Micro-RNA dysregulation in multiple sclerosis favours pro-inflammatory T-cell-mediated autoimmunity

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Pro-inflammatory T cells mediate autoimmune demyelination in multiple sclerosis. However, the factors driving their development and multiple sclerosis susceptibility are incompletely understood. We investigated how micro-RNAs, newly described as post-transcriptional regulators of gene expression, contribute to pathogenic T-cell differentiation in multiple sclerosis. miR-128 and miR-27b were increased in naïve and miR-340 in memory CD4⁺ T cells from patients with multiple sclerosis, inhibiting Th2 cell development and favouring pro-inflammatory Th1 responses. These effects were mediated by direct suppression of B lymphoma Mo-MLV insertion region 1 homolog (BMI1) and interleukin-4 (IL4) expression, resulting in decreased GATA3 levels, and a Th2 to Th1 cytokine shift. Gain-of-function experiments with these micro-RNAs enhanced the encephalitogenic potential of myelin-specific T cells in experimental autoimmune encephalomyelitis. In addition, treatment of multiple sclerosis patient T cells with oligonucleotide micro-RNA inhibitors led to the restoration of Th2 responses. These data illustrate the biological significance and therapeutic potential of these micro-RNAs in regulating T-cell phenotypes in multiple sclerosis.

Keywords: multiple sclerosis; miRNA; autoimmune T cells; Th1; Th2

Abbreviations: IL = interleukin; miRNA = micro-RNA; UTR = untranslated region

Introduction

Multiple sclerosis afflicts over two million people worldwide (Rosati, 2001) and is the primary cause of non-traumatic neurological disability in young adults (Frohman et al., 2006). In multiple sclerosis, inflammatory attack of the CNS causes myelin and axon damage, resulting in the nerve conduction defects that underlie multiple sclerosis symptoms. About 80% of patients present with relapsing–remitting multiple sclerosis, characterized by disability attacks interspersed with periods of recovery, which is followed by secondary progressive multiple sclerosis in which progressive disability continues without remissions. Other patients present with primary progressive multiple sclerosis, characterized by steadily progressing neurological disability from the onset (Compston and Coles, 2008). Although significant progress has been made in the management of this disease, the understanding
of multiple sclerosis pathogenesis, discovery of useful disease biomarkers and development of new therapies remain important challenges.

A conundrum in multiple sclerosis pathogenesis is that myelin-specific T cells exist in all individuals (Giegerich et al., 1992; Lovett-Racke et al., 1998). While these cells remain tolerant in healthy individuals, they display an activated phenotype (Allegretta et al., 1990; Lovett-Racke et al., 1998) with pro-inflammatory Th1 (Olsson et al., 1990; Balashov et al., 1997; Pelfrey et al., 2000; Crawford et al., 2004; Couturier et al., 2011) and/or Th17 (Lock et al., 2002; Kebir et al., 2007; Tzartos et al., 2008) features in multiple sclerosis, in contrast to more benign Th2 responses, ultimately leading to CNS inflammation and demyelination. The factor(s) that predispose multiple sclerosis naïve T cells to become activated and cause inflammation in multiple sclerosis remain largely unknown, with a single report demonstrating how a multiple sclerosis-associated polymorphism in the tyrosine kinase 2 gene can drive Th1 responses (Couturier et al., 2011).

Both genetic and environmental factors contribute to the pathogenesis of multiple sclerosis (Ebers, 2008). While genome-wide association studies have demonstrated genetic associations with multiple sclerosis (Oksenberg et al., 2008; Baranzini et al., 2009a, b; De Jager et al., 2009), a large portion of hereditary susceptibility remains unknown (Oksenberg and Baranzini, 2010). Micro-RNAs (miRNAs) have recently emerged as important regulators of gene expression (Ambros, 2004; Bartel, 2004) and their expression can be influenced by both genetic and environmental factors, making them attractive candidates in multiple sclerosis pathogenesis.

miRNAs are small RNAs, 19–24 nucleotides in length, that bind the 3′-untranslated region (UTR) of target messenger RNAs, thereby inhibiting translation or inducing messenger RNA degradation (Ambros, 2004; Bartel, 2004). miRNAs contribute to disease susceptibility, are useful susceptibility biomarkers and have provided novel therapeutic targets, particularly in cancer (Croce, 2009). The role of miRNAs in autoimmunity is just beginning to be elucidated, but several miRNAs have been associated with rheumatic diseases (Alevisos and Illei, 2010). Studies in peripheral blood mononuclear cells have revealed miRNA dysregulation in multiple sclerosis (Keller et al., 2009; Otaegui et al., 2009), and miR-326 has been associated with pro-inflammatory responses (Du et al., 2009). However, it is unclear whether these miRNA differences reflect differences in peripheral blood mononuclear cell composition or activation state during active multiple sclerosis, or whether they underlie the aetiopathogenesis of the disease.

