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SIRT1 is decreased during relapses in patients with multiple sclerosis

Cosmin A. Tegla [b,a], Philippe Azimzadeh [a], Maria Andrian-Albescu [a], Alvaro Martin [a], Cornelia D. Cudrici [a], Richard Trippe III [a], Adam Sugarman [a], Hegang Chen [d], Dallas Boodhoo [a], Sonia I. Vlaicu [a], Walter Royal III [a,e], Christopher Bever [a,b,e], Violeta Rus [c], Horea Rus [a,b,e,*]

[a] Department of Neurology, University of Maryland, School of Medicine, Baltimore, MD, USA
[b] Research Service, Veterans Administration Maryland Health Care System, Baltimore, MD, USA
[c] Department of Medicine, Division of Rheumatology and Clinical Immunology, University of Maryland, School of Medicine, Baltimore, MD, USA
[d] Department of Epidemiology and Public Health, University of Maryland, School of Medicine, Baltimore, MD, USA
[e] Veterans Administration Multiple Sclerosis Center of Excellence, Baltimore, MD, USA

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ABSTRACT

SIRT1 is a member of the histone deacetylase (HDAC) class III family of proteins and is an NAD-dependent histone and protein deacetylase. SIRT1 can induce chromatin silencing through the deacetylation of histones and can modulate cell survival by regulating the transcriptional activities. We investigated the expression of SIRT1 in multiple sclerosis (MS) brains and in peripheral blood mononuclear cells (PBMCs) obtained from patients with relapsing–remitting multiple sclerosis. We found that SIRT1 was expressed by a significant number of cells in both acute and chronic active lesions. We also found that $CD4^+$, $CD68^+$, oligodendrocytes (OLG), and glial fibrillar acidic protein $(GFAP)^+$ cells in MS plaques co-localized with SIRT1. Our results show a statistically significant decrease in SIRT1 mRNA and protein expression in PBMCs during relapses when compared to the levels in controls and stable MS patients. On the other hand, HDAC3 expression was not significantly changed during relapses in MS patients. SIRT1 expression correlated with that of histone H3 lysine 9 acetylation (H3K9ac) and methylation (H3K9me2). SIRT1 mRNA expression was significantly reduced after RGC-32 silencing, indicating a role for RGC-32 in the regulation of SIRT1 expression. Furthermore, we investigated the role of SIRT1 in the expression of FasL and found a significant increase in FasL expression and apoptosis after inhibition of SIRT1 expression. Our data suggest that SIRT1 may represent a biomarker of relapses and a potential new target for therapeutic intervention in MS.

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Introduction

Multiple sclerosis (MS) is a demyelinating disease characterized by chronic inflammation of the central nervous system in which many factors (genetic and environmental) may act together to influence disease susceptibility and progression (Frohman et al., 2006; Keegan and Noseworthy, 2002). While a large body of work has enhanced our understanding of the fundamental nature of MS, basic research into its etiology, pathophysiology, and treatment faces enormous challenges, and this may in part be due to the great variability in the clinical presentation and course of MS (Compston and Coles, 2008; Frohman et al., 2006; Keegan and Noseworthy, 2002).

Epigenetic regulators such as histone deacetylases (HDACs) and histone acetyltransferases are increasingly being implicated as direct or indirect components of the regulation of expression of neuronal, immune, and other tissue-specific genes (Koch et al., 2013a; Wang et al., 2013). Post-translational modifications of histone proteins have the ability to affect chromatin structure and regulate gene expression (Koch et al., 2013b; Sengupta and Seto, 2004). Recently, the HDAC inhibitor trichostatin A (TSA) was shown to ameliorate the disease course in experimental autoimmune encephalomyelitis (EAE). Using microarrays and real time-PCR to assess in vivo spinal cord gene regulation by this HDAC inhibitor, multiple genes were found to be up-regulated by TSA in the spinal cords of EAE mice, including anti-oxidant, neuroprotective, and neuronal differentiation genes (Camelo et al., 2005). In addition, the effect of sirtuin 1 (SIRT1) on EAE has recently been investigated (Nimmagadda et al., 2013). SIRT1 is a member of the HDAC class III family of proteins (Smith et al., 2000). It is an NAD^+-dependent histone and protein deacetylase (Penberthy and Tsunoda, 2009; Smith et al., 2000) that catalyzes the removal of acetyl groups from a variety of protein

