied. Chemical transfection of DLNCs, SCICs and $CD4^+$ T cells was performed by instructions provided by the manufacturer (Santa Cruz Biotechnology, Dallas, TX). 0.6×10^6 cells were seeded in a 24-well plate in 1.4 ml of RPMI-1640 medium without antibiotics and serum. The transfection complexes were formed by mixing 6 μl of antagomiR-155 (20 μM) or 6 μl of negative control (20 μM) with the same volume of the lipofectamine transfection reagent (Santa Cruz Biotechnology, Dallas, TX or Invitrogen, Carlsbad, CA) in 100 μl of RPMI-1640 medium. After incubation for 15–20 min at room temperature, the transfection complexes were added onto the cells. The final concentration of antagomiR-155 and negative control was 80 nM. Alternatively, 6 h after applying the transfection reagent, medium was replaced with fresh medium containing antibiotics and 2% RS (for DLNC) or 5% FCS (for $CD4^+$ T cells). The cells were cultivated at 37 °C, 5% CO2 for 20 h.

2.4. Reverse transcription—real time polymerase chain reaction

Total RNA was isolated using mi-Total RNA Isolation Kit (Metabion, Martinsried, Germany). For analysis of miR-155 gene expression each sample was split in two equal parts and reverse transcribed using miR-155 Stem loop specific primer (Invitrogen, Carlsbad, CA) or random hexamer primers (Fermentas, Vilnius, Lithuania). The Stem loop primer for reaction of reverse transcriptase was: 5′-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC CCC TA-3′. For analysis of cytokine mRNA expression only random hexamer primers were used. Reverse transcriptase was performed with MMLV (Moloney Murine Leukemia Virus) reverse transcriptase (Fermentas), according to the manufacturer's instructions. Prepared cDNAs were amplified by Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) according to the recommendations of the manufacturer in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR primers (Metabion) were as

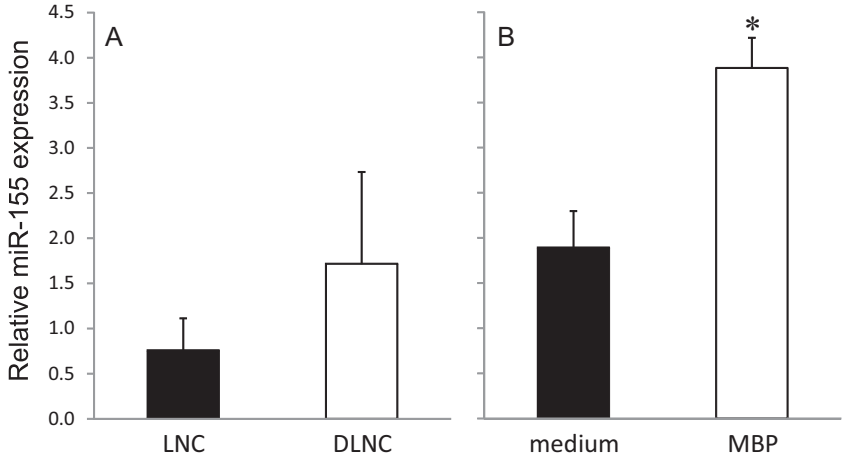

Fig. 1. miR-155 expression in lymph node cells. Lymph node cells were obtained from non-immunized rats (LNC) or from rats immunized with MBP7 days after the immunization (DLNC). miR-155 expression was determined immediately after isolation of the cells (A) or DLNC were cultivated in the absence (medium) or presence of MBP (10 μg/ml) for 24 h (B) before RNA isolation. Data from 5 (A) or 6 (B) rats per group are presented as mean +/− SD. *$p < 0.05$, statistical significance between medium and MBP culture.