Here, we investigated miRNA expression in purified naïve CD4+ T cells of patients with multiple sclerosis. The naïve CD4+ T-cell population represents cells that have not been activated, allowing us to take a snapshot of how T cells in patients with multiple sclerosis are poised to differentiate into pro-inflammatory phenotypes. We report that miR-128 and miR-27b levels were increased in naïve CD4+ T cells of patients with multiple sclerosis. In addition, miR-340 was upregulated in memory CD4+ T cells of patients with multiple sclerosis. These miRNAs acted in concert to suppress Th2 differentiation and set the stage for pro-inflammatory Th1 autoimmune responses, illustrating how these miRNAs may contribute to multiple sclerosis pathogenesis.

Materials and methods

Human subjects

Blood was obtained by leukapheresis from healthy donors or patients with multiple sclerosis after informed consent. Patients were in clinical remission and were treatment-naïve for immunomodulatory drugs. Peripheral blood mononuclear cells isolated over a Ficoll gradient were stored in liquid nitrogen until further use. This study was performed under OSU Internal Review Board protocol # 2006H0235 and 2010H0195.

Mice

Mice were bred in specific pathogen-free conditions at the OSU University Laboratory Animal Resources, under protocol # 2009A0142.

Human naïve and memory CD4+ T cell isolation

Naïve CD4+CD45RA+ cells were isolated on an AUTOMACSPro with the Dead Cell Removal Kit (Miltenyi) followed by the negative selection naïve T cell isolation Kit II (Miltenyi) by depleting cells expressing CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγδ, HLA-DR and CD235a (Glycophorin A). More than 95% CD4+CD45RA+ pure samples were used in this analysis. This population was ~99% CCR7+CD27+ and 99% CD25−CD45RO+, without any differences observed between healthy donors and multiple sclerosis (Supplementary Fig. 1). The mean ± standard deviation (SD) per cent purity of CD4+CD45RA+ cells in the various populations was as follows: healthy donors (96.8 ± 1.3), primary progressive multiple sclerosis (98 ± 0.8), relapsing–remitting multiple sclerosis (97 ± 1.47) and secondary progressive multiple sclerosis (96.4 ± 1.9). There were no significant differences in purity between multiple sclerosis groups and healthy donors. Re-analysis of the Taqman miRNA data limited to samples with a CD4+CD45RA+ purity of 99% confirmed the upregulation of miR-128 and miR-27b: fold change ± SD: miR-128 [healthy donors (2.756 ± 2.750, n = 2), multiple sclerosis (9.072 ± 0.5854, n = 4)]; miR-27b [healthy donors (1.055 ± 1.045, n = 2), multiple sclerosis (8.915 ± 2.748, n = 4)].

Memory CD4+CD45RO− T cells were isolated similarly, albeit using the Human Memory CD4+ T cell isolation kit (Miltenyi) by depleting cells expressing CD45RA, CD8, CD14, CD16, CD19, CD34, CD36, CD123, anti-TCRγδ and CD235a (glycophorin A). More than 95% CD4+CD45RO− pure samples were used in this analysis. The mean ± SD percent purity of CD4+CD45RO− cells in the various populations was as follows: healthy donors (98.08 ± 0.9), primary progressive multiple sclerosis (98 ± 1.1), relapsing–remitting multiple sclerosis (97.7 ± 0.7) and secondary progressive multiple sclerosis (98.5 ± 0.5). There were no significant differences in purity between multiple sclerosis groups and healthy donors.

RNA isolation

RNA was isolated with the mirVana kit (Ambion) total RNA isolation protocol, according to the manufacturer’s instructions, and stored at −80°C until analysis.
Naïve CD4+ T-cell miRNA Taqman array

Naïve CD4+ T-cell miRNA expression profiling was performed at the OSU Nucleic Acid Shared Resource with the TaqMan Array Human miRNA Panel (v.2, Applied Biosystems) containing 667 miRNA targets and endogenous controls. RNA (50 ng) was complementary DNA-transcribed and underwent 14 pre-amplification cycles. A 1:20 dilution of this reaction was loaded onto the Taqman array microfluidic cards. Initial analysis of raw data, stable normalization selection and statistical analysis were performed using the high-throughput real-time polymerase chain reaction analysis software Statminer (Integromics) at OSUCCC Biomedical Informatics Shared Resource. The small nucleolar RNAs RNU44 and RNU48 were confirmed to be stably expressed in our sample set and their mean used as the normalizer value. A relative fold change expression was calculated relative to the average C$_v$ value for the healthy donors group.

Memory CD4+ T-cell miRNA analysis

Memory CD4+ T-cell miRNA analysis was performed with the novel Nanostring nCounter technology at the OSU Nucleic Acid Shared Resource. This highly sensitive and specific methodology is based on the direct digital detection and enumeration of RNA transcripts after binding and labelling by specific capture and colour-coded reporter probes (Geiss et al., 2008). The nCounter miRNA assays can accurately distinguish between highly similar miRNAs with great specificity, without the need of enzymatic amplification.