Abbreviations: MS, Multiple sclerosis; RR, Relapsing–remitting; HDAC3, Histone deacetylase 3; p-HDAC3, Phosphorylated histone deacetylase 3; SIRT1, Sirtuin 1; p-SIRT1, Phosphorylated sirtuin 1; EAE, Experimental autoimmune encephalomyelitis; TSA, Trichostatin A; CNS, Central nervous system; NAD, Nicotinamide adenine dinucleotide; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; FOXO, Forkhead box gene; PBMC, Peripheral blood mononuclear cells; FasL, Fas Ligand; RGC-32, Response gene to complement-32; GFAP, Glial fibrillar acidic protein; MBP, Myelin basic protein; LFB, Luxol fast blue; NAGM, Normal-appearing gray matter; NAWM, Normal-appearing white matter; OLG, Oligodendrocyte.

* Corresponding author at: University of Maryland, School of Medicine, Department of Neurology, 655 W Baltimore St, BRB 12-016, Baltimore, MD 21201, USA. Fax: +1 410 706 0186.
E-mail address: hrus@umaryland.edu (H. Rus).

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substrates (Turner, 1998), including histones H1, H3, and H4 (Turner, 1998; Wang et al., 2011; Zhang and Kraus, 2010). In addition, SIRT1 has been found to promote histone H3K9 methylation, resulting in epigenetic gene silencing (Imai et al., 2000; Vaquero et al., 2004, 2007). SIRT1 is involved in the regulation of a number of cellular processes, including transcription, metabolism (Chen et al., 2008; He et al., 2012), DNA repair, and aging (Guarente, 2011). SIRT1 can induce chromatin silencing through the deacetylation of histones (Baur, 2010) and can modulate cell survival by regulating the transcriptional activities of p53 (Luo et al., 2000), NF-κB (Yeung et al., 2004), FOXO proteins (Brunet et al., 2004; Motta et al., 2004), and p300 (Bouras et al., 2005). Recently, resveratrol, a SIRT1 activator, was shown to ameliorate the disease course in experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Fonseca-Kelly et al., 2012; Imler and Petro, 2009; Petro, 2011). Studies have shown the ability of resveratrol to trigger apoptosis in activated T cells and also to induce a decrease in spinal cord inflammation during EAE (Singh et al., 2007). Another study has demonstrated that resveratrol has immunomodulatory effects, altering the percentage of IL-17-positive T cells in the periphery and central nervous system (CNS) following long-term treatment in the relapsing–remitting EAE model (Imler and Petro, 2009). In addition, resveratrol was found to be neuroprotective (Shindler et al., 2010), and the mechanism for its immunomodulatory and neuroprotective effects involved the activation of SIRT1 (Nimmagadda et al., 2013). Little is known about the changes that occur in SIRT1 expression or in the acetylation and methylation of histones in the PBMC and T cells from MS patients. In addition, the expression of SIRT1 in MS patients has not been investigated.

In the present study, we investigated the expression of SIRT1 and HDAC3 in MS patients and compared them to the expression in healthy controls. SIRT1 was found to be expressed in MS brains by inflammatory cells, OLG, and astrocytes. We also found that SIRT1 levels were significantly reduced in MS patients with relapses as compared to control patients. In addition, an increase in histone H3K9 acetylation was found during relapses in MS patients. We also found that SIRT1 levels in PBMCs were significantly decreased after RGC-32 silencing and that SIRT1 also regulated FasL expression and apoptosis. Decreased expression of SIRT1 in PBMCs during relapses and might represent a marker of disease activity in patients with MS.

