MicroRNA-155 modulation of CD8+ T-cell activity personalizes response to disease-modifying therapies of patients with relapsing-remitting multiple sclerosis

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Abstract. Multiple sclerosis (MS) is a chronic autoimmune disease where activated immune cells can attack oligodendrocytes causing damage to the myelin sheath. Several molecular mechanisms are responsible for the auto-activation of immune cells such as RNA interference (RNAi) through microRNAs (miRNAs or miRs). In the present study, the role of miR-155 in regulating CD8+ T-cell activity in patients with relapsing-remitting multiple sclerosis (RRMS) was investigated, in terms of its migratory functions with regard to intracellular adhesion molecule-1 (ICAM1) and integrin subunit β2 (ITGB2), and its cytotoxic proteins, perforin and granzyme B. Gene expression of miR-155, ICAM1, ITGB2, perforin and granzyme B was evaluated following epigenetic modulations using reverse transcription-quantitative polymerase chain reaction in CD8+ T-cells isolated from blood samples of patients with RRMS and compared to healthy controls. The ectopic expression of miR-155 resulted in a persistent downregulation in all genes of interest related to CD8+ T-cell activation that were positively correlated with the Expanded Disability Status Scale of patients. The present study revealed the interplay between miR-155, ICAM1, and ITGB2, shedding light on their beneficial use as possible therapeutic regulators and diagnostic biomarkers of disease. Moreover, epigenetic modulations enhancing the efficacy of disease-modifying therapies (DMTs) may be employed as personalized therapy, to decrease the side effects of DMTs and improve the outcomes of patients.

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Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS), characterized by recurrent episodes of inflammatory demyelination resulting in damage of axons present in the brain, optic nerve, and spinal cord (1,2). There are four types of MS: Clinically isolated syndrome, relapsing-remitting, secondary progressive, and primary progressive (1). A recent disease burden study in Egypt published in 2019, estimated an average of 59,671 patients nationwide (3).

Disease pathogenesis is known to be initiated through the activation of peripheral B and T-cells towards self-antigens resulting in damage to the myelin sheath and nerve block (4). One of the main key players in disease activity is cytotoxic T-cells as they are found to be abundant in MS lesions compared to other subsets of immune cells (5). CD8+ T-cells are known for their killing ability as they produce serine protease granzyme B, responsible for apoptosis in target cells due to loss of cellular integrity (6). This is complemented by the presence of perforin pores facilitating the exit of granzyme B from CD8+ T-cells and its attack on target cells (7). Moreover, CD8+ T-cells express surface receptors such as intracellular adhesion molecule-1 (ICAM1) and integrin subunit β2 (ITGB2/CD18). ICAM1 and ITGB2 provide the secondary signal needed for cellular activation following antigen presentation along with their role in migration through the blood-brain barrier (BBB) (8).

Unfortunately, current immunomodulatory approaches have severe side effects and complications for the patients, since they become more prone to infections due to immune response suppression (9). To overcome the limitations, modern approaches need to target specifically excessive immune responses against self-antigens in autoimmune diseases such as MS by the administration of self-antigens in high doses (10).

A promising therapeutic approach is personalized therapy, that could be achieved through the use of RNA interference, which involves gene silencing at the messenger RNA (mRNA) level mediated by small complementary non-coding RNA species such as small interfering RNAs (siRNAs) or microRNAs (miRNAs or miRs) (11). Upon investigating

promising epigenetics in MS pathogenesis, miR-155 was identified to be a favorable therapeutic target as it was reported to be upregulated in peripheral blood mononuclear cells (PBMCs), the spinal cord, and white matter lesions of patients with MS compared to healthy controls (12-14). Moreover, miR-155 was reported to regulate immune cell activity of innate and adaptive immunity (15). However, miR-155 was revealed to be downregulated in the serum samples of patients with MS in remission compared to patients with post-acute attack MS and upregulated in the PBMCs of patients with MS in remission compared to patients with relapsed MS and healthy controls (16,17). This raises the question as to the role of miR-155 in regulating $CD8^+$ T-cell activity in MS pathogenesis of patients with RRMS. Previous research indicated that a deficiency of miR-155 caused decreased $CD8^+$ T-cell responses, whereas miR-155 overexpression increased $CD8^+$ T-cell responses during inflammation (18). Moreover, $CD8^+$ T-cells lacking miR-155 exhibited reduced frequency of interferon (IFN)-γ production, reduced ability to lyse targets, reduced antigen-specific $CD8^+$ T-cells in cases of viral infection and impaired primary response, hence, the decreased viral clearance (15,19). The aim of the present study was to investigate the role of miR-155 on $CD8^+$ T-cell activity through the monitoring of ICAM1 and ITGB2 levels reflecting migration and activation, along with perforin and granzyme B levels reflecting cytolytic activity on oligodendrocytes.

Materials and methods

Sample collection. Blood samples were collected from 25 patients with RRMS and 10 healthy controls, according to the inclusion and exclusion criteria. Patients diagnosed with RRMS, without treatment with steroids in the past 3 months, were included in the present study. Patients were recruited from May 2019 to May 2020. The mean age of patients was 39.12 years with an age range of 28-55 years, while the mean age of controls was 30.3 years with an age range of 24-50 years. All subjects involved provided their written informed consent, and the Ethics Review Committee of the German University in Cairo (Cairo, Egypt) approved the study (approval no. PTX-2018-11-HET). The study followed the ethical guidelines of the 1975 Declaration of Helsinki. PBMCs were isolated from whole blood using Ficoll density gradient technique. All samples were stored at -80˚C until further use. The clinical characteristics of patients and controls are presented in Tables I, SI and SII.

Ficoll density gradient technique. PBMCs were isolated using Ficoll (Greiner Bio-One International GmbH), as per the manufacturer's instructions. Harvested cells were washed twice in Roswell Park Memorial Institute Medium-1640 (RPMI-1640; cat. no. SR263-10L; Serox GmbH) supplemented with L-glutamine, phenol red, 10% fetal bovine serum (FBS; cat. no. 10270098) and 1% penicillin/streptomycin (cat. no. 15140122; both from Applied Biosystems; Thermo Fisher Scientific, Inc.), and viable cells were counted using a hemocytometer. Cells were frozen at -80˚C at a density of 10^7 cells/ml in 50% v/v supplemented media, 40% v/v FBS and 10% v/v dimethyl sulfoxide (DMSO; cat. no. D12345; Applied Biosystems; Thermo Fisher Scientific, Inc.) for later use. Samples were stored at -80˚C for a maximum of 6 months and after thawing, viability was verified using 0.4% Trypan blue (cat. no. 15250061; Thermo Fisher Scientific, Inc.) with an acceptable viability of >80%.

