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Malat1 long noncoding RNA regulates inflammation and leukocyte differentiation in experimental autoimmune encephalomyelitis

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ABSTRACT

In this study, we investigated the contributions of the *MALAT1* long noncoding RNA to autoimmune neuroinflammation in central nervous system tissues from patients with multiple sclerosis (MS) and mice with experimental autoimmune encephalomyelitis (EAE). Expression of *MALAT1* was decreased in the spinal cords of EAE mice as well as in stimulated splenocytes and primary macrophages. *MALAT1* downregulation by specific siRNAs enhanced the polarization of macrophages towards the M1 phenotype. Interestingly, siRNA-mediated *MALAT1* downregulation shifted the pattern of T-cell differentiation towards a Th1/Th17 cell profile and decreased differentiation towards a Tregs phenotype. Proliferation of T-cells was also increased following *MALAT1* downregulation. These data point to a potential anti-inflammatory effect for *MALAT1* in the context of autoimmune neuroinflammation.

1. Introduction

Noncoding RNAs (ncRNAs) represent an important class of RNA transcripts with diverse biological properties. NcRNAs with a sequence length of > 200 nucleotides have been labeled 'long noncoding RNAs' (lncRNAs) (Esteller, 2011). Transcriptomic studies combined with bioinformatic analyses have revealed numerous lncRNA species in mammalian cells and functional aspects of these molecules is under intense investigation. Cell biology studies have chiefly associated lncRNAs with regulatory functions including chromatin-modification, control of splicing and regulation of transcription and translation (Rinn and Chang, 2012). Altered expression and function of lncRNAs have been reported in different categories of human disease, including cancer, infectious disease, inflammatory and autoimmune disorders (Liu et al., 2014; Shi and Yang, 2016; Wapinski and Chang, 2011; Sigdel et al., 2015; Zemmour et al., 2017). *MALAT1* (metastasis associated lung adenocarcinoma transcript 1) is a lncRNA which is present within cell nuclei and is known to regulate the expression of various genes by organizing ribonucleoprotein complexes and influencing RNA transcription and maturation (Eissmann et al., 2012). Malat1 is highly expressed in several tissues including bone marrow, gut, endocrine organs, lung and the nervous system (Tano et al., 2010; Bernard et al., 2010). Indeed, its expression regulates cell proliferation, differentiation and migration in these tissues (Tano et al., 2010; Zhang et al., 2017b). As the gene name implies, this lncRNA has been largely studied in the context of cancer where it is known to affect invasiveness and metastasis (Zhang et al., 2017b; Liu et al., 2017a). Nonetheless, some studies have also reported *MALAT1*'s pathogenic role in inflammatory diseases including diabetes mellitus (Liu et al., 2014) and systemic lupus erythematosus (Yang et al., 2017). These reports are consistent with *MALAT1*'s role in regulating leukocyte differentiation and function (Ma et al., 2015). Despite above pathogenic roles for *MALAT1* in inflammatory diseases, its neuroprotective role also has been studied in spinal cord ischemic injury through miR-204 regulation (Wang et al., 2018).

Multiple sclerosis (MS) is a chronic inflammatory disease of central nervous system (CNS) which is associated with demyelination and axonal injury (Sospedra and Martin, 2016). Both adaptive and innate immune elements are involved in MS immunopathogenesis (Trapp and Nave, 2008). Infiltration of autoreactive T lymphocytes with a Th1/Th17 phenotype is the key adaptive immune component in disease process (Selmi et al., 2016; Sospedra and Martin, 2005). Once inside the CNS, infiltrating autoreactive T cells can activate and be activated by innate immune cells, i.e. resident microglia and infiltrating monocytes,

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initiating pathogenic neuroinflammatory pathways with ensuing demyelination and neural cell injury. Despite searches and reported associations with genetic and environmental factors, it is unknown what activates the complex pathogenic cascade in MS. At the molecular level, altered expression of different genes has been reported in neural and immune cells in the beginning as well as in progressive phases of disease (Lock et al., 2002). These changes in gene expression are mostly believed to be a consequence of disease process, nonetheless they can also contribute to the progression of pathogenesis through autocrine and paracrine mechanisms. Numerous reports have highlighted the role of epigenetic factors, i.e. DNA methylation, histone modifications and microRNAs in neural/immune cell gene expression in MS (Koch et al., 2013; Huynh and Casaccia, 2013). Less information exists regarding the impact of other noncoding RNAs, e.g. lncRNAs, as potential regulators of neural/immune cell biology and neuroinflammatory processes in MS (Fitzgerald and Caffrey, 2014).

Given the high expression levels of *MALAT1* in CNS tissues (as reported in lncRNA database- http://www.lncrnadb.org/Malat1), as well as reported effects of this lncRNA in brain development, glial cell biology and immune cell functions, we investigated potential contributions of this lncRNA in the pathogenesis of autoimmune demyelination. First, we examined CNS tissues from MS and nonMS patients as well as animals with experimental autoimmune encephalomyelitis (EAE) animals at different time points after disease induction. Additionally, the expression analysis of *MALAT1* was assessed in activated lymphocytes and macrophages. We then investigated the role of *MALAT1* in leukocyte biology by altering the expression of *MALAT1* in primary macrophages. Likewise, experiments were performed to examine the effects of *MALAT1* in differentiation of CD4 + T cells towards different phenotypes as well as their proliferative response following activation.

2. Methods

2.1. Human brain studies

The use of autopsied brain tissues was approved by the University of Alberta Human Research Ethics Board (Biomedical, protocol number 2291), and written informed consent was obtained for all samples collected from age and sex-matched subjects including non-MS patients $n = 5$; mean age = 48.3 ± 6.8 years; male:female, 3:2; diagnoses at death: cancer, hydrocephaly, infectious neurodegenerative disease and ischemic encephalopathy) and patients with MS ($n = 5$; mean age = 51 ± 3 years; male:female, 2:3; diagnoses at death: secondary progressive MS ($n = 3$), primary progressive MS ($n = 1$) and relapsing-remitting MS (n = 1). All tissue samples were stored at −80 °C as previously reported (Noorbakhsh et al., 2006; Ellestad et al., 2009). In each MS patient, LFB and H&E staining were performed on multiple brain sections, and tissue samples were collected from normal appearing white matter (NAWM) juxtaposed to the lesions.