Materials and methods

Brain tissue

Frozen brain tissue specimens acquired at autopsy from six patients with a definitive diagnosis of MS were obtained from the Human Brain and Spinal Fluid Resource Center, Veterans Affairs West Los Angeles Health Care Center. Active lesions contained abundant infiltrates consisting of T cells and macrophages, with detectable myelin degradation products. Inflammation was restricted to the lesion margins in chronic active lesions. Regions of normal-appearing white matter (NAWM) and normal-appearing gray matter lesions (NAGM) that lacked macroscopic or histological evidence of demyelination were also used. The samples were derived from patients between the ages of 38 and 51, with a mean age of 47. Four healthy control brain samples from patients between the ages of 36 and 72, with a mean age of 49, were obtained from the Cooperative Human Tissue Network, Charlottesville, VA.

Immunohistochemical staining for SIRT1 and HDAC3

Immunohistochemical staining of brains from MS patients was performed as previously described (Cudrici et al., 2007). The air-dried cryostat brain sections (4–6 μm) were fixed for 10 min in acetone containing 0.3% H_2O_2 to remove endogenous peroxidase. Tissue sections were blocked for 10 min with 2.5% horse serum, then incubated overnight at 4 °C with rabbit anti-SIRT1 (Cell Signaling, Danvers, MA) or mouse monoclonal anti-SIRT1 (Active Motif, Carlsbad) as previously described (Fosbrink et al., 2005). For HDAC3 immunolocalization, we used a mouse monoclonal anti-HDAC3 (BD Biosciences, San Jose, CA). For phosphor-SIRT1 we have used an antibody targeting serine 47(Cell Signaling) and for phosphor-HDAC3 we used an antibody targeting serine 424 (Assay Biotech, Sunnivale, CA) (Supplementary Table S1). The slides were washed three times for 3 min each with PBS, pH 7.4, and then incubated with biotinylated pan-specific universal antibody (Vector Labs, Burlingame, CA), followed by streptavidin/peroxidase complex reagent (Vector Labs). Specific reactions were developed using NovaRED (Vector Labs) as the substrate; slides were then counterstained with Harris's hematoxylin (Sigma, St. Louis, MO) and mounted with permanent mount. For CD4 and CD68 detection, brain cryosections were processed as described above and then incubated with mouse monoclonal anti-CD4 antibody (NovoCastra, Newcastle upon Tyne, U.K.), diluted 1/50, for 2 h at room temperature (RT) or with mouse monoclonal CD68 (Dako, Carpenteria, CA), diluted 1/200, for 1 h at RT. The sections were washed with PBS, pH 7.4, and then incubated for 1 h at RT with HRP-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA). Specific reactions were again developed using NovaRED. The immunostained samples were analyzed by two independent scientists.

Double-staining immunohistochemistry

Frozen sections of the brains from patients with MS were double-stained for SIRT1 and CD3, CD4, CD68, MAB328, or GFAP as previously described (Tegla et al., 2013). Cryosections were initially processed for SIRT1 immunostaining as described above, and the reactions developed with NovaRED. Then slides were incubated with anti-CD4 mouse monoclonal antibody (NovaCastra), diluted 1/50 or with mouse monoclonal antibody anti-CD68 (Dako) was followed by alkaline phosphatase-conjugated goat anti-mouse (Sigma), diluted 1/400. The reaction was developed using a Vector alkaline phosphatase substrate kit III (Vector Labs). Similar reactions were performed for oligodendrocytes using a monoclonal antibody against oligodendrocytes/myelin (MAB328, Chemicon, Temecula, CA). For the double staining GFAP/SIRT1 the sections were first incubated with mouse monoclonal monoclonal anti-GFAP (Dako), diluted 1/1000 ON at 4 °C. The slides were washed several times in PBS and reacted with goat anti-mouse antibody (Santa Cruz Biotech) for 1 h at RT. The slides were then exposed to NovaRED then exposed to the rabbit IgG anti-SIRT1 followed by alkaline phosphatase conjugated anti-rabbit IgG (Vector Labs). The reaction was developed using a Vector alkaline phosphatase substrate kit III (Vector Labs). Control sections were prepared by immunostaining without the primary antibody or by using control isotype IgG instead of the primary antibody. The immunostained slides were independently evaluated by two investigators.