Real-time polymerase chain reaction

RNA (300 ng) from profiled samples was complementary DNA transcribed using random primers. Human (h)BMI1 Sybr Green real-time polymerase chain reaction was performed using hBMI1 and 18S control primers, as described (Godlewski et al., 2008). Taqman hGATA3 real-time polymerase chain reaction was performed using Applied Biosystems hGATA3 and hHPRT primer sets. Results were analysed using the comparative C$_v$ method after confirmation of similar amplification efficiency for test and control genes. Relative fold change expression was calculated relative to the average healthy donors group.

Luciferase assay

A fragment of the human BMI1 gene (NM_005180.6: 2404-2661), GATA3 gene (NM_001002295.1: 1943-2522) or the entire interleukin-4 (IL-4) gene 3'-UTR were cloned into the XbaI site on the PGL3 control vector (Promega). The miR-27 site or the two miR-340 binding sites in the 3'-UTR of the human BMI1 gene were mutated (miR27 site: CGTACTGTGTTTA; miR-340 site 1: TACGCGC, miR-340 site 2: CCTC). The miR-340 binding site in the human IL-4 gene (ATAAAAATTTAGAGTCAGAGGA) was deleted using the QuickChange XL kit (Agilent). The miR-340 binding site in the human BMI1 gene (ATAAAAATTTAGAGTCAGAGGA) was deleted using the QuickChange XL kit. Vector (150 ng) and 200 pmol of precursor nonsense (NS1) or test miRNA (Applied Biosystems) were transfected with Lipofectamine (Invitrogen) overnight into cos-7 cells. After a 44-h incubation, cells were lysed in Cell Culture Luciferase Lysis Buffer (Promega). Luciferase activity in the lysate was measured using the Luciferase Assay System (Promega) in a FLUOstar Optima plate reader (BMG Labtech). Luciferase activity was normalized to protein content and percent relative luciferase units calculated relative to the NS1 control.

miRNA transfection and T-cell activation

Human peripheral blood mononuclear cells or mouse T cells/splenocytes were transfected overnight with 1350 pmol of double-stranded miRNA mimics or inhibitors (Dharmacon) using the TransIt-TKO Reagent (Mirus). Mimic inhibitor sequences were nonsense control (NS) (CTATGTCATCCGCTCCAC) or miR-27 (UUCACUGUCUAA GUUCCUGC, miR-128 (UCACAGUGACCGGCUU C) and miR-340 (UUAUAACGCAAUGACUGA U). For human T-cell activation, transfected cells were plated onto anti-hCD3/CD28-coated plates for 48 h in the absence of exogenously added cytokines (Th neutral conditions: Th, harvested, rested for 7 days and restimulated with anti-hCD3/CD28 for 72 h. Mouse T-cell activation, Tcr-tg splenocytes were stimulated with irradiated wild-type splenocytes (1:5 T cells:feeders) and 2 µg/ml MBPAc1-11 for 72 h in the absence of exogenously added cytokines, rested 7 days and restimulated in the same conditions. To estimate the minimum transfection efficiency in human T cells, a fluorescein isothyocyanate (FITC)-labelled small RNA was transfected into CD4+ T cells and analysed by flow cytometry. The observed FITC-positive CD4+ T cells was 39% (Supplementary Fig. 2). The expected transfection efficiency of untagged miRNA would be significantly higher since the large FITC molecule would impede transfection and this was confirmed in biological assays in which miRNA targets were analysed.

Human T-cell activation for assessment of IL-4 secretion

Purified (>95% CD4+CD45RA+) naïve human T cells were stimulated for 48 h on anti-CD3/CD28 (1 µg/ml) coated plates in Th neutral conditions. Cells were then rested for a total of 7 days in the presence of rhIL-2 (5 ng/ml), prior to restimulation with Phorbol myristate acetate (PMA)/Ionomycin (5 h) in the presence of GolgiStop (BD) for the last 3 h of the incubation.

Intracellular staining

After a 15-min Fc block reagent incubation, cells were stained with surface antibodies for 20 min at 4°C, fixed with E-bioscience (Bmi-1 and GATA3 staining) or BD (IL-4 staining) FixPerm Reagent (30 min, 4°C) and blocked with Fc block for 15 min prior to staining with intracellular antibodies (30-45 min, 4°C). Data were acquired on a FACSCantill flow cytometer. The antibodies used were hCD3, hCD4 and hIL-4 (BD), hCD45RA (Biolegend), mCD4 and mCD62L(BD), mCD44 and m/hGATA3 (eBioscience) and hBMI-1 (R&D).

Adoptive transfer experimental autoimmune encephalomyelitis

Myelin basic protein peptide Ac1-11-specific αβ TCR-tg B10.PL mouse splenocytes (Goverman et al., 1993) were transfected overnight with NS or miR-27, miR-128 and miR-340 mimics. Cells were harvested, washed and stimulated with 2 µg/ml of MBPAc1-11 in the presence of irradiated B10.PL splenocytes for 72 h in Th neutral conditions. A suboptimal number of cells (4 × 10^6/mouse) was transferred intraperitoneally into B10.PL recipients. As expected, experimental
autoimmune encephalomyelitis severity in control miRNA-transfected mice was mild due to the Th negative conditions used during T-cell activation as well as the suboptimal number of adoptively transferred cells. Experimental autoimmune encephalomyelitis was scored on a scale of 0–6: 0 (no clinical disease), 1 (limp/flaccid tail), 2 (moderate hind limb weakness), 3 (severe hind limb weakness), 4 (complete hind limb paralysis), 5 (quadruplegia or premoribund state) and 6 (death).