2.2. EAE induction in mice

8 week-old female C57BL/6 mice were purchased from Pasteur Institute of Iran and maintained in the animal facility of Tehran University of Medical Sciences. At 12 weeks of age, EAE was induced using a MOG35–55/CFA Kit (EK-2110, Hooke Kit™ MOG35–55/CFA Emulsion PTX). For this purpose, mice ($n = 30$) were injected subcutaneously with MOG35–55 peptide emulsified in complete Freund's adjuvant (CFA) at two different sites (0.1 ml of emulsion/site) followed by two intraperitoneal injections of pertussis toxin (200 ng/mouse for each injection) administered on the day of immunization and 24 h later. Control mice received similar injections except for the MOG antigen. EAE scoring was performed daily for 30 days post-immunization using a 0 to 15-point scoring scale (Giuliani et al., 2005). All experiments were performed in accordance with guidelines from Animal Care Committee of Tehran University of Medical Sciences. Spleen and CNS tissues were obtained from EAE mice at three time points following disease induction including: pre-onset (before the appearance of symptoms; Day 10 post-immunization), acute phase (at the peak of the disease), and a late phase here after called the post-peak phase (Days 24–30 post-immunization). Spinal cord tissue samples from EAE and control mice were stored at −80 °C.

2.3. RNA extraction, cDNA synthesis and real-time PCR

Total RNA was isolated from tissue samples and cells using miRNeasy Mini Kit (Qiagen). RNA concentration was determined with a Nanodrop (Thermo Scientific) and stored at −80 °C. First-strand cDNA synthesis was performed with 1 μg total RNA using TAKARA kit for mRNA expression analyses according to the manufacturer's instructions (TAKARA). Real-time RT-PCR was performed on an Applied Biosystems machine using Syber Green method using Solis Biodyne Real-time PCR mix. Gene expression levels were normalized against beta-actin and Gapdh for mouse and against beta-tubluin for human samples. Primer sequences used for mRNA expression analyses are shown in Supplementary Table S1.

2.4. Splenocyte culture and stimulation

To perform in vitro stimulation experiments, splenocyte cultures were prepared from 6- to 8-week-old C57BL/6 mice. Mice were sacrificed and spleens were removed under sterile conditions. Spleen tissues were homogenized in PBS and splenocytes were isolated using Ficoll-Hypaque density gradient centrifugation. 2×10^6 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 5% FBS (Gibco) and were stimulated with anti-CD3 (1 μg/ ml) and anti-CD28 (1 μg/ml) antibodies (eBioscience) for different time points followed by RNA extraction and gene expression analysis.

2.5. MALAT1 knock down experiments

Malat1 knock down was performed using Smart Pool Malat1 siRNA, which includes a mixture of 4 siRNAs as well as scrambled sequences as negative control (Dharmacon). Cells were transfected with Malat1 siRNA or scrambled sequences using Attractene transfection reagent according to manufacturer's instructions (Qiagen). Briefly, 1.5 μl of Attractene transfection reagent was added to 100 μl of serum-free DMEM medium containing Malat1 siRNA or negative control at a final concentration of 100 nM and was incubated in room temperature for 30 min. The mixture was then added drop wise to cells maintained in serum free media in 24-well plates. Medium was replaced with DMEM-10% FBS after 4 h. To evaluate the efficiency of down regulation, expression of Malat1 in transfected cells was measured by Real Time RT-PCR (Fig. S1 in Supplementary Material).

2.6. Macrophage culture and differentiation

Bone marrow-derived macrophages were prepared from C57BL/6 mice, as previously described (Tsutsui et al., 2004; Talebi et al., 2017). Differentiated macrophages were then treated with lipopolysaccharide (LPS) (10 and 100 ng/ml) for 12 h at 37 °C before RNA extraction. For Malat1 knockdown, 10^6 differentiated macrophages in 600 μl of serum free medium were transfected with Malat1 siRNA or scrambled sequences for 4 h, as described above. After 48 h, total RNA was extracted for gene expression analysis. To polarize macrophages towards the M1 phenotype, LPS (100 ng/ml) and recombinant IFN-γ (50 ng/ml) were added to cells 4 h post transfection and cells were incubated for 48 h. Treatment with recombinant IL-4 (10 ng/ml) was used to polarize cells towards the M2 phenotype.

2.7. T cell culture and differentiation

Naive CD4+ T cells were isolated from spleens of C57BL/6 mice using naïve CD4+ T cell isolation by negative selection kit (mouse CD4+ T cell isolation kit, Miltenyi Biotec). 1×10^5 cells were cultured in each well of 96-well plates and were then transfected with Malat1 siRNA or scrambled sequences (100 nM); as described above. After 4 h, transfected cells were stimulated with anti-CD3 (1 ug/ml) and anti-CD28 (0.5 ug/ml) antibodies (eBioscience) and differentiated to Treg, Th1, and Th17 cells, as described before (Ghorbani et al., 2017). Th0 cells were used as controls. For regulatory T (Treg) cells, transfected cells were cultured in RPMI containing IL-2 (20 ng/ml) and TGF-β1 (50 ng/ml) (BioLegend) for 96 h. For differentiation towards Th1 cells, transfected cells were cultured in RPMI containing IL-2 (20 ng/ml), IL-12 (50 ng/ml) and anti-IL-4 antibody (10 ng/ml) (BioLegend) for 96 h. To differentiate cells towards Th17 phenotype, transfected cells were cultured in in the presence of TGF-β (5 ng/ml), IL-6 (100 ng/ml), anti-IFN-γ (10 ng/ml), anti-IL-4 (10 ng/ml) and IL-23 (50 ng/ml) (BioLegend) for 96 h. To produce Th0 cells, transfected cells were cultured in the presence of IL-2 (20 ng/ml), anti-IFN-γ (10 ng/ml) and anti-IL-4 (10 ng/ml) (BioLegend) for 96 h.

2.8. Intracellular staining and flow cytometry

Flow cytometry with intracellular staining was used to analyze differentiation of T cells towards Th1, Th17 and Treg phenotypes, using methods that have been described previously (Ghorbani et al., 2017). APC-labeled anti-mouse CD4 (eBioscience, 17-0042-81) was used for surface staining. PE-labeled anti-mouse IL17A (eBioscience, 12-7177-81), PE-labeled anti-mouse IFN-γ (eBioscience, 12-7311-81) or PE-labeled anti-mouse Foxp3 antibodies (eBioscience, 12-5773-80) were used for intracellular staining. APC Rat IgG2a κ Isotype Control antibody (eBioscience, 17-4321-41) and PE Rat IgG2a κ Isotype Control (eBioscience, 12-4321-41) were used as isotype controls. Stained cells were analyzed using a FACSCalibur Flow Cytometer (BD Biosciences) and results were analyzed with FlowJo software.