Isolation of $CD8^+$ T-cells by negative depletion using magnetic nanobeads. Frozen PBMCs were thawed at 37˚C and transferred to 10 ml of supplemented media and centrifuged at 300 x g for 5 min at room temperature. Cells were isolated to obtain $CD8^+$ T-cells by negative depletion using MojoSort™ Human $CD8^+$ T-cell Isolation Kit (cat. no 480012), MojoSort Buffer (cat. no 480017) and MojoSort Magnet (cat. no 480019; all Biolegend, Inc.) as per manufacturer's instructions. Collected pure $CD8^+$ T-cells were centrifuged (at 300 x g for 5 min at room temperature) and re-suspended in culture media.

Flow cytometry. Confirmation of $CD8^+$ T-cell isolation was performed using flow cytometry on the isolated population, and CD8-PE antibody (product no. IM0452U; Beckman Coulter, Inc.) for 30 min at room temperature, followed by a washing step and acquisition. Samples were analyzed by flow cytometry (CytoFLEX benchtop flow cytometer; Beckman Coulter Inc.) gating for the CD8-PE-positive population. Fluorescence data were acquired and analyzed using the CytExpert software (version 2.3.3.84; Beckman Coulter Inc.) to determine the purity of the sample, as shown in Fig. S1 and previously described (20). With regard to the isolation process and the size scatter of the resultant populations, of note, a small percentage of cells (30%), were remaining monocytes and other T-cells that were not completely depleted.

Cell culture. Isolated $CD8^+$ T-cells were incubated in supplemented media at 37˚C with an atmosphere of 5% CO_2 and 95% humidity. The cultured cells were then screened for miR-155, ICAM1, ITGB2, perforin, and granzyme B expression.

Transfection. Before transfection, seeding of $4\text{-}7x10^4$ isolated $CD8^+$ T-cells per well of a 96-well plate was performed. The cells were incubated under normal growth conditions (37˚C and 5% CO_2). Isolated $CD8^+$ T-cells were transfected for 5-10 min at room temperature, with mimics of miR-155 (syn-hsa-miR-155-5p miScript miRNA mimic; cat. no. MSY0000646) and antagomirs of miR-155 (anti-hsa-miR-155-5p miScript miRNA inhibitor; cat. no. MIN0000646), along with both siRNAs of ICAM1 (Hs_ICAM1_3 FlexiTube siRNA; cat. no. SI00004347) and ITGB2 (Hs_ITGB2_3 FlexiTube siRNA; cat. no. SI00004571; all from Qiagen GmbH), in addition to a negative control. The mass of miR-155 mimics and antagomirs, as well as all siRNAs including all negative controls was 250 ng. The negative controls for miRNA mimics and antagomirs were purchased from Invitrogen; Thermo Fisher Scientific, Inc. (cat. nos. AM17110 and AM17010, respectively) and transfected similar to miR-155 mimics and antagomirs. The negative control for siRNA was purchased from Qiagen GmbH (cat. no. 1022076) and was transfected similarly to ICAM1 and ITGB2 siRNA. All transfection experiments were performed in triplicate using HiPerfect Transfection Reagent (cat. no. 301704; Qiagen

Table I. Characteristics of patients and healthy controls.

A, Patients (n=25)	Percentage (%)
Sex	
Female (17/25)	68
Male (8/25)	32
Age	
<50 years old (22/25)	88
≥50 years old (3/25)	12
Family history	
Positive family history (1/25)	4
Negative family history (24/25)	96
Type	
RRMS (15/25)	100
PRMS (0/25)	0
PPMS (0/25)	0
SPMS (0/25)	0
CSF findings	
+ve Oligoclonal antibodies (25/25)	100
Protein (0/25)	0
Treatment	
Untreated (naïve) (3/25)	12
DMT-treated (22/25)	88
IFNβ-1a (6/25)	24
IFNβ-1b (6/25)	24
Fingolimod (10/25)	40

B, Controls (n=10)	Percentage (%)
Sex	
Female (7/10)	70
Male (3/10)	30
Age	
<50 years old (9/10)	90
≥50 years old (1/10)	10

GmbH) according to the manufacturer's instructions, and experiments were repeated three times. Cells exposed to transfection reagent only were designated as mock cells, cells transfected with miR-155 mimics and antagomirs were designated as mimics and antagomirs, respectively, and cells transfected with ICAM1 and ITGB2 siRNA were designated as siICAM1 and siITGB2 cells. Negative controls transfected with pre-miR negative control, anti-miR negative control and negative control siRNA were designated as pre-miR NC, anti-miR NC and siRNA NC, respectively. siRNA NC was not utilized in silencing experiments as it is widely interchanged with pre-miRNA negative controls (as they have the same makeup), hence the data obtained from the pre-miRNA were proof enough. This was followed by RNA extraction, screening for miR-155, ICAM1, ITGB2, perforin, and granzyme B expression, and finally, comparison to $CD8^+$ T-cell mock cells, 48 h after transfection.

RNA isolation. RNA was isolated from cultured $CD8^+$ T-cells using RNeasy Minikit (cat. no. 74104; Qiagen GmBH) as per the extraction protocol. RNA was stored at -80˚C until further use. RNA concentration was calculated using Nanodrop and RNA purity was evaluated using A260/280 with an acceptable range of 1.9-2.2. Total RNA used per sample was 30-50 ng.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA extracted was reverse-transcribed into single-stranded cDNA using the high-capacity cDNA reverse transcription kit (cat. no. 4368814; Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative expression of ICAM1, ITGB2, perforin and granzyme B, with β-actin (as a housekeeping gene for normalization), along with miR-155 and RNU6 (as a housekeeping gene for normalization) was quantified and amplified using TaqMan RT-quantitative polymerase chain reaction (qPCR; Assay IDs: Hs00164932_m1, Hs00164957_m1, Hs00169473_m1, Hs00188051_m, and Hs99999903_m1 respectively for genes of interest along with 002623 and 001093 for miR-155 and RNU6, respectively; Applied Biosystems; Thermo Fisher Scientific, Inc.) on a StepOne™ Real-Time PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). For every sample, a reaction mix was prepared according to the manufacturer's instructions, and 4 μl of the respective cDNA was added. The RT-qPCR run was performed in the standard mode, consisting of two stages: A first 10-min stage at 95˚C where the Taq-polymerase enzyme was activated, followed by a second stage of 40 amplification cycles (15 sec at 95˚C and 60 sec at 60˚C). qPCR runs with negative controls as undetermined were taken into account, relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (21). All PCR reactions including controls were run in triplicate.