Cytokine enzyme-linked immunoabsorbent assay

Unless otherwise indicated, cytokines were detected in 72-h supernatants post-stimulation by a sandwich enzyme-linked immunosorbent assay (ELISA). Human and mouse interferon (IFN)-γ and IL-4 reagents were from BD and human and mouse IL-5 reagents from R&D DuoSet ELISA. ELISA was performed as previously described (BD) (Yang et al., 2009) or following the manufacturer's instructions (R&D).

Th2 cell line generation

The Th2 cell line was generated with MBPAc1-11-specific TcR-tg naïve CD4+ T cells isolated by magnetic bead sorting and stimulated with 2 μg/ml of MBPAc1-11 peptide and irradiated syngeneic feeder cells in the presence of mouse (m) IL-4 (1500 U/ml), anti-IFN-γ (10 μg/ml) and anti-mIL-12 (10 μg/ml). Three days post-primary stimulation, cells were split and received human (h) IL-2 (5 ng/ml). Cells were subsequently restimulated every 7–10 days with 1 μg/ml MBPAc1-11 with irradiated feeders and rIL-2. Transfection with miR-340 mimic was performed 18 h prior to the fourth or later rounds of restimulation.

Statistical analysis

Statistical significance was determined with Statminer software parametric moderated Limma test optimized to adjust significance for the large datasets of Taqman array data (healthy donors to all multiple sclerosis or healthy donors to specific multiple sclerosis subtype comparisons in Taqman miRNA profiling). The parametric Limma test computes the statistical significance of the detection modifier moderating the standard errors across all the detectors using a simple Bayesian model (Smyth, 2004). The effectiveness of this approach has been demonstrated on differential expression data sets (Tadesse et al., 2005). A false discovery rate (FDR) q-value of 0.05 was used as a cut-off to identify the miRNAs differentially regulated in multiple sclerosis versus healthy donors. In the remaining comparisons, Student's t-test (single treatment to control comparisons), Dunnett's post hoc test after a significant one-way ANOVA (multiple comparisons of treatment to control groups) or Mann–Whitney t-test (experimental autoimmune encephalomyelitis control to treatment group comparisons) were performed using GraphPad Prism software.

Results

Increased miR-128 and miR-27 expression in naïve CD4+ T cells of patients with multiple sclerosis

In order to determine if there is a baseline miRNA dysregulation in patients with multiple sclerosis, we isolated naïve CD4+ CD45RA+ T cells (≥95% pure, Supplementary Fig. 1A) from peripheral blood mononuclear cells of healthy donors (n = 16) or treatment-naïve patients with multiple sclerosis (n = 22), falling into the primary progressive (n = 5), relapsing–remitting (n = 12) or secondary progressive (n = 5) categories (Supplementary Table 1). The naïve status of the CD4+ T cells was verified by post-purification analysis of CD45RA, CD45RO, CD25, CCR7 and CD27 (Supplementary Fig. 1B–C). A Taqman array of 667 miRNAs was performed.

The profiling data were analysed by comparing healthy donors to either the entire multiple sclerosis patient population or individual multiple sclerosis subtypes. There were 85 miRNAs differentially expressed (P < 0.05, FDR q < 0.05) in the comparison of healthy donors to all patients with multiple sclerosis, irrespective of multiple sclerosis subtype (primary progressive, relapsing–remitting and secondary progressive; Supplementary Table 2). Individual multiple sclerosis subtype versus healthy donors analysis yielded 61, 106 or 55 significantly different miRNAs (P < 0.05) in primary progressive, relapsing–remitting or secondary progressive, respectively (Supplementary Table 3).

Although primary progressive multiple sclerosis is clinically very different from relapsing–remitting/secondary progressive multiple sclerosis, they all involve immune attack on the CNS and a miRNA commonly dysregulated in these three multiple sclerosis subtypes may regulate a critical pathway in encephalitogenicity. Furthermore, miR-128 has recently been shown to repress expression of BMI1 (Godlewski et al., 2008), which stabilizes the Th2 transcription factor GATA3 in T cells (Hosokawa et al., 2006). We reasoned that increased miR-128 in multiple sclerosis naïve CD4+ T cells could target BMI1 to inhibit Th2 and promote pro-inflammatory Th1 differentiation and further tested this hypothesis.