2.9. T cell proliferation assay

To asses T cell proliferation capacity after Malat1 down regulation, isolated naive CD4+ T cells were stained with CFSE dye using CFSE Cell Division Tracker Kit (Biolegend, 423,801) based on manufacturer's instructions. Briefly, 10^6 naive CD4+ T cells were resuspended in 1 ml RPMI 1640 medium and mixed with CFSE working solution (5 μM). After 20 min of incubation at 37 °C in dark, CFSE was quenched by addition of 1 ml FBS for 10 min at 4 °C followed by 3 times washing with ice -cold RPMI 1640 containing 10% FBS. CFSE-labeled cells were then transfected with Malat1 siRNA or scrambled sequence followed by stimulation with anti-CD3 and anti-CD28. After 72 h, CFSE dilution in cells was analyzed by a FACS Calibur Flow Cytometer (BD Biosciences).

2.10. Statistical analyses

Statistical analyses were performed using SPSS software, Version 20. Student's t and Mann–Whitney *U* tests were used for parametric and non-parametric mean comparisons between the two groups. Kruskal–Wallis tests were performed for non-parametric mean comparisons between multiple groups. Data are shown as mean + SEM. A *p*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Malat1 is down-regulated in the CNS during autoimmune neuroinflammation

To examine the expression of *MALAT1* in the CNS during MS disease, we performed a quantitative expression analysis on white matter samples obtained from autopsied brain tissues of MS patients and nonMS controls. In MS cases, samples were taken from white matter tissue juxtaposed to the lesions. Real time RT-PCR revealed a reduction in *MALAT1* level in MS brain samples compared with nonMS controls, although this difference showed a *p* value of 0.08, using Mann-Whitney *U* test (Fig. 1a). We then investigated the expression of *Malat1* in the lumbar spinal cord tissues obtained from control and EAE mice. As described in the Methods section, EAE was induced in 30 animals which were divided into three groups for tissue extraction after the induction of disease. The first time point was approximately day 10 post-induction and before the development of neurological signs (pre-onset); the second time point was at the peak of the disease that varied between days 18 and 20 for mice in the group (peak of disease); and the third time point was at day 25 post-induction (post-peak phase). We have previously shown enhanced expression of inflammation related genes such as *Tnfa* and *Il6* in EAE lumbar spinal cords at the pre-onset phase of disease along with increased expression of *Cd3e* (lymphocyte marker), *Gfap* (astrocyte marker) and *f4/80* (monocytoid cell marker) at the peak and post-peak phases of disease, compared with control animals' spinal cords (Ghorbani et al., 2017; Talebi et al., 2017). Analyzing the expression of *Malat1* in lumbar cord tissue samples from EAE mice showed significant reduction at the peak of disease compared with control mice. Interestingly, when we analyzed the expression levels in pre-onset or chronic EAE cases there was no significant difference compared with controls (Fig. 1b). Altered expression of various genes in inflamed CNS (or any other) tissue could be a reflection of leukocyte infiltration into the tissue. However, in case of *Malat1*, it does not seem that decreased levels of the transcript is merely a reflection of cell infiltration, as a significant difference in Malat1 expression also existed between acute and chronic phases of disease. These phases are more or less similar in terms of the degree of cellular infiltrates, while they are different in terms of the activation status of the infiltrating cells (Muja et al., 2011; Zorzella-Pezavento et al., 2013; Prinz et al., 2015).

3.2. Malat1 expression is altered in activated splenocytes and macrophages

Different cell types are involved in the neuroinflammatory process in MS/EAE. Infiltrating monocytes/macrophages and locally-activated microglia represent innate immune and autoreactive T cells represent adaptive immune players in MS/EAE neuroinflammation. We asked whether macrophages and/or T cells express *Malat1* and if so whether their activation might affect *Malat1* expression levels. To address this question, we prepared primary macrophage cultures as well as splenocyte cultures from C57BL/6 mice. LPS stimulation of cells was used as a model that could recapitulate some of the features of monocyte/glial cell activation during disease (Chen et al., 2010; Gresa-Arribas et al., 2012). Treatment of cells with 10 ng/ml of LPS led to a mild non-significant reduction in expression, however treatment of cells with 100 ng/ml of LPS led to a significant decrease in Malat1 levels compared to unstimulated macrophages (Fig. 1c).

We next examined the expression of *Malat1* in splenocyte cultures. To this end, expression of *Malat1* was measured at multiple times points in T cells polyclonally activated by anti-CD3 and anti-CD28 antibodies. The analyses revealed a two-fold increase in *Malat1* levels 1 h after stimulation of splenocytes, however the expression levels showed substantial reduction after 12, 24 and 48 h (Fig. 1d). These reductions in *Malat1* expression in two important groups of leukocytes seem to be consistent with diminished *Malat1* expression in the acute phase of EAE.

3.3. Malat1 regulates activation and polarization of macrophages

LncRNAs are known to influence the biology of different cell types by regulating the expression of multiple genes. In the context of inflammatory disorders, polarization of monocytoid cells towards the