Statistical analysis. All data were expressed in relative quantitation (RQ). One Way ANOVA was employed, followed by Dunnett's multiple comparison test to compare the basal expression of two different studied groups. Unpaired t-test was used to compare the effect of manipulations within each group (compared to mock). Data were expressed as the mean ± standard error of the mean (SEM). Correlation analyses were performed using Spearman's correlation coefficient, denoted by a rho value, indicating that when the strength of the correlation approaches 1, the degree of correlation increases. Analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, Inc.). All experiments were performed in triplicate. $P<0.05$ was considered to indicate a statistically significant difference.

Bioinformatics analysis. Target prediction was performed using Tools for miRs (https://tools4mirs.org/software/target_prediction/), which included the algorithm Probability of Interaction by Target Accessibility (PITA), and TargetSpy (http://webclu.bio.wzw.tum.de/targetspy/index.php?search=true). Hits found between miR-155 and genes of interest are reported in Table SIII.

Results

Effect of miR-155 overexpression and knockdown on the mRNA expression of ICAM1, ITGB2, perforin and granzyme B in cytotoxic T-cells of patients with RRMS. First, to understand the

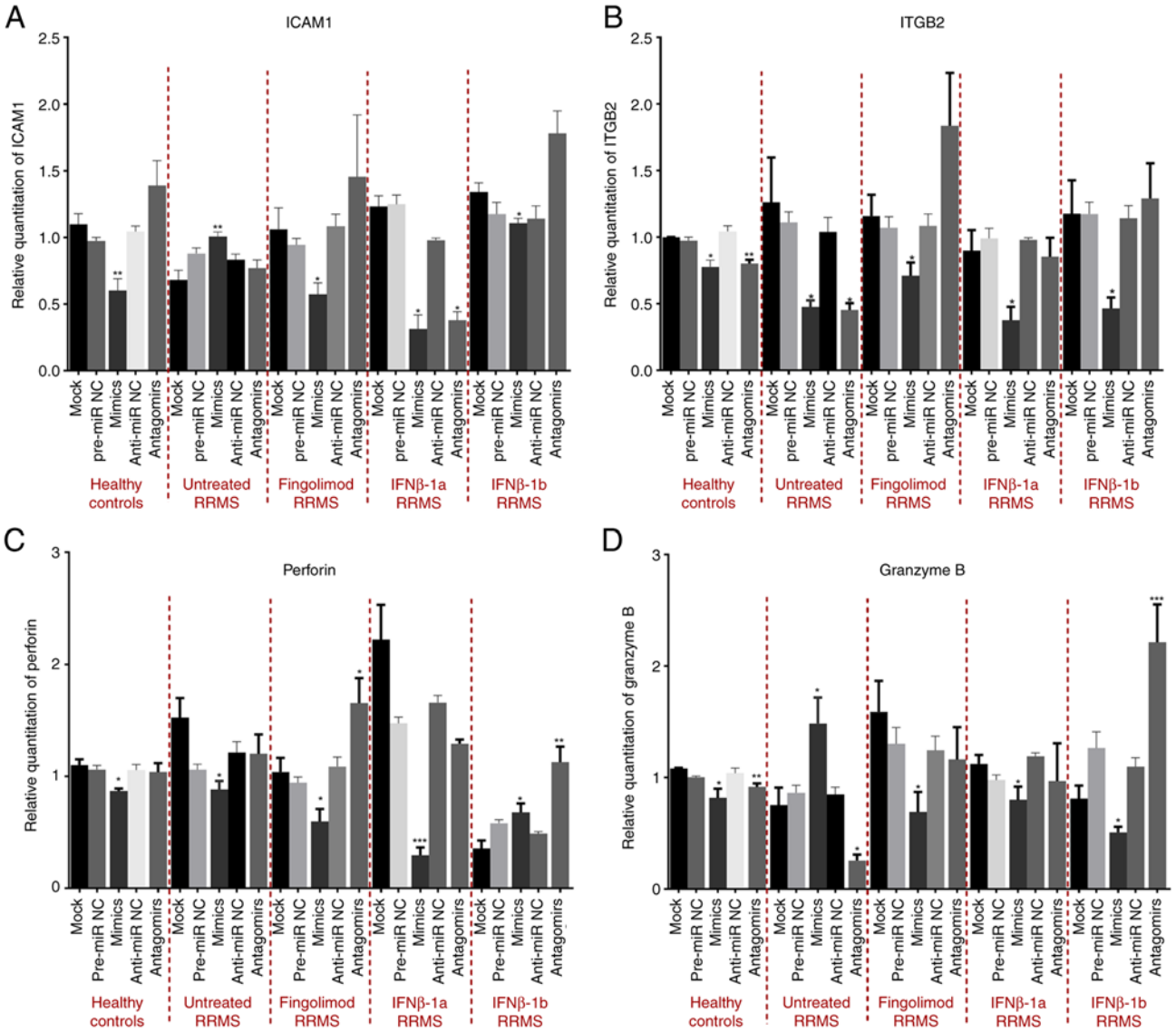

Figure 1. Effect of miR-155 overexpression and knockdown on the mRNA levels of ICAM1, ITGB2, perforin, and granzyme B in CD8+ T-cells isolated from different treatment groups of patients with relapsing-remitting multiple sclerosis and healthy controls. (A-D) Effect of miR-155 overexpression and knockdown on the mRNA levels of (A) ICAM1, (B) ITGB2, (C) perforin and (D) granzyme B compared to the mock group. *P<0.05, **P<0.01 and ***P<0.001. Data are presented as the mean ± standard error of the mean. miR-155, microRNA-155; ICAM1, intracellular adhesion molecule-1; ITGB2, integrin subunit β2; RRMS, relapsing-remitting multiple sclerosis; NC, negative control.