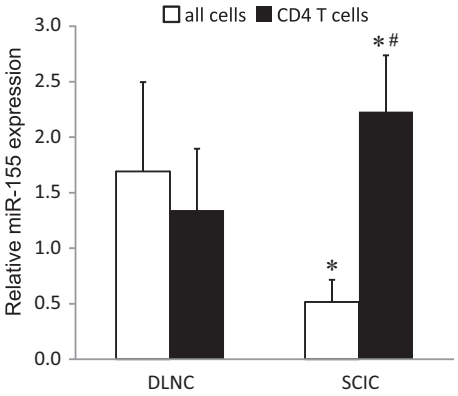

Fig. 2. miR-155 expression in lymph nodes and the CNS of the immunized rats. DLNC and SCIC were isolated from MBP-immunized rats at day 7 or days 13–15 after the immunization, respectively. RNA was isolated from the cells immediately after the isolation (all cells) or $CD4^+$ T cells were purified from DLNC and SCIC and RNA was isolated immediately after the purification. Data from 6 rats per group are presented as mean +/− SD. $^*p < 0.05$, statistical significance between correspondingDLNC and SCIC samples.$^\#p < 0.05$, statistical significance between all cells and $CD4^+$ T cells.

follows: β-actin forward primer 5′-GCT TCT TTG CAG CTC CTTCGT-3′; β-actin reverse primer 5′-CCA GCG CAG CGA TAT CG-3′; miR-155 forward primer 5′-GGA GGT TAA TGC TAA TTG TGA TAG-3′; miR-155 reverse primer 5′-GTG CAG GGT CCG AGG T-3′; IFN-γ forward primer 5′-TGG CAT AGA TGT GGA AGA AAA GAG-3′; IFN-γ reverse primer 5′-TGC AGG ATT TTC ATG TCA CCA T-3′; IL-17 forward primer 5′-ATC AGG ACG CGC AAA CAT G-3′; IL-17 reverse primer 5′-TGATCGCTGCTGCCTTCA C-3′; IL-10 forward primer 5′-GAA GAC CCT CTG GAT ACA GCT GC-3′; IL-10 reverse primer 5′-TGC TCC ACT GCC TTG CTT TT-3′. Accumulation of PCR products was detected in real time and the results were analysed with 7500 System Software (AB). Relative RNA expression is presented as 2^{-dCt}, where dCt is the difference between Ct values of a gene of interest and the endogenous control (β-actin).

2.5. Statistical analysis

The results are presented as mean ± SD of values obtained in repeated experiments or in individual rats. A Student's *t* test (two-tailed) was performed for statistical analysis. A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. miR-155 expression in DLNC of MBP-immunized rats

Expression of miR-155 was determined in lymph node cells of non-immunized rats and in DLNC of rats immunized with MBP. Although miR-155 expression was higher in DLNC, the difference was without statistical significance (Fig. 1A). DLNC were also re-stimulated with MBP *in vitro*, and this antigen-specific re-activation significantly increased miR-155 expression in comparison to un-stimulated DLNC (Fig. 1B). The increase was also