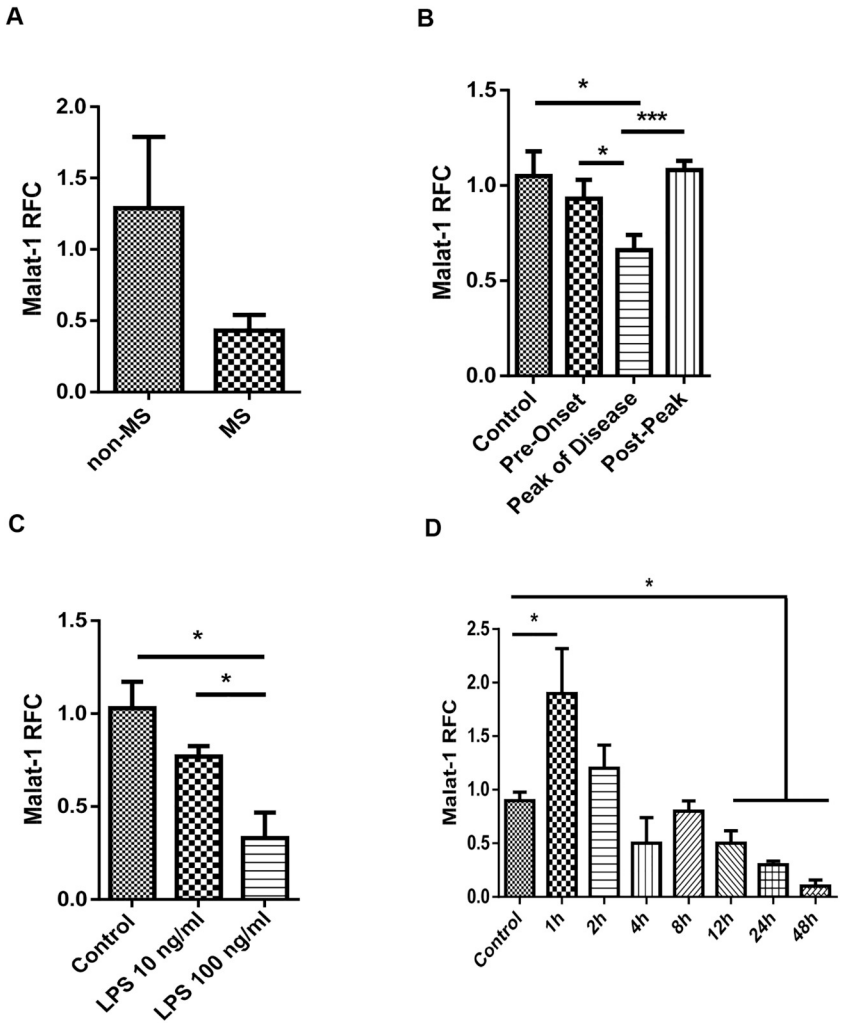


Fig. 1. Malat1 expression levels in human brain tissue samples and EAE spinal cords and activated leukocytes. Expression of Malat1 was measured in autopsy brain tissues from MS patients ($n = 5$) and non-MS controls (n = 5) by real-time RT-PCR (A). EAE was induced in C57BL/6 mice and spinal cord tissues were extracted at three time points after disease induction. Expression of Malat1 was measured using real time RT-PCR (B). Bone marrow-derived macrophages were stimulated with two concentrations of LPS for 12 h, before expression analysis by RT-PCR (C). Splenocytes were stimulated with anti-CD3 and anti-CD28 for indicated time points and expression levels of Malat1 were analyzed (D). Data are shown as mean ± SEM. Number of mice in each group = 10,* $p < .05$, Mann-Whitney and Kruskal–Wallis tests.

classically-activated M1 or the alternatively-activated M2 phenotypes is considered a pivotal pathogenic determinant (Wang et al., 2018). To investigate the functional significance of *Malat1* down-regulation in activation and polarization of macrophages, we established primary macrophage cultures from the bone marrow of C57BL/6 mice (BMDMs). To polarize macrophages, BMDM cells were initially treated with either LPS/interferon-γ or IL4 (as described in Methods), to generate M1-like or M2-like cells, respectively. Untreated macrophages were considered as cells with an M0 phenotype. The expression of M1 markers, i.e. *Il1b*, *Il6* and *iNos* and M2 markers, *Mrc1* and *arginase* were evaluated in cells 48 h after polarization (Fig. 2a). Quantitative real-time PCR revealed a substantial increase in *Il6* and *iNos* expression in M1-like cells along with decreased expression of *Mrc1* compared with M0 cells. Likewise, the expression of *Mrc1* and *arginase* were significantly increased in M2-like cells compared with undifferentiated cells. We next investigated whether *Malat1* down regulation might affect M1/M2 polarization. To this end, primary macrophages were transfected with a mixture of *Malat1*-specific siRNAs or with control scrambled sequences, followed by polarization towards M1 or M2 cells, or untreated M0 cells. Transfection with siRNAs led to approximately 50% reduction in Malat1 transcript levels (Supplementary Fig. S1a). In cells with an M0 phenotype, *Malat1* down-regulation resulted in higher expression of *Il6* gene compared to cells transfected with scrambled sequences but other markers did not show any significant differences (Fig. 2b). Interestingly, suppression of *Malat1* expression in M1 differentiated cells led to significant induction in Il1b and *Il6* levels with a mild but significant reduction in *Mrc1* levels (Fig. 2c). In macrophages receiving M2 polarizing regimen, *Malat1* down regulation resulted in approximately 8-fold increase in *iNos* gene expression, although expression of M2 markers (*mrc1* and *arginase*) were also increased around 2-folds (Fig. 2d). Altogether, these data indicate that Malat1 could act as a regulator of macrophage activation and differentiation; *Malat1* down-regulation in cells that were exposed to M1-differentiating factors enhanced macrophage differentiation towards the proinflammatory M1 phenotype. Thus, the consequence of *Malat1* suppression in M2 polarized cells resulted in upregulation of both M1 and M2 markers, with a greater increase in the levels of M1 marker, *iNOS*.

3.4. *Malat1* down regulation affects Th1, Th17 and Treg differentiation

CD4+ T-cells are considered a major pathogenic variable in the context of MS/EAE. Differentiation towards IFN-γ-producing Th1 or IL-17-producing Th17 phenotypes is generally believed to promote disease and worsen disease outcomes, while differentiation towards T regulatory (Treg) cells is regarded as a modulator of the disease process. To evaluate the potential role of *Malat1* in differentiation of T helper cells, Naïve CD4+ T-cells were purified from mouse splenocytes, with a purity of > 95% (Supplementary Fig. S2). Purified naïve CD4+ T- cells were transfected with *Malat1*-specific siRNAs or scrambled sequences and cultured in Th1, Th17, or T regulatory polarizing conditions for a period of 4 days, as described in Methods section. As expected, exposure of T-cells to Th1 polarizing conditions in the absence of transfection enhanced the frequency of IFNγ immunopositive cells compared with

undifferentiated (Th0) cells (Fig. 3). Likewise, frequency of IL17 and FoxP3 immunopositive cells were enhanced significantly in Th17 and Treg polarizing conditions, respectively (Fig. 3). Transfection with *Malat1*-targeting siRNAs led to a > 50% reduction in *Malat1* transcript levels, compared with cells transfected with the scrambled control sequences (Supplementary Fig. 1b). Interestingly, *Malat1* suppression in cells exposed to Th1-polarizing regimen increased the frequency of IFNγ immunopositive cells (Fig. 3b and c). Moreover, *Malat1* down-regulation in cells exposed to Th17 polarizing conditions, also led to enhanced frequency of IL-17 producing cells (Fig. 3b and d). *Malat1* suppression did not affect the baseline frequency of IFNγ- or IL-17-producing cells in T-cells grown under Th0 conditions (data not shown). When studying T-cells grown in T regulatory polarizing conditions, Malat1 suppression reduced the frequency of FoxP3 immunoreactive cells (Fig. 3b and e). The baseline frequency of Foxp3-positive cells also showed a decrease in T-cells grown under Th0 conditions (data not shown). Overall, these findings suggested that a decrease in *Malat1* levels in CD4+ T cells can influence their differentiation and tip the balance of cell phenotype towards pathogenic Th1 and Th17 and away from protective Treg phenotype.