In order to achieve their biological effects, miRNAs often act in complex networks, with multiple miRNAs targeting the same gene (Wu et al., 2010) or a single miRNA targeting multiple genes in the same pathway (Boettger et al., 2009). Therefore, we investigated whether other overexpressed miRNAs in multiple sclerosis T cells identified in the miRNA profiling target BMI1 or other Th2 pathway genes. RNA hybrid software predicted targeting of BMI1 by two highly conserved miRNAs upregulated in multiple sclerosis: miR-128 and miR-27b (Table 1). miR-27b was increased 5-fold in the pooled multiple sclerosis population (Supplementary Table 2, Fig. 1A) and particular multiple sclerosis groups (Supplementary Table 3, Fig. 1B). miR-27a and miR-27b are highly homologous, they share the same predicted targets, but are encoded by two different chromosomes. We therefore analysed miR-27a in these samples and observed a modest increase in patients with multiple sclerosis (fold change = 2.2) that was not significant (P = 0.07; Fig. 1A). Chromosome 9 encodes miR-27b and thus, is the source of the overexpressed miR-27 observed in CD4+ T cells of patients with multiple sclerosis. miR-27 shares the third best
that these miRNAs may be targeting two critical elements in the miR-27 and miR-128 were predicted to target GATA3, suggesting targeted by multiple sclerosis-associated miRNAs, we found that miR-128, which is the top candidate (Table 1). In addition, when context score among conserved miRNAs for BMI1 targeting after miR-128, which is the top candidate (Table 1). In addition, when we investigated whether other genes of the Th2 pathway were targeted by multiple sclerosis-associated miRNAs, we found that miR-27 and miR-128 were predicted to target GATA3, suggesting that these miRNAs may be targeting two critical elements in the Th2 differentiation pathway. Since IL-4 is the signature cytokine of Th2 cells, we analysed whether any miRNAs were predicted to target IL-4. miR-340 was one of only a few miRNAs predicted to target IL-4 and interestingly, miR-340 was also predicted to target BMI1. Although miR-340 was not significantly overexpressed in the naïve CD4<sup>+</sup> T cells in patients with multiple sclerosis, it was highly (36-fold change) and significantly (P < 10<sup>−5</sup>) overexpressed in resting memory CD4<sup>+</sup>CD45RO<sup>+</sup> T cells in relapsing–remitting multiple sclerosis and secondary progressive multiple sclerosis (Fig. 1C), where it could regulate IL-4 production. Importantly, the expression of these miRNAs was independent of sex or donor age, as confirmed by both linear regression analysis and Pearson’s correlation analysis (Supplementary Fig. 3 and Supplementary Table 4 and data not shown), ruling out age as a factor affecting miR-128, miR-27 and miR-340 expression in patients with multiple sclerosis. Overall, it appeared that there could be collaborative targeting of the Th2 pathway by several miRNAs upregulated in naïve and memory CD4<sup>+</sup> T cells of patients with multiple sclerosis.

**Multiple sclerosis-associated miRNAs target genes of the Th2 pathway**

To validate target prediction analyses, we determined whether the BMI1, GATA3 and IL-4 transcripts were bona fide direct targets of miR-128, -27 or -340. A luciferase vector containing the 3′-UTR of the hBMI1 transcript was transfected into cos-7 cells along with NS or test miRNAs. miR-128, -27 and -340 significantly downregulated luciferase expression. Mutating the 3′-UTR miRNA-binding sites for miR-27 or miR-340 restored luciferase activity (Fig. 2A), confirming direct and specific targeting of BMI1 by these miRNAs. The specificity of miR-128 binding to the BMI1 3′-UTR by restoration of luciferase activity upon miR-128 site mutation has already been proven (Godlewski et al., 2008).

![Figure 1](https://example.com/figure1.png)

**Figure 1** miR-128, miR-27 and miR-340 are overexpressed in multiple sclerosis CD4<sup>+</sup> T cells. (A) miR-128 (left) and miR-27a or miR-27b (right) expression in purified naïve CD4<sup>+</sup> T cells from healthy donors (HD; n = 16) and multiple sclerosis (MS; n = 22, including five primary progressive, 12 relapsing–remitting and five secondary progressive) by Taqman real-time polymerase chain reaction array. (B) Distribution of miR-128 and miR-27b expression by Taqman array in primary progressive (PP) multiple sclerosis, relapsing–remitting (RR) multiple sclerosis and secondary progressive (SP) multiple sclerosis subtypes. (C) miR-340 expression counts in purified memory CD4<sup>+</sup> T cells from healthy donors (n = 17), all multiple sclerosis (n = 19), primary progressive multiple sclerosis (n = 4), relapsing–remitting multiple sclerosis (n = 11) or secondary progressive multiple sclerosis (n = 4) by Nanostring nCounter detection. miRNA fold-changes were calculated relative to healthy donors C<sub>l</sub> value geometric mean and geometric mean values for healthy donors and patients with multiple sclerosis are shown by the lines (A and B). Statminer’s Limma test P-values for the healthy donors to multiple sclerosis groups comparisons shown in A–C.

<table>
<thead>
<tr>
<th>Predicted miRNA</th>
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Lower (more negative) TargetScan context scores indicate higher predicted binding. m.f.e. = mean free energy.