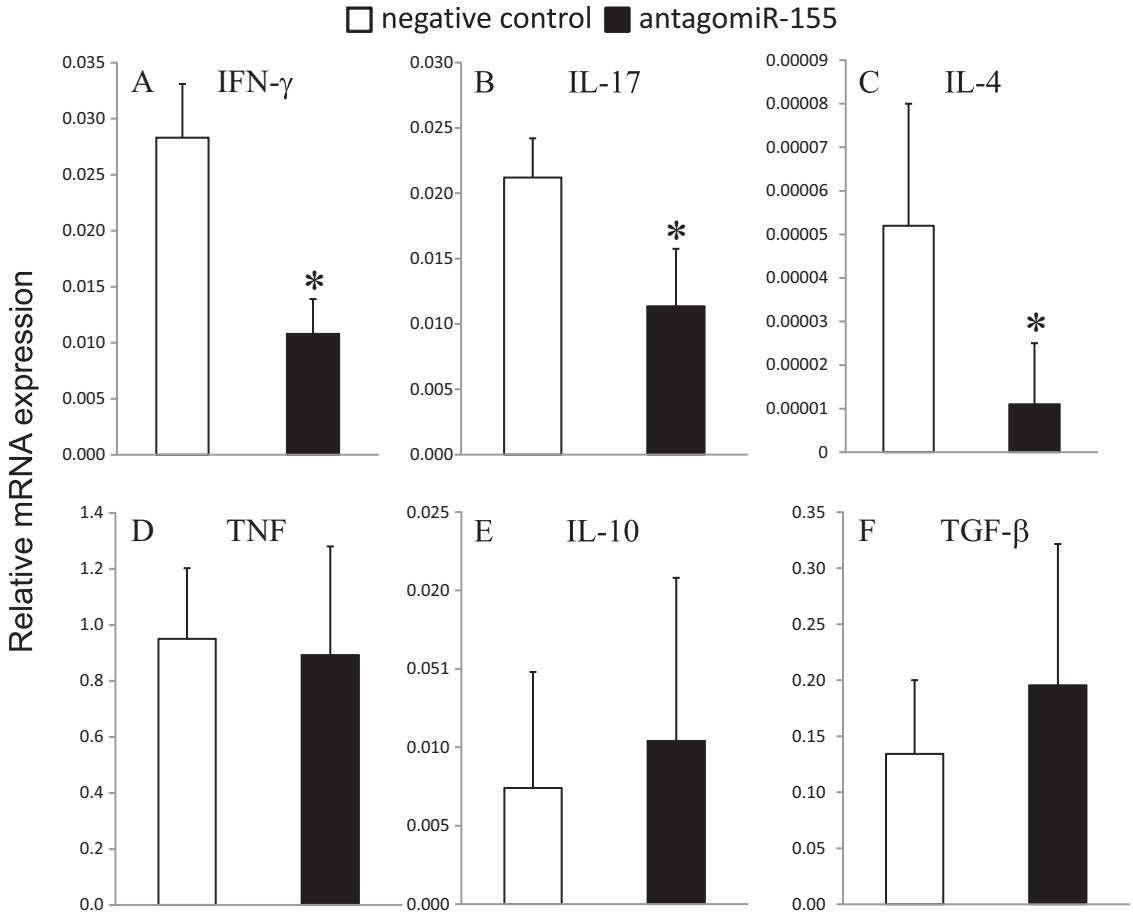

Fig. 3. Effects of miR-155 inhibition on cytokine expression in DLNC. DLNC were isolated from MBP-immunized rats 7 days after the immunization. DLNC were transfected with antagomiR-155 or with negative control and they were stimulated with MBP. RNA was isolated after 20 h of incubation and cytokine mRNA expression was determined by RT-PCR. Data from 5 independent experiments are presented as mean +/− SD. $^*p < 0.05$, statistical significance between antagomiR-155 and negative control.

significantly higher in comparison to lymph node cells obtained from non-immunized rats.

3.2. miR-155 expression in CD4$^+$ T cells isolated from DLNC and SCIC

CD4$^+$ T cells were purified from DLNC isolated at the inductive phase of EAE and from SCIC isolated at the peak of EAE. miR-155 expression was determined in DLNC, SCIC and in the purified CD4$^+$ T cells. While miR-155 expression was significantly higher in DLNC than in SCIC, it was significantly lower in CD4$^+$ T cells purified from DLNC in comparison to CD4$^+$ T cells purified from SCIC (Fig. 2). Also, CD4$^+$ T cells isolated from SCIC had significantly higher expression of miR-155 than the whole SCIC population.

3.3. Inhibition of miR-155 activity reduces IFN-γ and IL-17 expression in DLNC

miR-155 activity was inhibited in DLNC and CD4$^+$ T cells purified from DLNC by a specific antagomiR-155. DLNC were re-stimulated *in vitro* with MBP and CD4$^+$ T cells with a cocktail of anti-CD3 and anti-CD28 antibodies. Importantly, mRNA expression of IFN-γ IL-17 and IL-4, but not of tumour necrosis factor (TNF), IL-10 and transforming growth factor (TGF)-β was significantly lower in the cultures treated with antagomiR-155 in comparison to those treated with irrelevant (negative control) oligonucleotide, both in DLNC (Fig. 3) and in CD4$^+$ T cells (Fig. 4).