3.5. *Malat1* affects CD4+ T cells proliferation in vitro

Given that Malat1 down regulation alters CD4+ T-cells differentiation towards Th1 and Th17, we decided to examine whether *Malat1* knock down also affects proliferative ability of T-cells following activation. To this end, purified CD4+ T-cells from the splenocytes of C57BL/6 mice were labeled with CFSE prior to transfection with *Malat1* siRNA or scrambled control siRNAs. The cells were then stimulated with anti-CD3 and anti-CD28 and after 4 days were evaluated for their CFSE levels. Flow cytometry analyses showed higher frequency of CFSE-low cells in *Malat1* siRNA-transfected cells, compared with cells transfected with control scrambled sequences. This indicated that *Malat1* suppression could enhance the proliferative ability of T cells (Fig. 4a, b). However, we did not observe any differential proliferation of specific CD4+ T-cell subtypes in *Malat1*-suppressed cells (data not shown).

Overall, our differentiation and proliferation analyses indicated that *Malat1* down-regulation might affect T-cell biology in a manner that is concordant with enhanced neuroinflammation and exacerbation of disease.

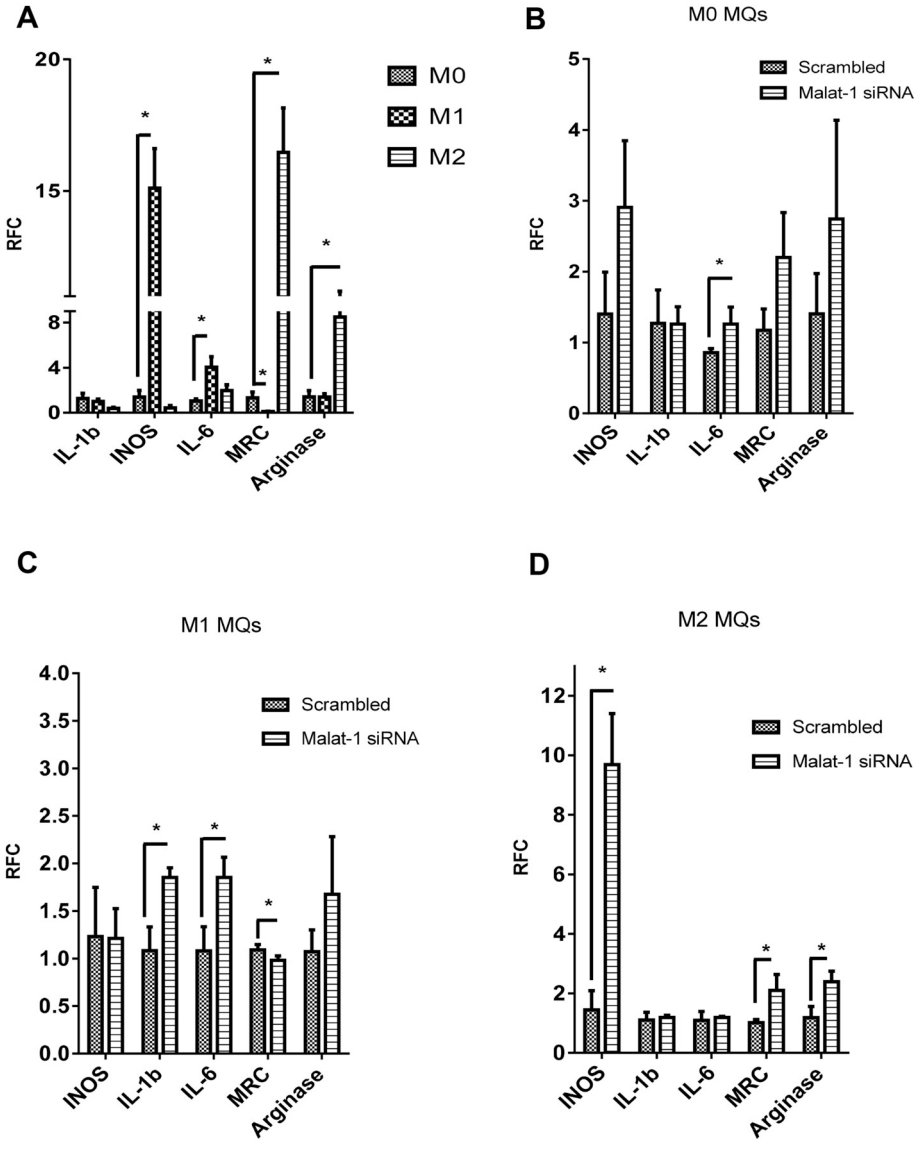


Fig. 2. Malat1 influences macrophage differentiation. In vitro polarization of macrophages from bone marrow derived precursors. Primary macrophages were differentiated towards M1 or M2 cells using LPS (100 ng/ml) and IFNγ (50 ng/ml) or IL-4 (10 ng/ml) respectively. The expression of inflammatory cytokines Il1 and Il6 together with M1/M2 markers iNOS, Mrc1, and arginase were analyzed (A). Effect of Malat1 down regulation on macrophage polarization. Primary macrophages were transfected with Malat1 siRNAs or negative control sequences (scrambled) and were then differentiated towards M0 (B), M1 (C) or M2 cells (D). The expression of inflammatory cytokines Il1, and Il6 together with M1/M2 markers iNOS, Mrc1, and arginase were analyzed after 48 h. Data are shown as mean ± SEM, *$p < .05$. Kruskal–Wallis test.