relationship between miR-155 and the genes of interest, bioinformatics studies were performed and interactions between miR-155 and genes of interest were found and reported in Table SIII. The expression profile of miR-155, ICAM1, ITGB2, perforin and granzyme B in cytotoxic T-cells isolated from different treatment groups of patients with RRMS is presented in a previous study (20). Subsequently, the effect of miR-155 on the expression of ICAM1, ITGB2, perforin and granzyme B was studied through the overexpression and knockdown of miR-155 *ex vivo*. Efficient overexpression of miR-155 was confirmed in cultured cells as shown in Fig. S2 (P=0.0008). As a result of miR-155 overexpression using mimics, a significant downregulation of ICAM1 mRNA was observed in healthy controls, and all patients with RRMS, treated with fingolimod, IFNβ-1a, and IFNβ-1b (P=0.0048; P=0.0161; P=0.0097; and P=0.0248; respectively) compared to mock. However, cells from naïve RRMS patients exhibited a significant increase in ICAM1 mRNA following miR-155 overexpression (P=0.0073) compared to the mock group. Of note, anti-miR-155 produced no significant changes except in IFNβ-1a-treated patients, with anti-miR-155 exhibiting similar effects to mimics (Fig. 1A). Moreover, miR-155 mimic-transfection resulted in significant consistent downregulation of ITGB2 mRNA in healthy controls and all patients with RRMS, including naïve-, fingolimod-, IFNβ-1a-, and IFNβ-1b-treated patients (P=0.0133; P=0.04011; P=0,0250; P=0.0224; and P=0.0214; respectively), compared to the mock group. Conversely, anti-miR-155 caused no significant changes except for healthy controls and RRMS-naïve patients, where anti-miR-155 exhibited similar effects to mimics (Fig. 1B). In addition, miR-155 mimic-transfection induced a downregulation in perforin mRNA levels in healthy controls and RRMS naïve-, fingolimod-, and IFNβ-1a-treated patients (P=0.0137; P=0.0153; P=0.0206; and P=0.0001) with an unexpected upregulation the the mRNA

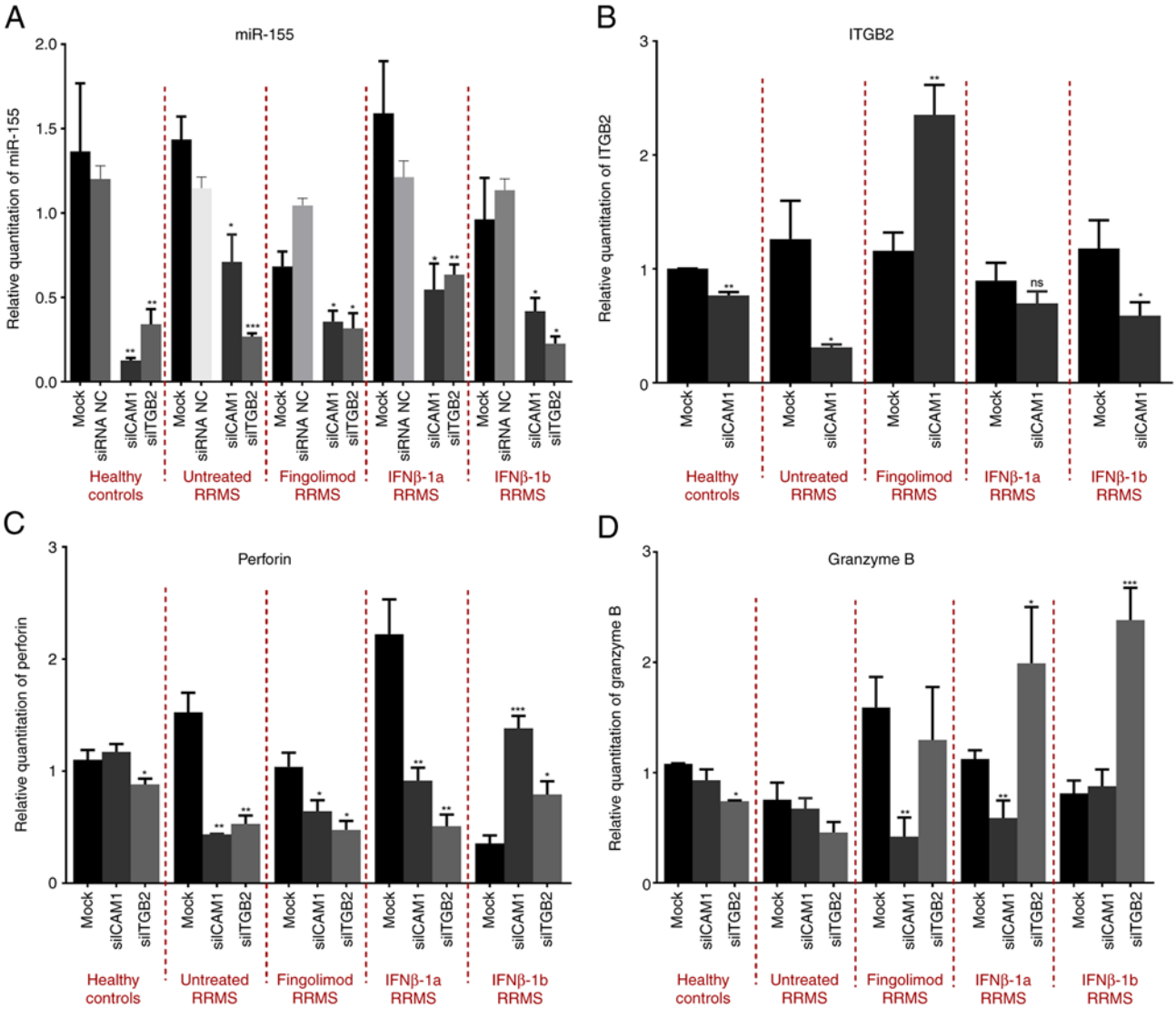

Figure 2. Effect of ICAM1 and ITGB2 knockdown on the mRNA levels of miR-155, ITGB2, perforin, and granzyme in $CD8^+$ T-cells isolated from different treatment groups of patients with relapsing-remitting multiple sclerosis and healthy controls. (A-D) Effect of ICAM1 and ITGB2 knockdown on the mRNA levels of (A) miR-155, (B) ITGB2, (C) perforin and (D) granzyme B compared to the mock groups. *P<0.05, **P<0.01 and ***P<0.001. Data are presented as the mean ± standard error of the mean. ICAM1, intracellular adhesion molecule-1; ITGB2, integrin subunit β2; miR-155, microRNA-155; siRNA or si, small interfering RNA; NC, negative control; RRMS, relapsing-remitting multiple sclerosis; ns, not significant.

levels of perforin in IFNβ-1b-treated patients (P=0.0340) compared to the mock group. Anti-miR-155 caused no significant changes, except for fingolimod-treated patients where a significant opposite effect to mimics was observed and in IFNβ-1b patients, where anti-miR-155 exhibited a similar effect to mimics (Fig. 1C). Finally, overexpression of miR-155 caused a significant decrease in granzyme B mRNA in healthy controls and all patients with RRMS, treated with fingolimod, IFNβ-1a and IFNβ-1b (P=0.0345; P=0.0118; P=0.0334; and P=0.0397; respectively) except for naïve patients with RRMS, where a significant increase in granzyme B expression was observed following miR-155-mimic transfection (P=0.0405) (Fig. 1D).