Table 1: Th2 pathway miRNA targeting
Dysregulated miRNAs repress BMI1 and GATA3 in T cells, exacerbating experimental autoimmune encephalomyelitis

We hypothesized that the increase in miR-128 and miR-27 in multiple sclerosis naïve CD4+ T cells would inhibit Th2 differentiation and miR-340 in multiple sclerosis memory T cells and would alter effector T-cell function. To test this, myelin basic protein-specific T-cell receptor transgenic (TcR-tg) naïve mouse splenocytes were transfected with miRNAs prior to activation. Indeed, miR-340 overexpression would be expected to inhibit Th2 cell differentiation. Overall, these results suggest that the dysregulated miRNAs in patients with multiple sclerosis can suppress the Th2 pathway through repression of BMI1 and IL-4 (Fig. 3).

Importantly, we observed an inverse correlation of miR-128 expression and BMI1 transcripts in the profiled naïve CD4+ T cells (Fig. 2B) and miR-128, miR-27 and miR-340 repressed BMI1 protein expressed in glioma cells (Supplementary Fig. 4). In contrast, none of the predicted GATA3 suppressing miRNAs repressed luciferase expression from the GATA3-3′-UTR-containing vector (Fig. 2C), indicating GATA3 is not a direct target of these miRNAs.

The predicted targeting of IL-4 by miR-340 (Table 1) was confirmed by the efficient repression of luciferase activity from an IL-4-3′-UTR-tagged luciferase vector (Fig. 2D). This shows direct and specific targeting of the IL-4 gene by miR-340 and indicates that, besides targeting the Th2 pathway at the differentiation stage, miR-340 may additionally target the effector stage via inhibition of IL-4 production. To confirm this, miR-340 was transcribed into a fully differentiated IL-4-producing Th2 cell line, resulting in a reduction in IL-4 secretion (Fig. 2E). This reduction of IL-4 in the Th2 cell line was not secondary to loss of Th2-commitment, as shown by maintenance of GATA3 expression and IL-5 secretion (Fig. 2E and Supplementary Fig. 5), yet in naïve T cells miR-340 overexpression would be expected to inhibit Th2 cell differentiation. Overall, these results suggest that the dysregulated miRNAs in patients with multiple sclerosis can suppress the Th2 pathway through repression of BMI1 and IL-4 (Fig. 3).

**Dysregulated miRNAs repress BMI1 and GATA3 in T cells, exacerbating experimental autoimmune encephalomyelitis**

We hypothesized that the increase in miR-128 and miR-27 in multiple sclerosis naïve CD4+ T cells would inhibit Th2 differentiation and miR-340 in multiple sclerosis memory T cells and would alter effector T-cell function. To test this, myelin basic protein-specific T-cell receptor transgenic (TcR-tg) naïve mouse splenocytes were transfected with miRNAs prior to activation. Indeed, the percentage of BMI1+ GATA3+ and BMI1+ GATA3+ CD4 T cells representative of Th2 differentiation was decreased upon transfection with individual miRNAs (Fig. 4A–B). The largest

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**Figure 2** Multiple sclerosis-associated miRNAs target Th2 pathway genes. (A) Relative luciferase units (RLU) from a luciferase vector carrying the predicted multiple sclerosis-associated miRNA wild-type (WT) or mutant binding sites (mut) from the hBMI1 gene 3′-UTR (indicated above bars) in cos-7 cells transfected with multiple sclerosis-associated miRNAs. Mean relative luciferase units ± SEM are shown as percentage of the control NS miRNA. Results from three independent experiments are shown. The miR-128 wild-type data have been previously reported (Godlewski et al., 2008) and are shown here for confirmation and comparison of the relative effects of each miRNA. The miR-128 mutant data have also been published (Godlewski et al., 2008). (B) Non-linear regression Pearson’s correlation analysis of the relationship between BMI1 and miR-128 expression in naïve CD4+ T cells from healthy donors and patients with multiple sclerosis. Pearson P (indicating statistical significance) and r (indicating negative correlation) values shown.

**Figure 2 Continued**

(C) Luciferase assay with a hGATA3 3′-UTR luciferase vector after transfection of cos-7 cells with multiple sclerosis-associated miRNAs predicted to bind GATA3 messenger RNA. Mean relative luciferase units ± SEM results from multiple replicates of three independent experiments shown. (D) Luciferase assay with a hIL-4 3′-UTR luciferase vector after transfection with miR-340. Mean relative luciferase units ± SEM results from multiple replicates of two independent experiments shown. (E) IL-4 (left) and IL-5 (right) expression determined by ELISA in 24-h supernatants from a myelin basic protein Ac1-11-specific Th2 cell line transfected overnight with NS miRNA or miR-340 prior to stimulation with myelin basic protein Ac1-11 peptide. Mean ± SEM results shown are intra-experimental replicates, representative of two independent experiments. Dunnett’s post hoc (A and C–E) or t (E) test P-value: **P < 0.005. RU = relative expression units.
reduction was observed when we combined all miRNAs to better mimic the phenotype of multiple sclerosis T cells. BMI1 expression was reduced on a per-cell basis, while the effects on GATA3 were primarily visible as a reduction of the GATA3+ population rather than its mean fluorescence intensity (Fig. 4C). These results are consistent with miR-27, miR-128 and miR-340 primarily inhibiting BMI1 expression while loss of the GATA3+ population, indicative of Th2 phenotype, would be a consequence of BMI1 inhibition and reduced Th2 differentiation. To determine if the reduced number of BMI1+GATA3+ T cells correlated with an enhanced pathogenic phenotype, MBPAc1-11 TcR-tg cells transfected with the miRNA combination were transferred into mice and monitored for signs of experimental autoimmune encephalomyelitis (Fig. 4D). Both the incidence and severity of experimental autoimmune encephalomyelitis were enhanced in the mice that received the miRNA-transfected T cells, illustrating that these miRNAs regulate genes that modulate T-cell pathogenicity.