4. Discussion

Long noncoding RNAs have recently emerged as key regulators of gene expression and their potential role in human disease has attracted the attention of basic and clinical scientists. In this study, we provide evidence pointing to the contribution of the Malat1 lncRNA to inflammatory responses in autoimmune demyelination. We show diminished expression of Malat1 transcripts in CNS tissues from mice with EAE at the acute phase of disease, compared with control tissues. Our preliminary gene expression studies on human brain autopsy tissues also displayed a trend towards diminished *Malat1* expression in tissues derived from MS patients compared with non-MS controls

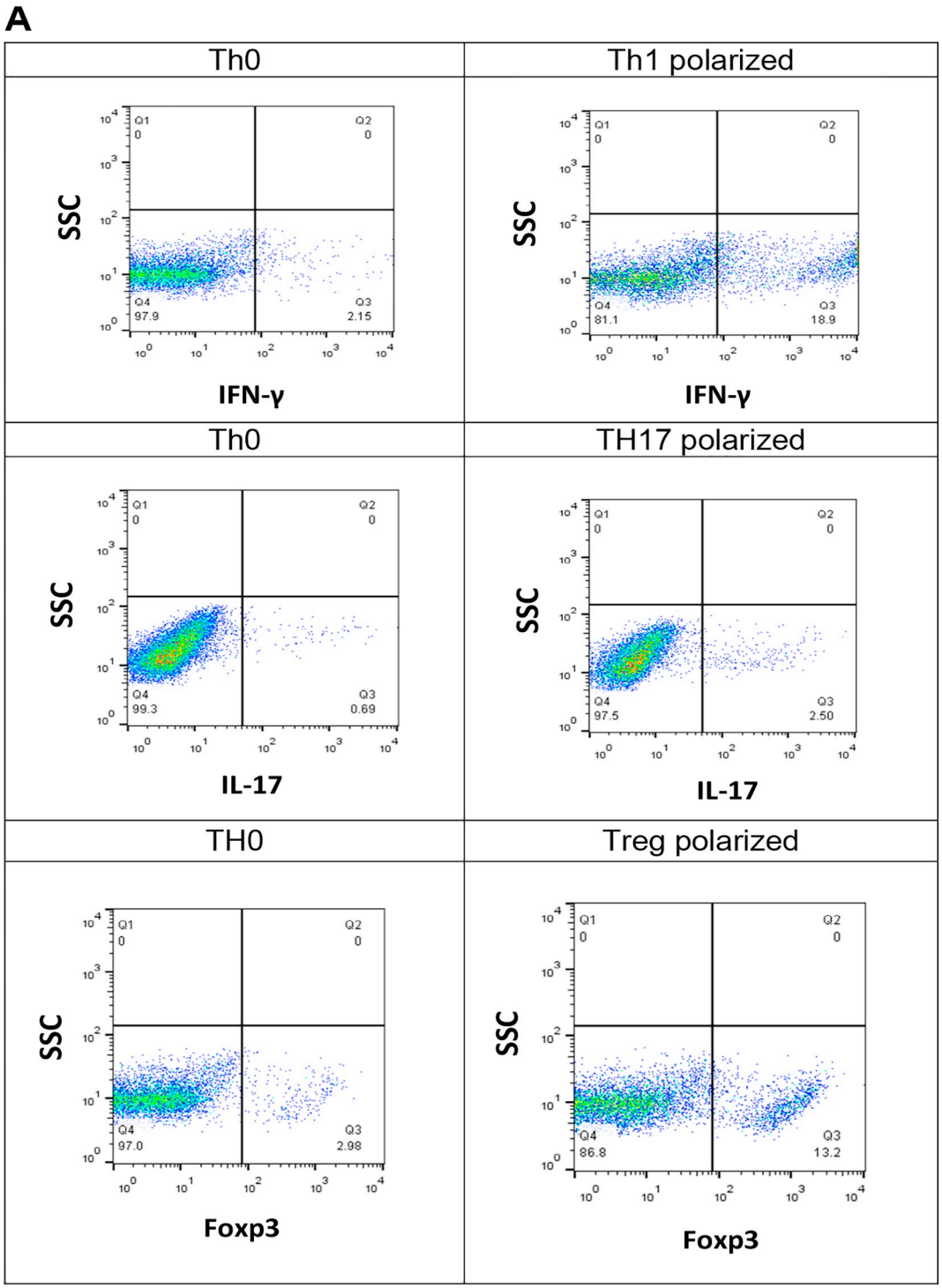


Fig. 3. Malat1 down regulation affects differentiation of CD4+ T cells. Purified naïve CD4+ T cells were activated and polarized towards Th1, Th17 or Treg phenotypes. The frequencies of IFNγ, IL17 or FoxP3 positive cells were determined by flow cytometry (A). Purified naïve CD4+ T cells were transfected with Malat1 siRNA or negative control sequences. Cells were then activated and polarized towards Th1, Th17 or Treg phenotypes. The frequencies of IFNγ, IL17 or FoxP3 positive cells were determined by flow cytometry. Representative dot plots show the frequency of IFNγ positive Th1 cells, IL17 positive Th17 and FoxP3 positive Treg cells (B). Quantification of cell frequencies is shown in the bar graphs (C, D and E). Percentages of positive cells in CD4+ T cells are presented as mean ± SEM ($n = 3$). Data are presented from a single experiment representative of three independent experiments (*$p < .05$. Kruskal–Wallis test).