MS patients and controls

We enrolled a total of 29 patients with relapsing–remitting MS (15 patients with stable disease and 14 with relapses). The criteria for inclusion of MS patients in the study were: (i) age 18 to 65 years; (ii) fulfillment of McDonald criteria for definite MS (McDonald et al., 2001; Polman et al., 2005); (iii) relapsing–remitting course; (iv) having newly diagnosed MS, or MS not treated with currently used immunomodulatory drugs (interferon-β or glatiramer acetate) for 3 months prior to study entry; (v) no exacerbations in the 4 weeks before the study; (vi) no iv or po steroids for 4 weeks prior to study enrollment; (vii) no treatment with Tysabri, Gilenya, Tecfidera, mitoxantrone, cyclophosphamide, or investigational drugs during the past year; and (viii) a disability score of 0–5.5, as defined by the expanded disability status scale (EDSS) (Kurtzke, 1983). Exclusion criteria for MS patients were: (i) a history of autoimmune disorders, vascular disease, or active acute

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or chronic infections; (ii) use of antibiotics in the last 30 days; (iii) a history of intracranial or intraspinal tumor or metabolic myelopathy; or (iv) a history of alcohol or drug abuse. Fifteen healthy, age-, gender-, and race-matched healthy controls were also enrolled in the study. Exclusion criteria for controls were: the presence of (i) overt acute or chronic disease(s) or (ii) other autoimmune disease(s).

Collection of PBMCs, total RNA purification, and cDNA synthesis

PBMCs were collected using BD Vacutainer CPT tubes (Becton Dickinson, Franklin Lakes, NJ). The mononuclear cells were isolated from fresh blood as previously described (Tegla et al., 2013). RNA isolation and cell lysate preparation for protein analysis were performed the same day (Niculescu et al., 1997). Total RNA was purified using the RNeasy Mini Kit (Qiagen, Santa Clarita, CA) according to the manufacturer's instructions. RNA (0.5 μg per sample) was mixed with RT buffer, dNTP, and oligo-dT primer (Invitrogen). RNA was denatured by incubation at 65 °C for 5 min. The reverse transcriptase (Promega) and RNase inhibitor (Invitrogen) were then added, and the reaction mixture was incubated at 37 °C for 1 h. The reaction was terminated by incubating the mixture at 95 °C for 5 min (Tegla et al., 2013).

Real-time PCR

Real-time PCR was performed using a StepOne real-time PCR system (Applied Biosystems, Foster City, CA). The primers for the genes investigated were designed and synthesized by IDT (Coralville, IA) (Table 1) and used in conjunction with LightCycler FastStart SYBR Green Master (Roche) according to the manufacturer's protocol. As a negative control for each real-time PCR assay, the same reaction was performed in the absence of cDNA or reverse transcriptase. For each gene, the cycle threshold (C_T) values were determined in the exponential phase of the amplification plot and normalized by subtraction of the C_T value for 18S (generating a ΔC_T value). The results were normalized to L13 ribosomal protein. A standard curve was generated using serial dilutions of qPCR Reference Total cDNA (Clontech, Mountain View, CA), and the normalized mRNA value (NRV) was calculated according to the following formula for relative expression of target mRNA: NRV (TarS/L13), where TarS represents the level of mRNA expression of the target gene, and L13 corresponds to that of the amplified L13 mRNA. In some cases, the -fold change in target gene samples, after normalization with the housekeeping gene (18S), was calculated using the $2^{-\Delta\Delta CT}$ value, where $\Delta\Delta C_T = \Delta C_T$ (sample) $- \Delta C_T$ (control) and ΔCT is the C_T value of target gene normalized to the C_T value of the housekeeping gene (Tegla et al., 2013).