Effect of ICAM1 and ITGB2 knockdown on the expression levels of miR-155, perforin and granzyme B. Secondly, to understand the relationship between ICAM1, ITGB2 with miR-155, perforin, and granzyme B, the effect of ICAM1 and ITGB2 knockdown on the expression of miR-155, perforin and granzyme B was investigated. Efficient knockdown of ICAM1 and ITGB2 was confirmed as shown in Fig. S3 (P=0.0367 and P=0.0105, respectively). Silencing of ICAM1 resulted in a significant downregulation of miR-155 expression in healthy controls and all patients with RRMS, including naïve, fingolimod, IFNβ-1a and IFNβ-1b (P=0.0013; P=0.0142; P=0.0243; P=0.0143; and P=0.0405; respectively) treatment groups compared to the mock group. Moreover, knockdown of ITGB2 caused a significant downregulation in miR-155 expression in cells isolated from healthy controls and all patients with RRMS, including naïve, fingolimod, IFNβ-1a and IFNβ-1b treatment groups (P=0.0053; P=0.0001; P=0.0458; P=0.0074; and P=0.0425; respectively) compared to the mock group (Fig. 2A). Investigation of the effect of ICAM1 silencing on ITGB2 revealed a significant downregulation of ITGB2 in healthy controls, naïve and IFNβ-1b patients with RRMS (P=0.0015; P=0.0201; and P=0.0494; respectively) compared

to the mock group. By contrast, a significant increase was observed in the fingolimod-treated RRMS patients (P=0.0019) with a non-significant decrease in IFNβ-1a-treated RRMS patients compared to the mock group (Fig. 2B). In addition, the effect of ICAM1 and ITGB2 knockdown on perforin mRNA was investigated. ICAM1 knockdown caused no significant change in the mRNA levels of perforin in healthy controls, however, it did induce a significant increase in IFNβ-1b-treated RRMS patients (P=0.0006), and a significant decrease in the naïve, fingolimod and IFNβ-1a treatment groups (P=0.0033; P=0.0422; and P=0.0067; respectively) compared to the mock group. Furthermore, ITGB2 knockdown exerted a significant decrease in perforin mRNA levels in healthy controls and naïve patients with RRMS, as well as groups treated with fingolimod and IFNβ-1a (P=0.0311; P=0.0022; P=0.0125; and P=0.0019; respectively), and a significant increase in IFNβ-1b-treated patients with RRMS (P=0.0352) compared to the mock group (Fig. 2C). With regard to the effect of ICAM1 silencing on granzyme B mRNA levels, no significant change in healthy controls, naïve and IFNβ-1b-treated RRMS patients was observed, while a significant decrease was observed in the groups treated with fingolimod and IFNβ-1a (P=0.0057 and P=0.0075, respectively) compared to the mock group. Furthermore, silencing of ITGB2 resulted in a decrease in granzyme B mRNA levels in healthy controls, a non-significant change in naïve and fingolimod-treated RRMS patients and a significant increase in RRMS patients treated with IFNβ-1a and IFNβ-1b (P=0.0213 and P<0.0001, respectively) compared to the mock group (Fig. 2D).

Correlation analysis between the effect of miR-155 overexpression on target genes and the expanded disability status scale (EDSS) score of patients. In a previous study, correlation analyses revealed a positive correlation between miR-155 and ITGB2 with the EDSS of patients and a negative correlation between ICAM1, perforin, and granzyme B with the EDSS (20). Moreover, the correlation between miR-155 and genes of interest *ex vivo* showed a consistent negative correlation between miR-155 and genes of interest in patients with RRMS (20). Finally, to investigate whether the clinical score of a patient could affect the manipulation outcome, correlation analysis was performed between the fold change (RQ) in the genes of interest following miR-155 overexpression and the EDSS of patients, using Spearman's correlation coefficient. The analysis revealed a consistent positive correlation between the effect of miR-155 on ICAM1, ITGB2, perforin and granzyme B with the EDSS of patients (P=0.0082; P=0.0188; P=0.0003; and P=0.0045; respectively and rho=0.5738; rho=0.4489; rho=0.6586; and r=0.4697, respectively), raising the question as to whether patients with high clinical scores may be responding differently to treatments than patients with low EDSS scores (Fig. 3).

Discussion

MS is a chronic neuroinflammatory disease and considered one of the leading causes of disability worldwide. Due to the heterogeneity of the disease, an optimized targeted therapeutic approach is required to achieve efficient treatments for the diverse subpopulations of the disease. Molecular proteins of interest to regulate are CD8+ T-cell surface receptors, ICAM1 and ITGB2, along with cytotoxic proteins produced by the cells, perforin and granzyme B (7,22). Accumulating evidence has demonstrated non-coding RNAs as pivotal tools in targeting the molecular make-up of MS pathogenesis and miR-155 has multiple roles in innate and adaptive immunity (15,23,24). Its role in carcinogenesis has been studied previously in various cancers such as hepatocellular carcinoma (HCC), and its immunomodulatory role in regulating the programmed cell death protein 1 (PD-1), programmed death ligand 1 (PDL-1) pathway has been highlighted (25,26). Specific upregulation of miRNA-155 is witnessed in various immunopathologic conditions including MS (27), rheumatoid arthritis (28), and systemic lupus erythematosus (29,30) where it affects both T lymphocyte and blood-brain barrier functions (31).