4. Discussion

Despite numerous studies, etiopathogenesis of multiple sclerosis is still not completely understood. Also, there is a necessity for the highly efficient therapeutics for the treatment of the disease. Accordingly, further molecular dissection of the disease pathogenesis and identification of novel therapeutic targets are needed. Along the line, this study on the role of miR-155 in re-activation of encephalitogenic T cells has been performed. Results presented here suggest that miR-155 contributes to this essential process in the disease pathogenesis.

miR-155 is expressed in various immune cells, including T and B lymphocytes, dendritic cells and macrophages [4]. This miR is involved in process of myelopoiesis, as well as in functional regulation of myelopoietic cells. Importantly, miR-155 is important in skewing Th cells towards Th1 and Th17 phenotype and inhibiting Th2 differentiation [4,9,11]. Also, miR-155 stimulates inflammatory activity of microglia, while it inhibits anti-inflammatory functions of astrocytes [7,8]. All of these data clearly imply that miR-155 is a pro-inflammatory regulator in the immune response. However, it has also been demonstrated that miR-155 is very important for regulatory T cell differentiation. Its expression is induced by a major regulatory T cell transcription factor – FoxP3 and mir-155 deficient animals have reduced numbers of regulatory T cells. Although it seems that miR-155 deficiency affects regulatory T cell fitness, it does not seem to have any effect on their functionality [9].

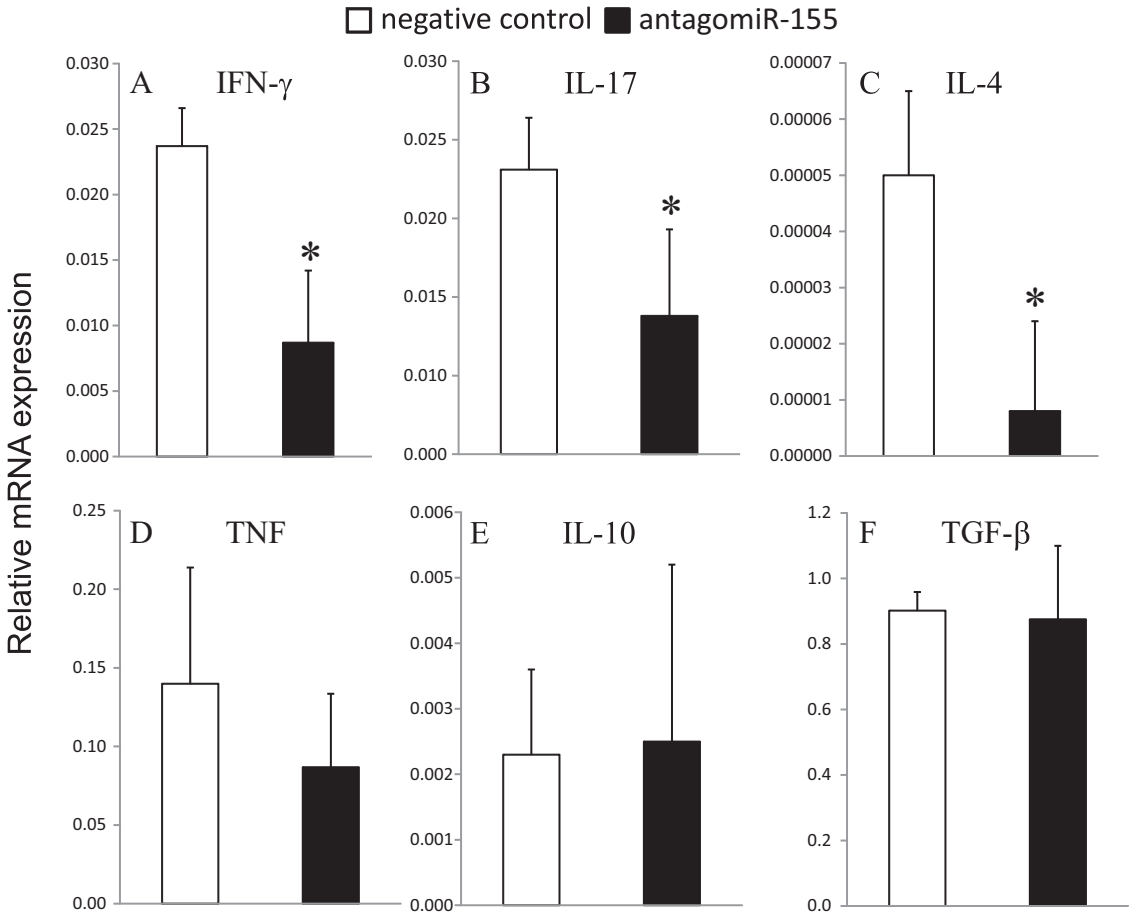

Fig. 4. Effects of miR-155 inhibition on cytokine expression in DLNC. CD4$^+$ T cells were purified from DLNC that were isolated from MBP-immunized rats 7 days after the immunization and. CD4$^+$ T cells were transfected with antagomiR-155 or with negative control and they were stimulated with anti-CD3+anti-CD28 antibody. RNA was isolated after 20 h of incubation and cytokine mRNA expression was determined by RT-PCR. Data from 5 independent experiments are presented as mean +/− SD. *$p < 0.05$, statistical significance between antagomiR-155 and negative control.