RGC-32 silencing and transfection of cells

Transfection of PBMCs

PBMCs were plated in RPMI-10% FBS in 12-well plates 24 h prior to viral infection. After 24 h, the medium from the plate wells was removed and replaced with 1 ml of polybrene/RPMI-10% FBS medium mixture per well. Cells were then infected by adding the RGC-32 shRNA lentiviral particles (Santa Cruz Biotech) or control lentiviral particles (Santa Cruz Biotech) to the culture medium overnight, as previously described (Tegla et al., 2013). The next day, the culture medium was removed and replaced with 1 ml of RPMI-FCS 10%, and after 24 h, the transfected cells were analyzed for the expression of RGC-32, SIRT1, and L13 mRNA by real-time PCR as described above.

Annexin V Assay

PBMCs and Jurkat cells were plated in RPMI-10% FBS in 12-well plates 24 h prior to Annexin V FITC FACS analysis. Some of the cells were treated with sirtinol (200 μM) and the binding of Annexin V-FITC to cells was compared with that of cells exposed to vehicle (DMSO) only. Annexin V assay was performed according to manufacturer's instructions (BD Biosciences) and only Annexin V FITC positive, propidium iodide negative cells were used in our evaluations since they are reflecting early apoptosis.

Western blotting

Western blotting was performed as previously described (Rus et al., 1996a, 1996b). PBMC were washed with PBS, then lysed in a buffer consisting of 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 1% Triton X-100, 0.1% SDS, 100 mM NaCl, 10% glycerol, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, and complete mini protease inhibitor mixture (Roche Applied Science, Indianapolis, IN), which was added just prior to use. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL). Lysates (30 μg of protein) were fractionated on 10% gradient SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA). The following primary antibodies were used: rabbit IgG anti H3K9ac (Cell Signaling, Danvers MA) and mouse monoclonal anti-SIRT1 and rabbit IgG anti-H3K9me2 (both from Active Motif Carlsbad, CA). Goat anti-rabbit or goat anti-mouse IgG HRP-conjugated Ab (Santa Cruz Biotech., Santa Cruz, CA), as appropriate, was used as a secondary antibody. For detection we used enhanced chemiluminescence (ECL, Pierce). Membranes were stripped using Restore Western Blot Stripping Buffer (Pierce) and reprobed for the expression of β-actin (Rockland Immunochemicals, Rockville, MD). The radiographic band density was measured using UN-SCAN-IT software (Silk Scientific, Orem, UT) and results expressed as ratio to β-actin.

Statistical analysis

Comparisons between multiple groups were performed using two-way ANOVA. P values <0.05 were considered significant. Pearson correlation analysis was conducted to examine the association between variables. Statistical analysis was performed using SAS software, version 9.2. All values are shown as means ± SEM and are representative of three experiments unless otherwise noted.

Results

Immunohistochemical localization of SIRT1 and HDAC3 in MS brain

Since effector T cells migrate into the brain at the time of an MS relapse (Costantino et al., 2008; Martinez-Pasamar et al., 2013), we investigated the expression of SIRT1 in MS brains in relation to that of T cells and macrophages. We first examined the localization of SIRT1 in 20 areas from 8 patients with MS (Table 2). MS brain samples from active lesions contained abundant inflammatory cell infiltrates, consisting of $CD4^+$ and $CD8^+$ T cells as well macrophages. Acute active lesions contained inflammatory cells throughout the entire lesion, whereas

Table 1
Primers used for Real-Time PCR.

Gene symbol	Primers sequence	Product (bp)
SIRT1	For: 5′-TGGCAAAGGAGCAGATTAGTAG-3′	159
	Rev: 5′-GGCATGTCCCACTATCACTGT-3′	
HDAC3	For: 5′-CATGCACCTAGTGTCCAGATTC-3′	182
	Rev: 5′-CACTCTTAAATCTCCACATCGC-3′	
RGC-32	For: 5′-AGGAACAGCTTCAGCTTCAG-3′	152
	Rev: 5′-GCTAAAGTTTTGTCAAGATCAGCA-3′	
FasL	For: 5′-GCCCATTTAACAGGCAAGTC-3′	110
	Rev: 5′-ATCACAAGGCCACCCTTCTT-3′	
L13	For: 5′-CGTGCGTCTGAAGCCTACA-3′	227
	Rev: 5′-GGAGTCCGTGGGTCTTGAG-3′	

Abbreviations used in the table: For, forward primer; Rev, reverse primer; Bp, base pairs; RGC-32, response gene to complement 32; FasL, Fas ligand; SIRT1, Sirtuin 1; HDAC3, Histone Deacetylase 3; and L13, ribosomal protein L13.