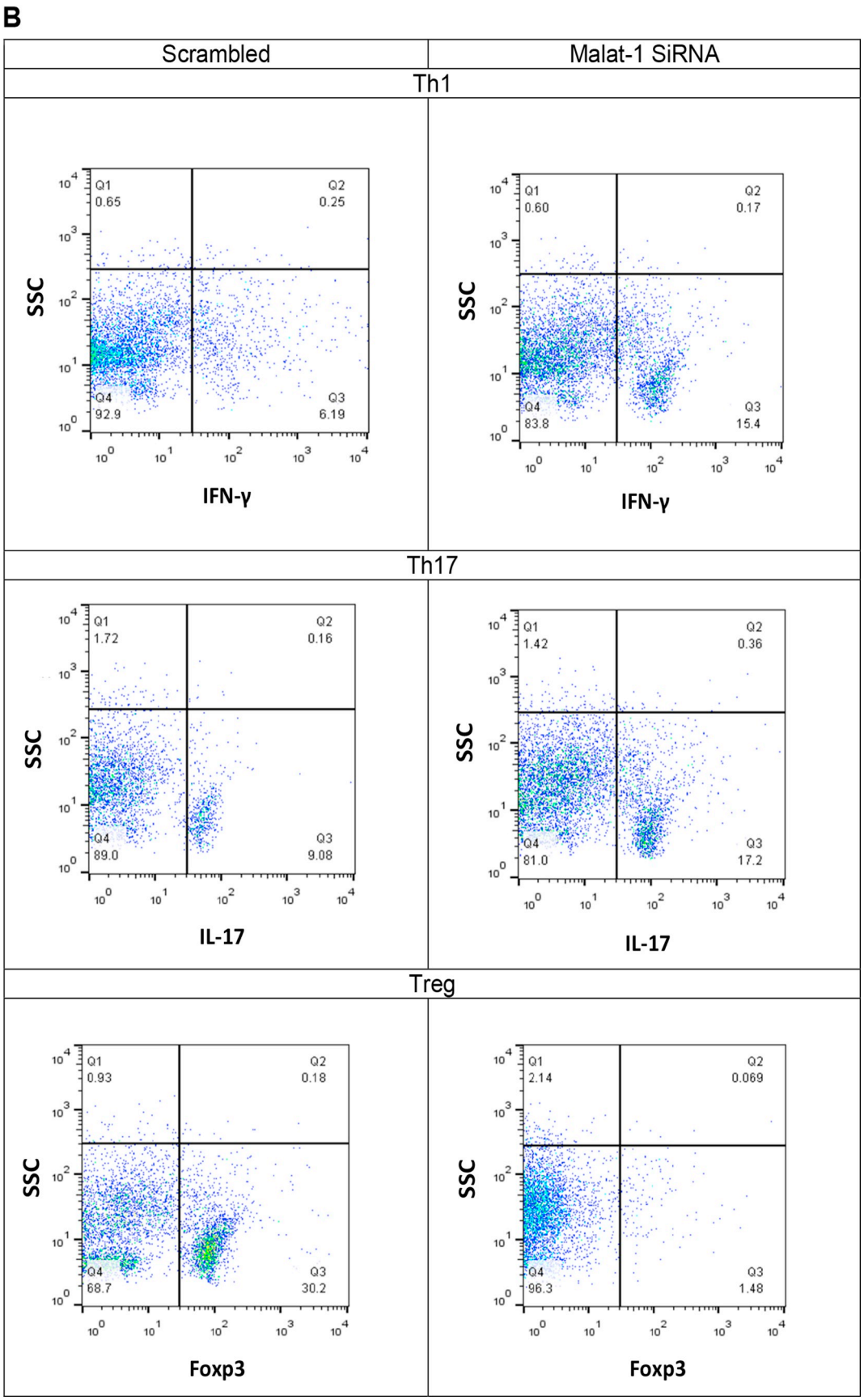
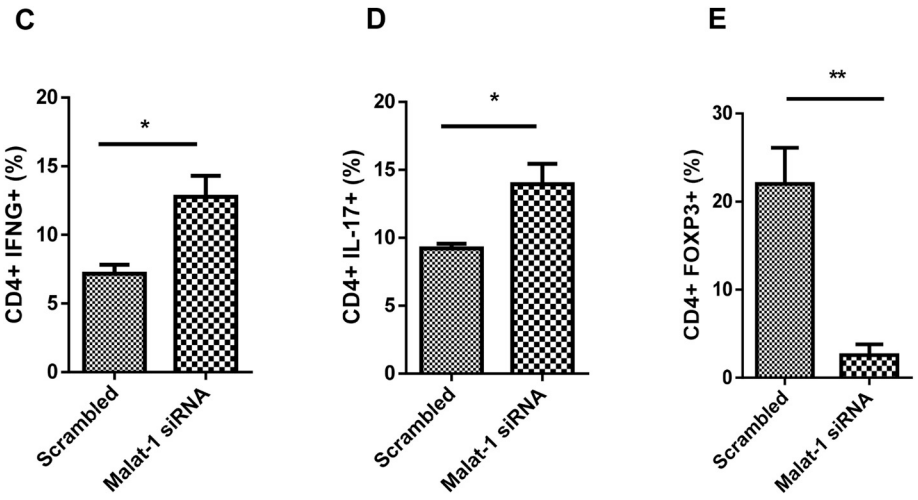


Fig. 3. (*continued*)



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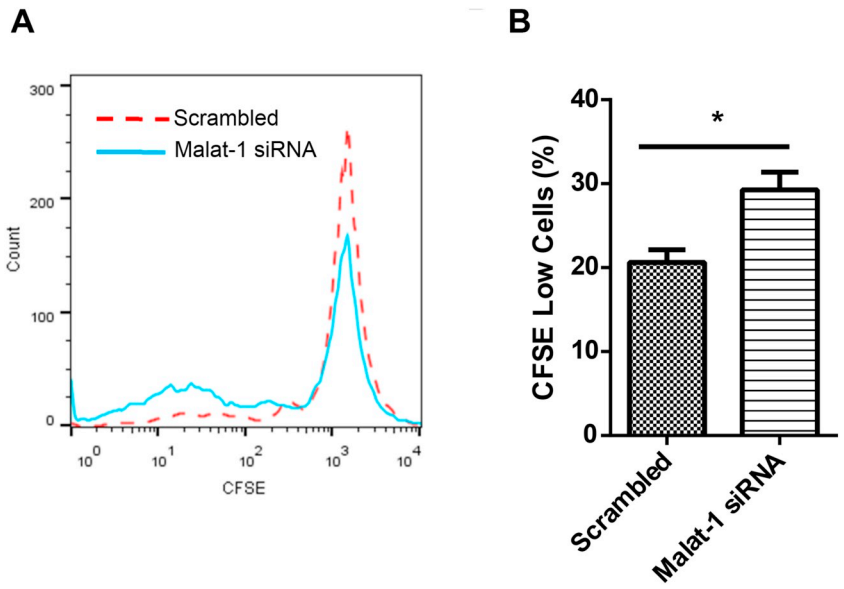

Fig. 4. Malat1 down regulation affects Proliferation of CD4 + T cells. Malat1 or negative control sequences (scrambled) were transfected into CFSE labeled naïve CD4 + T cells, which were then activated using anti-CD3 and anti-CD28. The percent of proliferated cells were analyzed based on CFSE dilution by flow cytometry 4 days later. Representative histogram overlays (A) and corresponding bar graphs are shown. (C). data are presented as mean ± SEM, n = 3 (*p < .05. Kruskal–Wallis test).

(Fig. 1a). Of note, previous microarray analyses on MS brain lesions support decreased *Malat1* expression in diseased CNS tissues (Han et al., 2012) (NCBI Geo database GDS4218). Following experiments on tissue samples, we observed decreased expression levels of *Malat1* in activated primary macrophages and splenocytes. Treatment of macrophages with Malat1-specific siRNAs altered the balance of cell differentiation towards the pro-inflammatory M1 phenotype. Moreover, Malat1 down-regulation in CD4 + T-cells promoted the differentiation of these cells towards pathogenic Th1 and Th17 phenotype and away from Tregs, and it also enhanced proliferative responses of T cells, both effects being consistent with a pro-inflammatory role for Malat1 suppression.