It has been shown previously that mir-155 deficient mice are highly resistant to EAE [10]. Differentiation of auto-reactive Th1 and Th17 cells is impeded in miR-155 knockouts [12]. Moreover, dendritic cell activity needed for the development of the effector Th cell subsets is impaired in these mice. Also, it has recently been demonstrated that MOG35-55-specific, miR-155 deficient Th17 cells are inefficient in inducing adoptive EAE in mice [11]. Low expression of IL-23R seems to be important for the inefficiency of the cells. These two studies clearly demonstrate that miR-155 is very important for EAE pathogenesis. However, we have to be cautious with interpretation of the results obtained with knockout mice in EAE, as in such animals "one may expect many diverse changes in several physiological processes, and one might find that after all is done, that another gene and its product can replace the function of the gene that was disrupted" [13]. Although originally this statement is on cytokine knockouts, the same could be applicable to miR deficient animals, as well. Also, knockout mice used in the studies of EAE were made through deletion of B cell integration cluster (BIC) from which primary transcript for miR-155 is produced [14]. Although functional products of BIC transcription other than miR-155 have not been identified so far, there is a possibility that the used knockouts had some other deficiencies besides miR-155-related ones. Thus, our study, in which wild type animals were used and miR-155 was inhibited by an antagomir during the limited period, eliminates the possibilities of functional replacement and un-specific interference. Also, our results were obtained in rats, thus excluding an option that the effects of miR-155 function in CNS inflammatory disorders are species-specific. Finally, and the most importantly, the studies in mice did not investigate the role of miR-155 in the process of encephalitogenic T cell re-activation. In our study, it is shown that both *in vitro* and *in vivo* antigen-specific re-stimulation increases miR-155 expression in encephalitogenic T cells. Also, it is demonstrated that inhibition of miR-155 during the process of re-stimulation leads to impaired effector functions of encephalitogenic Th1 and Th17 cells, *i.e.,* reduced expression of IFN-γ and IL-17. Interestingly, the major Th2 effector cytokine IL-4 expression was also reduced under the inhibition of miR-155. Accordingly, it was previously shown that a miR-155 inhibitor decreased secretion of IL-4 in human peripheral blood mononuclear cells [15]. However, it has to be noted that IL-4 mRNA expression was extremely low in DLNC and $CD4^+$ T cells in our experiments. Thus, it is reasonable to argue that our system is not adequate for general comprehension of the miR-155-IL-4 interaction. Importantly, expression of TNF, as well as of anti-inflammatory IL-10 and TGF-β were not affected by the inhibition of miR-155 activity. This fact clearly implied that the effect of miR-155 inhibition on cytokine expression in encephalitogenic T cells was not general, but rather specific. Of particular interest for a potential therapeutic application of miR-155 inhibition is lack of its effects on IL-10 and TGF-β as these are the major cytokines that prevent and reduce excessive inflammatory T cell reactivity [16].

In conclusion, we show that miR-155 inhibition reduces major Th1 and Th17 effector cytokine generation during the process of re-stimulation of encephalitogenic cells. Having in mind the importance of re-activation of encephalitogenic Th cells for the pathogenesis of inflammatory CNS disorders [2,3], the observed involvement of miR-155 in the process supports an idea to utilize miR-155 targeting as a therapeutic approach in multiple sclerosis. This idea is based on the facts that miR-155 level is elevated both in peripheral blood mononuclear cells and in active brain lesions of multiple sclerosis patients [6,7]. Still, the idea has to be further tested in pre-clinical settings, especially in light of the observed effects of miR-155 in regulatory T cells [9]. Thus, although a significant knowledge has been accumulated on the role of miR-155 in multiple sclerosis pathogenesis, further research on the topic is warranted.

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