**Multiple sclerosis-associated miRNAs suppress Th2 and favour Th1 cytokine responses**

To determine if these miRNAs were influencing Th1 and Th2 differentiation, CD4+ T cells isolated from Th1-prone C57BL/6 (B6) mice were activated after transfection with miRNAs and miRNA inhibitors, and effector cytokine secretion was measured by ELISA. As expected, miR-27 and miR-128 increased IFN-γ production, while miR-27 and miR-128 inhibitors decreased it (Fig. 5A; no IL-17 was detected). In these Th neutral (no exogenously added cytokines) culture conditions, no IL-4 and only low levels of IL-5 were detected, consistent with the Th1 bias of B6 mice. To better visualize the effects of these miRNAs on Th2 cytokine production, ovalbumin-specific DO11.10 TcR-tg splenocytes from Th2-prone Balb/c mice were transfected with individual miRNAs, prior to two rounds of activation in Th neutral conditions, allowing differentiation into the Th2 pathway. IFN-γ production was increased in miR-27 transfected T cells, while both miRNAs suppressed Th2 (IL-4 and IL-5) cytokine production (Fig. 5B, Supplementary Fig. 6), indicating that these miRNAs influence T-cell phenotype.

**Dysregulated miRNAs repress BMI1 and GATA3 in human CD4+ T cells and their inactivation restores Th2 cytokine production**

To determine whether these findings could be recapitulated in humans, miRNAs were transfected into human peripheral blood
mononuclear cells prior to polyclonal stimulation. A decrease in the BMI1+, GATA3+ and BMI1 + GATA3+ T-cell populations with individual or combined miRNAs was observed (Fig. 6A and B), as seen in mice. The mean expression of both BMI1 and GATA3 was decreased (Fig. 6C). We hypothesized that overexpression of these miRNAs in peripheral blood mononuclear cells from healthy patients should recapitulate the phenotype of patients with multiple sclerosis, while inhibition of these miRNAs in T cells of patients with multiple sclerosis should recapitulate the healthy donors phenotype. To test this, healthy donors and multiple sclerosis patient samples were transfected with the miRNAs combination or their inhibitors, respectively, and Th2 cytokine production was analysed post-secondary stimulation. A reduction in IL-5 secretion was observed in healthy donors samples treated with multiple sclerosis-associated miRNAs, while miRNA inhibitor-treated multiple sclerosis samples increased IL-5 production (Fig. 6D). No IL-4 was detected at this early point of differentiation and the percentage of GATA3+ cells increased in correlation with IL-5. In addition, we determined whether there was a relationship between the endogenous miR-128 and miR-27 levels in humans and the propensity of naïve CD4+ T cells to differentiate into Th2 cells. Naïve CD4+ T cells were polyclonally activated under neutral conditions, rested, reactivated and analysed for IL-4 expression. There was an inverse relationship between the percentage of Th2 cells and the level of miR-128 and miR-27 (Fig. 6E). This illustrates that overexpression of these miRNAs in patients with multiple sclerosis may predispose to the development of a Th1 response and autoimmunity.
Discussion

Myelin-specific T cells exist in healthy individuals (Giegerich et al., 1992; Lovett-Racke et al., 1998) indicating that the mere presence of these cells does not result in the development of multiple sclerosis. However, in patients with multiple sclerosis these T cells have an activated phenotype (Allegretta et al., 1990; Olsson et al., 1990; Balashov et al., 1997; Lovett-Racke et al., 1998; Pelfrey et al., 2000; Crawford et al., 2004) and a tendency to differentiate into pro-inflammatory phenotypes (Windhagen et al., 1998; Couturier et al., 2011). Here, by comparing the miRNA profiles of CD4+ T cells of healthy donors and patients with multiple sclerosis, we found that three miRNAs overexpressed in patients with multiple sclerosis, miR-128, miR-27 and miR-340, had the ability to inhibit Th2 differentiation (Fig. 3) and favour pathogenic Th1 differentiation in mouse and human cells, ultimately enhancing the encephalitogenic capacity of myelin-specific T cells in adoptively transferred experimental autoimmune encephalomyelitis.