Gene expression alterations that underlie the pathogenesis of inflammatory and autoimmune disorders have long been investigated by researchers in different fields. Transcriptomic analyses performed on cells and tissues from MS patients in late 90s revealed numerous gene expression alterations in MS cells/tissues compared with controls. However, those studies were using cDNA or oligonucleotide microarrays that were chiefly representing protein-coding transcripts. With the advent of RNA-seq methods it became clear that, unlike the traditional view, a substantial fraction of genome-encoded transcripts are non-coding RNAs and alterations in these molecules also carry significant associations with various diseases, including neurological disorders. A PubMed search for “multiple sclerosis and non-coding RNA” retrieves several hundred citations, with an upward trend in the number of publications from 2010. Modifying the search to “multiple sclerosis and non-coding RNA” gives only a limited number of papers (21 papers at the time of writing of this manuscript). Nonetheless, reviewing these papers highlights potentially important roles for lncRNAs in MS disease process. In a study by Zhang et al., researchers performed microarray analyses on lncRNAs (as well as mRNAs) extracted from PBMCs of MS and control cases. These analyses showed hundreds of dysregulated lncRNAs in MS patients' PBMCs (Zhang et al., 2016). Another lncRNA expression profiling on serum samples from MS patients revealed altered expression of several lncRNAs in sera from RR-MS cases (Santoro et al., 2016). In another study Zhang et al. showed that Linc-MAF4 lncRNA facilitated differentiation of Th1 cells while it decreased differentiation of Th2 cells, by inhibiting MAF transcription factor (Zhang et al., 2017a). Other studies have revealed the role of lncRNAs in Th17 cell differentiation in MS/EAE (Guo et al., 2017; Zhang et al., 2018). A recently published work by Sun et al. has also demonstrated the role of GAS5 lncRNA in inhibiting the polarization of microglia towards M2 phenotype and that its blockade could reduce the severity of EAE (Sun et al., 2017). With regard to Malat1, there seems to be no previous reports examining its role in MS/EAE. Indeed, most studies exploring the link between Malat1 and neurological disorders have been performed in the context of neurological malignancies,

where Malat1 has been associated with both tumor suppressive and tumor-promoting functions (Han et al., 2016; Cao et al., 2016; Li et al., 2017; Xiang et al., 2016). Some studies have also indicated protective roles for Malat1 in the context of ischemic stroke (Zhang et al., 2017c; Zhang et al., 2017b). In the context of neurodegenerative disorders, upregulation of Malat1 has been reported in brains of patients with Parkinson's disease (PD) as well as in PD animal models, where it might promote neuronal apoptosis (Kraus et al., 2017; Liu et al., 2017b). Lower levels of Malat1 has also been reported in CSF samples from Alzheimer's patients (Yao et al., 2016). In the current study we detected decreased levels of *Malat1* transcripts in the spinal cord tissues of EAE mice using qPCR. It should be noted that PCR analyses demonstrate the average expression level of transcripts in different cells and provide no information about the cells responsible for altered expression. In situ hybridization (ISH) experiments would be required to determine the cellular localization of transcripts, including lncRNAs. While we did not perform ISH on tissue samples, previous studies have shown that monocytoid cells and lymphocytes express Malat1 at considerable levels. Our expression analyses on primary macrophage and splenocyte cultures also demonstrated expression of this lncRNA in these cells, both being important players in autoimmune neuroinflammation; hence, we focused the rest of our experiments on these cells. That said, the possibility of Malat1 expression in other neural cells (e.g. neurons and astrocytes) is also worth investigating.

Research on non-neurological autoimmune diseases has pointed to pathogenic properties for Malat1 (Liu et al., 2014; Yang et al., 2017). Studies on systemic lupus erythematosus (SLE) have shown increases in Malat1 levels in PBMCs and monocytes of SLE patients compared to healthy controls (Yang et al., 2017). Malat1 has also been considered as a regulator of inflammatory responses in diabetic retinopathy (Biswas et al., 2018). Studies on the role of Malat1 in leukocyte biology have led to controversial results. Zhao et al. have reported that Malat1 is upregulated in macrophages following LPS stimulation, but its knockdown increases LPS-induced expression of TNFα and IL-6 in macrophages. They found that Malat1 interacts with NF-κB in the nucleus, thus inhibiting its DNA binding activity and consequently decreasing the reduction of inflammatory cytokines (Zhao et al., 2016). In our experiments we detected a decrease in Malat1 levels after LPS treatment, but, similar to Zhao et al. study, Malat1 knockdown increased production of inflammatory cytokines. Also in line with our findings, Huang et al. have recently reported that exosomal Malat1 derived from oxLDL-treated endothelial cells could promote M2 macrophage polarization in the context of atherosclerosis (Huang et al., 2018). Conversely, some studies have reported that Malat1 might promote inflammation in different contexts. Of note, Dai et al. have demonstrated that Malat1 knockdown leads to inhibition of inflammatory responses in murine alveolar macrophages, by acting as a molecular sponge for miR-146a (Dai et al., 2018). These discrepancies might reflect multi-faceted nature of this lncRNA's function.

Perhaps the most important part of our results was illustrating the effects of Malat1 in T cell activation and differentiation. We observed that Malat1 suppression could enhance CD4+ T cells proliferative ability. This was associated with significant increases in numbers of IFN-γ-producing Th1 and IL-17-producing-Th17 cells, while the numbers of Foxp3-positive cells were decreased. Studies exploring the role of Malat1 in lymphocyte biology are limited to a few works on lymphocytic malignancies and there is very limited information regarding Malat1 effects on T cell activation and/or differentiation processes. That said, Malat1 has been illustrated to act as a miRNA sponge for several miRNAs, including miR-146, which have effects in T cell differentiation (Dai et al., 2018).

5. Conclusions

The present results suggest that Malat1 lncRNA is down regulated in the CNS of EAE animals at the peak of disease. Decreased Malat1 expression might exacerbate autoimmune neuroinflammation through changing the pattern of macrophage differentiation towards M1 phenotype as well as enhancing T cell differentiation towards Th1 and Th17 cells while impeding the differentiation of regulatory T cells. Altogether, these data introduce Malat1 as a new immunopathogenesis determinant of inflammatory demyelination and a potential target for future therapeutic interventions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2018.11.013.

Declarations of interest

All authors declare that they do not have any conflicts of interest regarding the present studies.

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