Prior to investigating the role of miR-155 in the regulation of crucial proteins for CD8+ T-cells, a screening step for the basal expression levels of miR-155, ICAM1, ITGB2, perforin, and granzyme B in CD8+ T-cells was performed to identify the endogenous levels of these genes. Significant downregulation of miR-155 was observed in CD8+ T-cells of patients with RRMS (20). The downregulation in miR-155 in isolated CD8+ T-cells coincides with previous studies reporting variation in miRNA expression in CD8+ T-cells during the differentiation process and an inverse correlation with activation status (32-34). Moreover, upregulation of ICAM1, ITGB2, perforin, and granzyme B was observed in all patients with RRMS compared to healthy controls (20). This upregulation of ICAM1 and ITGB2 [β sub-unit of lymphocyte function-associated antigen 1 (LFA-1)] is consistent with previous results showing overexpression of ICAM1 and LFA-1 on mononuclear cells from the blood of patients with RRMS compared to controls (35). Another interesting study by Fujii *et al* studied the levels of cytotoxic proteins perforin and granzyme B in patients administered fingolimod and found a significant increase in perforin and granzyme B expression in both relapse-free and relapsing patients with higher overexpression in the latter compared to the healthy controls (36). To the best of our knowledge, the present study is the first to investigate the interplay of the aforementioned genes of interest on CD8+ T-cells of MS patients.

The role of miR-155 on CD8+ T-cell auto-activity and cytotoxicity was studied by *ex vivo* overexpression and knockdown of miR-155 in isolated CD8+ T-cells of patients with RRMS of different treatments representing the effect of the epigenetic manipulation on the four target genes in each subtype of patients with RRMS. miR-155 mimic transfection induced a downregulation in the mRNA of ICAM1 in all subtypes except naïve RRMS patients (Fig. 1A) and a downregulation in the mRNA levels of ITGB2 in all subtypes of RRMS (Fig. 1B). The inconsistency in the effect of miR-155 on ICAM1 could be an indirect mechanism of the effect of miR-155 on leukocyte adhesion other than regulating gene expression of adhesion molecules as stated previously by Cerutti *et al* (37). Moreover, miR-155 mimic transfection caused a significant downregulation in the mRNA expression of perforin in all groups except for IFNβ-1b-treated RRMS patients (Fig. 1C) and a downregulation in the mRNA expression of granzyme B in all groups except for untreated patients with RRMS (Fig. 1D). An inconsistent pattern in the effect of miR-155 on pro-inflammatory

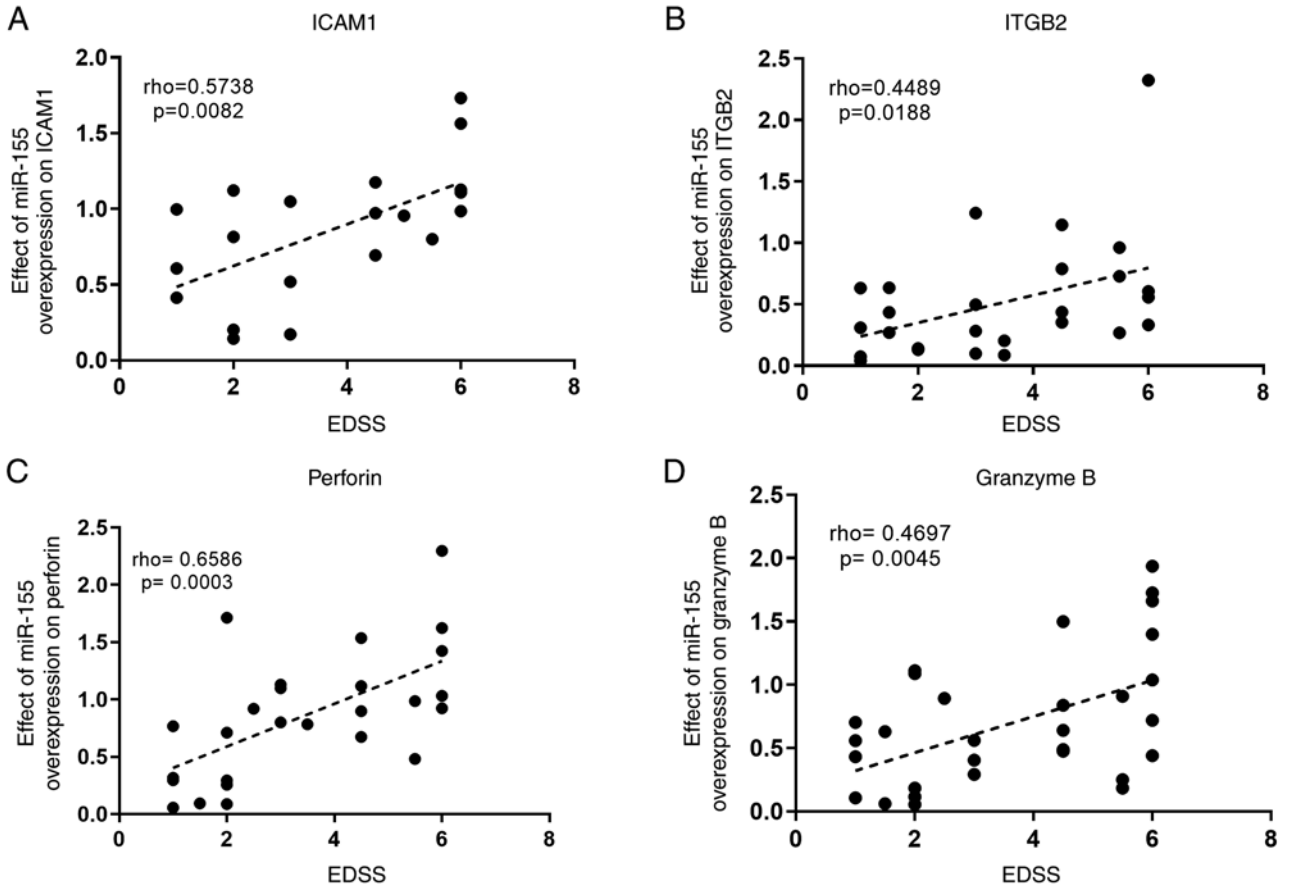

Figure 3. Correlation analysis between the relative expression of target genes following miR-155 overexpression, normalized to the mock groups, and the EDSS score of patients, determined using Spearman's correlation coefficient. (A-D) Correlation between the effect of miR-155 overexpression on (A) ICAM1, (B) ITGB2, (C) perforin and (D) granzyme B expression, as the RQ values of each gene normalized to the mock groups in $CD8^+$ T-cells isolated from patients with relapsing-remitting multiple sclerosis, and the EDSS score of patients. Correlation analysis was performed using Spearman's correlation coefficient, and the rho values and P-values are presented in each graph. miR-155, microRNA-155; ICAM1, intracellular adhesion molecule-1; ITGB2, integrin subunit β2; EDSS, Expanded Disability Status Scale.