The importance of miRNAs in multiple sclerosis is highlighted by the number of recent studies showing miRNA expression differences in multiple sclerosis. However, little or no overlap has been observed in the miRNAs identified by the different studies. This can best be explained by the different tissues and cell populations studied: brain (Junker et al., 2009), whole blood (Keller et al., 2009; Cox et al., 2010), peripheral blood mononuclear cells (Otaegui et al., 2009), total CD4+ T cells (Du et al., 2009; Lindberg et al., 2010) or CD4+CD25high T cells (De Santis et al., 2010). Since miRNA expression varies between cell types, miRNA changes may reflect different cellular composition or activation states. Our study is the first to analyse the miRNA profile of highly purified naïve CD4+CD45RA+ T cells. By focusing on naïve (i.e. never activated) CD4+ T cells, our experiments avoid nonspecific effects due to activation. In addition, this experimental design allowed us to identify dysregulated miRNAs that may cause or enhance susceptibility to multiple sclerosis. Interestingly, we observed some healthy individuals displaying high levels of these miRNAs, indicating they may have an increased risk for development of multiple sclerosis or perhaps other pro-inflammatory diseases. Besides miR-128 and miR-27, a number of additional miRNAs were differentially expressed in multiple sclerosis compared with naïve T cells of healthy donors, which may regulate additional pathways. In addition, the identification of another Th2 targeting miRNA, miR-340, as overexpressed in patients with multiple sclerosis memory T-cell population, provides a potential mechanism by which Th2 response inhibition is perpetuated in multiple sclerosis.

Interestingly, the incidence of other Th1-mediated autoimmune diseases, such as diabetes, thyroiditis and psoriasis, is increased in patients with multiple sclerosis relative to the general population (Roquer et al., 1987; Karni and Abramsky, 1999; Sloka, 2002; Annunziata et al., 2003; Nielsen et al., 2006). In fact, it is possible that the miRNA dysregulation observed in multiple sclerosis is not specific to multiple sclerosis, but common to several autoimmune diseases. In addition, multiple sclerosis has been associated with reduced Th2-associated diseases, such as allergy and asthma (Bergamaschi et al., 2009; Pedotti et al., 2009). The increased expression of miR-27, miR-128 and miR-340 may enhance susceptibility to inflammation in a target organ-independent manner while HLA genes, stochastic TcR rearrangement processes or epigenetic regulation and specific environmental triggers facilitate CNS targeting in multiple sclerosis. Thus far, no studies have been reported on the role of miRNAs in allergies, but one might predict that miR-27, miR-128 and miR-340 would be decreased in...
individuals with atopy. Although Th17 cells have also been implicated in multiple sclerosis, no IL-17 was detected using Th neutral conditions in any of our assays and thus, the effect of these miRNAs on IL-17 could not be assessed. However, the literature demonstrates that suppressing the Th2 pathway enhances Th17 differentiation, suggesting that overexpression of miR-27, miR-128 and miR-340 would enhance both Th1 and Th17 cell development in the appropriate inflammatory environment (Park et al., 2005).

Multiple mechanisms may contribute to altered expression of miRNAs in multiple sclerosis. miRNAs can be located in intergenic regions or within host gene introns, and can therefore be expressed using their own promoters or as bystanders when their host gene is expressed. The fact that none of the miRNA genes

![Figure 6](https://academic.oup.com/brain/article-abstract/134/12/3578/260063)
studied here were encoded in loci identified in multiple sclerosis genome-wide association studies published to date, suggests their differential expression in multiple sclerosis may not be due to a genetic change in the miRNA locus. However, some miRNA loci were adjacent or nearby to identified loci (Supplementary Table 5), which may affect chromatin structure and gene expression on nearby loci. If miR-128 dysregulation was extended to CNS tissues, it may be of significance in multiple sclerosis since miR-128 is enriched in brain, and several miR-128 targets important in neuronal physiology have been identified, including NTRK3, Reelin, DCX, E2F3a and SNAPP25 (Eletto et al., 2008; Evangelisti et al., 2009; Zhang et al., 2009; Guidi et al., 2010). It has also been shown that miRNAs are expressed in response to infections to silence viral replication (Pedersen et al., 2007), raising the possibility that miRNA expression modulation during infections contributes to increased multiple sclerosis risk. Thus, it will be interesting to determine whether the genetic variations and infections associated with multiple sclerosis are related to the observed miRNA expression changes.

The finding that miR-27, miR-128 and miR-340 are over-expressed in patients with multiple sclerosis opens the door to preventative and/or therapeutic strategies that inhibit these miRNAs. We found that treatment of T cells from patients with multiple sclerosis with miRNA inhibitors specific for miR-27, miR-128 and miR-340 was able to restore Th2 cytokine production. Although the efficiency of this therapeutic strategy may be highest early in the disease, the fact that miR-340 inhibitors are expected to relieve the inhibition of Th2 pathway end-products indicates that targeting these miRNAs may be able to inhibit pro-inflammatory effector T cells, not merely prevent their differentiation.

By analysing miRNA differences in the CD4^+ T-cell population, we have identified three miRNAs that target the Th2 pathway, contributing to the susceptibility of patients with multiple sclerosis to develop the pro-inflammatory myelin-specific T cells that mediate CNS pathology. These miRNAs have significant value as potential multiple sclerosis biomarkers and therapeutic targets (Ferracin et al., 2010).

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Supplementary material

Supplementary material is available at Brain online.

References

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