mediators was observed in previous studies on $CD8^+$ T-cells in viral infection settings, where miR-155 knockdown decreased IFN-γ production and had no effect on granzyme B and TNF-α levels (19). Nevertheless, another study revealed a significant decrease in IFN-γ and granzyme B levels in $CD8^+$ T-cells in miR-155-knockdown mice following viral infection induction (38). Building on the complex effects of miR-155, miR-155 knockdown in an RA mouse model had no effect on the levels of IFN-γ following induction of disease in those mice (39). Another study investigating the role of miR-155 in PBMCs isolated from juvenile systemic lupus erythematosus (SLE) patients reported an anti-inflammatory response as the upregulation of miR-155 relieved the immune modulator IL-2 from the inhibitory effect of PP2A (40). These conflicting results give rise to the hypothesis that miR-155 has a diverse, non-specific role in regulating $CD8^+$ T-cell immune response depending on the differentiation stage of the cell.

Furthermore, the role of ICAM1 and ITGB2 in the regulation of cytolytic proteins perforin and granzyme B as well as miR-155, was investigated. The silencing of ICAM1 and ITGB2 induced significant downregulation of miR-155 compared to the mock group in all patients with RRMS and healthy controls (Fig. 2A). Additionally, ICAM1 silencing caused an inconsistent downregulation of ITGB2, perforin and granzyme B (Fig. 2B-D). Moreover, ITGB2 silencing induced an inconsistent downregulation of ICAM1 and a consistent downregulation of perforin (Fig. 2C). The inconsistent increase in perforin mRNA levels in IFNβ-1b-treated patients following all manipulations could be due to the increase in the number of perforin-dependent $CD8^+$ T-cells in this subtype. A previous study investigating the effects of β integrins on other adhesion molecules revealed that stimulation of β1 integrin by cross-linking or ligation with matrix proteins reduced ICAM1 expression in lung cancer cell lines (41). If the same relationship applies herein, then the increase in the expression of ICAM1 or ITGB2 following the silencing of either, is expected. However, immune cells rather than cancer cells are in question hence, this could explain the different results.

As aforementioned, ICAM1 expression on antigen-presenting cells or T lymphocytes is crucial for antigen-specific interactions leading to $CD8^+$ T-cell activation, proliferation, and differentiation into effector T-cells (42). The results of ICAM1 silencing are consistent with previous studies indicating that ICAM-1 expression is critical on T-cells and other cell types for the development of demyelinating disease (43). Additionally, the previous deletion of Mac-1 (CD11b/CD18) resulted in profound protection in both active and adoptive-transferred EAE, indicating that Mac-1 (partially CD18) expression is

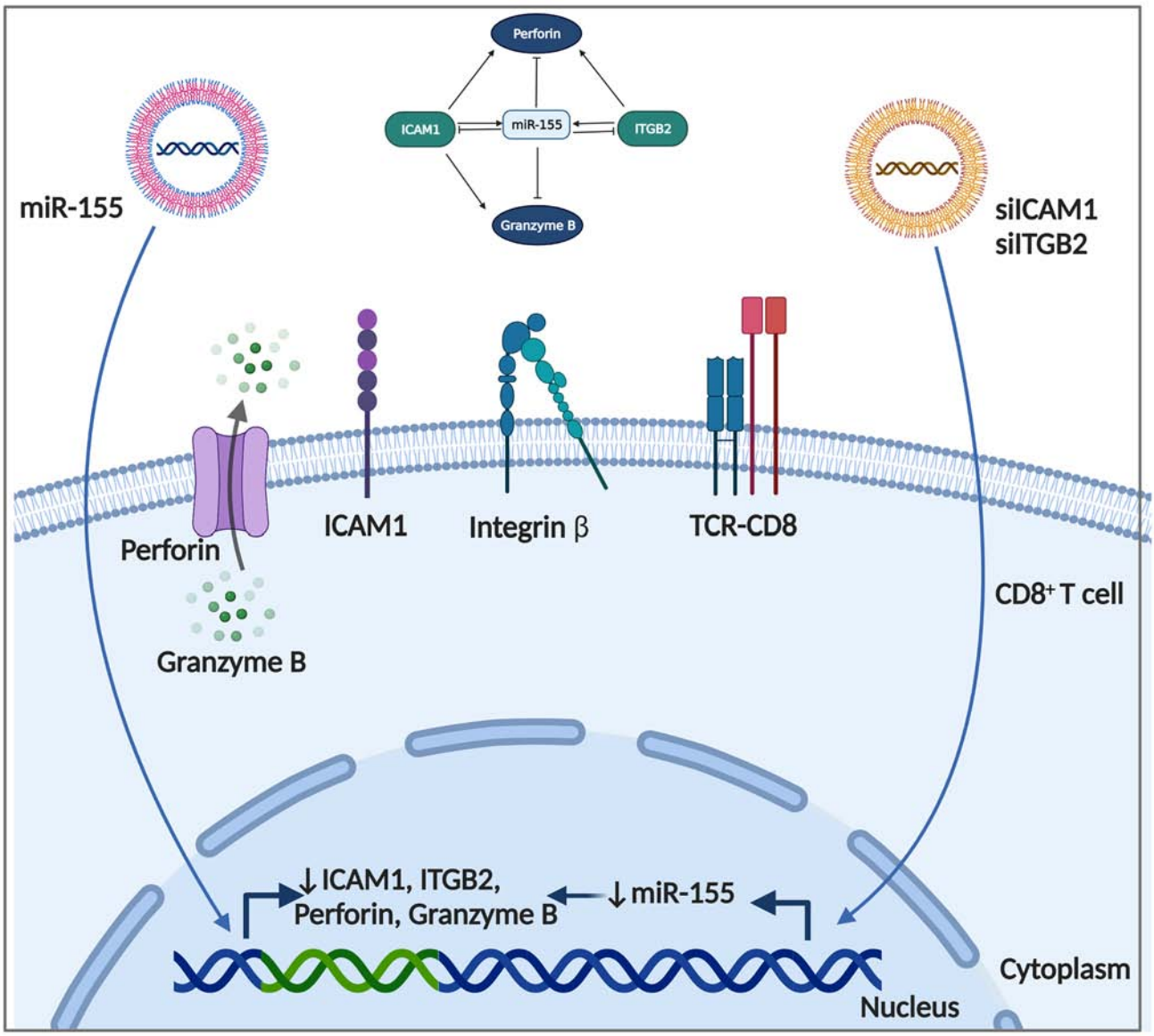

Figure 4. Summary of the regulatory roles of miR-155 mimics, siICAM1 and siITGB2 on ICAM1, ITGB2, perforin and granzyme B. The inhibitory effect of miR-155 on ICAM1, ITGB2, perforin and granzyme B, as well as that of siICAM1 on miR-155, ITGB2, perforin and granzyme B, and that of siITGB2 on miR-155 and perforin is illustrated. Created by Biorender. miR-155, microRNA-155; si, small interfering RNA; ICAM1, intracellular adhesion molecule-1; ITGB2, integrin subunit β2.

critical not only to phagocytic cells but also to T-cells for the development of demyelinating disease, concluding that Mac-1 is an important integrin target for MS immunotherapy (44). Collectively, the results confirm the hypothesis that silencing of ICAM1 and ITGB2 could be of therapeutic value in modulating cytotoxic T-cells of patients with MS.

Interestingly, ICAM1 silencing caused similar changes to miR-155 overexpression and miR-155 overexpression caused a decrease in ICAM1 expression in all treated subtypes which suggests that the modulations observed with miR-155 overexpression could be due to ICAM1 downregulation rather than miR-155 manipulation. This indicates that ICAM1 may have a dominant effect in modulating the aforementioned target genes in CD8+ T-cells of treated patients with MS. For further insight, it was also examined whether the disease state affects the manipulation outcomes, hence the same manipulations on CD8+ T-cells isolated from healthy controls were performed. The genetic and epigenetic manipulations performed caused similar outcomes in all diseased cells and healthy controls cells with two exceptions. First, the upregulation of ICAM1 in untreated naïve patients following miR-155-mimic transfection (Fig. 1A) could be due to the increased expression of the endogenous levels as observed in the previous screening of ICAM1 (20). Second, the upregulation of perforin following miR-155 overexpression as well as ICAM1 and ITGB2 knockdown in the IFNβ-1b-treated subtype (Figs. 1C and 2C) could be due to the increased upregulation of perforin in those samples before manipulation as observed in the previous screening of perforin (20).

Relating the experimental data obtained to the clinical data of the patients was intriguing, hence, correlation studies between mRNA expression of miR-155, target genes, and the EDSS of the patients were carried out. The positive correlation between miR-155, ITGB2, and EDSS, and the negative

one with ICAM1, perforin, and granzyme B, determined in a previous study by the authors, could be further exploited to enhance the use of these molecules as biomarkers for diagnostic and prognostic purposes (20). Moreover, in this previous study, the negative correlation between miR-155 and the target genes reflects the results observed during the *ex vivo* experiments of the present study (20). Considering the probability of personalized, optimized therapy, correlating the effect of miR-155 overexpression on the expression of target genes with the EDSS of patients revealed a significant positive correlation between the effect of miR-155 overexpression on all genes and the EDSS of patients (Fig. 3) leading to the theory that the manipulation of miR-155 could be more effective in patients with high EDSS. It is also worth mentioning that this is the first reported correlation study discussing miR-155 and the target genes. If miR-155 could really downregulate the expression of surface receptors responsible for migration and target attack, or cytolytic proteins responsible for destruction, then it could be one of the targets to be used to downregulate those key players in $CD8^+$ T-cells. This would decrease their migration through the BBB following activation and their attack on oligodendrocytes following migration. Regarding the vulnerability of patients to infections following $CD8^+$ T-cell manipulation, this is unfortunately the case with most immunomodulatory drugs. A method to tone down the activated immune system against the oligodendrocytes of patients may be a first approach until research discovers selective activation markers or auto-receptors present on immune cells activated against self-antigens only.

Considering the multi-target influence afforded by a single miRNA, it is reasonable to hypothesize that studies directed at establishing the effect of drugs on miRNA gene expression could disclose possible unrevealed, to date, modes of action of drugs (45). This explains the aim of screening for the expression of miR-155 throughout different treatments. The differences in results between treatment groups reveal the potential role of epigenetic modulations in treatment outcome and efficacy. Hence, a biomarker for treatment responses in MS would be of considerable clinical value. Thus, prospective studies using cohorts of patients with MS at different stages of disease would validate whether miR-155 could fulfill this additional role.

In conclusion, the *ex vivo* overexpression of miR-155 in $CD8^+$ T-cells caused significant downregulation of pro-inflammatory ICAM1, ITGB2, perforin, and granzyme B expression, indicating a probable anti-inflammatory role of the recognized to be pro-inflammatory miRNA (Fig. 4). Interestingly, the knockdown of ICAM1 and ITGB2 caused downregulation of miR-155 and a similar anti-inflammatory profile to that observed with miR-155 overexpression, suggesting that the changes observed during overexpression could be a result of ICAM1 downregulation rather than the direct effect of miR-155 modulation. Future recommendations involve a larger cohort in a longitudinal study setting, where patients are followed prior to and further into treatment, to identify cellular and molecular changes occuring due to treatments. The present study revealed the interplay between miR-155, ICAM1, and ITGB2, paving the road for their beneficial use as probable therapeutic regulators and diagnostic biomarkers of disease.

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Availability of data and materials

Data is contained within the article or supplementary material. The data presented in this study are available in Tables SI-III and Figs. S1-S3.

Authors' contributions

AAE carried out all the experiments, analyzed the data and contributed to the writing of the manuscript. DAZ is the clinical neurologist who provided all samples and clinical data, and contributed to the data acquisition and revision of manuscript drafts. HMET is the principal investigator and the main supervisor of this research work, and contributed to the conception and design of the work, revising and approving the drafts and final version of the manuscript. AAE and HMET confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was conducted according to the guidelines of the Declaration of Helsinki, and approved (approval no. PTX-2018-11-HET) by Ethics Committee of the German University in Cairo (Cairo, Egypt). All subjects involved provided their written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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