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Table 2
Expression of SIRT1, phospho-SIRT1, HDAC3 and phospho-HDAC3 in MS brain.

Case no. (Age, sex)	Lesion (no.)	Lesion Type	SIRT1		Phospho-SIRT1		HDAC3		Phospho-HDAC3	
			Peri- vascular	Paren- chymal	Peri-vascular	Paren-chymal	Peri-vascular	Paren- chymal	Peri-vascular	Paren-chymal
1	Occipital (3)	Chronic active	++	++	+++	+++	+/++	+	++	++
(53,F)		NAWM	++	++	+++	+++	+/++	++	++	++
		NAGM	+	++	++/+++	++/+++	++	+++	+	++
	Parietal (3)	Chronic active	++	++	++/+++	+++	++	+/++	ND	ND
		NAWM	++	+++	++/+++	++/+++	+/++	+++	+/++	++
		NAGM	++	++	+++	+++	+	++	+/++	++
2	Parietal (3)	Acute	++	+++	ND	ND	+/++	++	ND	ND
(68,M)		NAWM	++	+++	+++	+++	+++	++	ND	ND
		NAGM	+/++	++	+++	+++	++	++	+	+++/++
3	Parietal (3)	Acute	++	+++	ND	ND	++	++/+++	++	++
(62, M)		NAWM	+++	+++	ND	ND	+	++	++	++
		NAGM	+	++	ND	ND	+	++	++	+++
4	Frontal (3)	Chronic active	+	+/++	+++	+++	ND	ND	ND	ND
(38, F)		NAWM	+	++	+++	+++	+/++	++	ND	ND
		NAGM	+	+	+++	+++	+	+	ND	ND
5	Frontal (2)	Chronic active	+	++	+++	++/+++	+	++	+	++
(51, F)		NAGM	+	+/++	++	++	++	++	ND	ND
6	Parietal (3)	Acute	+/++	+++	+++	+++	+	+	ND	ND
(47, F)		NAWM	+/++	+++	+++	+++	+	+	ND	ND
		NAGM	++	++	+++	+++	+++	++	ND	ND

Abbreviations used in the table: F: female, M: male, NAWM: normal appearing white matter, NAGM: Normal appearing gray matter, ND: non-determined, + slightly positive, ++ positive, and +++ highly positive.

the inflammation was restricted to the lesion margins in chronic active lesions (Cudrici et al., 2007). We were able to show that SIRT1 was expressed in both acute and chronic active lesions (Figs. 1 and 2, Table 2). SIRT1 was also found to be present on cells in both perivascular (Fig. 1A, B) and parenchymal areas (Fig. 1C). The SIRT1 deposition not only was confined to MS plaques but also was present in areas of NAWM and NAGM. In general, the expression of SIRT1 was higher in the MS plaques than in NAWM and NAGM (Table 2). SIRT1 was not found to be expressed in normal brain, with the exception of rare neurons in the cortex. Controls for the immunoperoxidase reaction using isotype IgG instead of the primary antibody were all negative (Figs. 1D). We also investigated the expression of HDAC3 in MS brain, since this protein was previously reported to be expressed by PBMCs of MS patients (Zhang et al., 2011). Like SIRT1, HDAC3 was also found to be present in periventricular and parenchymal areas of MS brains (Fig. 2 A, B) (Table 2). The HDAC3 deposition not only was confined to MS plaques but also was present in areas of NAWM and NAGM (Table 2). Expression of HDAC3 was higher in the MS plaques than in the NAWM and NAGM. Since both SIRT1 and HDAC3 are regulated by phosphorylation (Sasaki et al., 2008; Zhang et al., 2005) we localized these phosphorylated proteins to MS brains. We found that SIRT1 phosphorylated (p-SIRT1) at Ser 47 and HDAC3 phosphorylated (p-HDAC3)

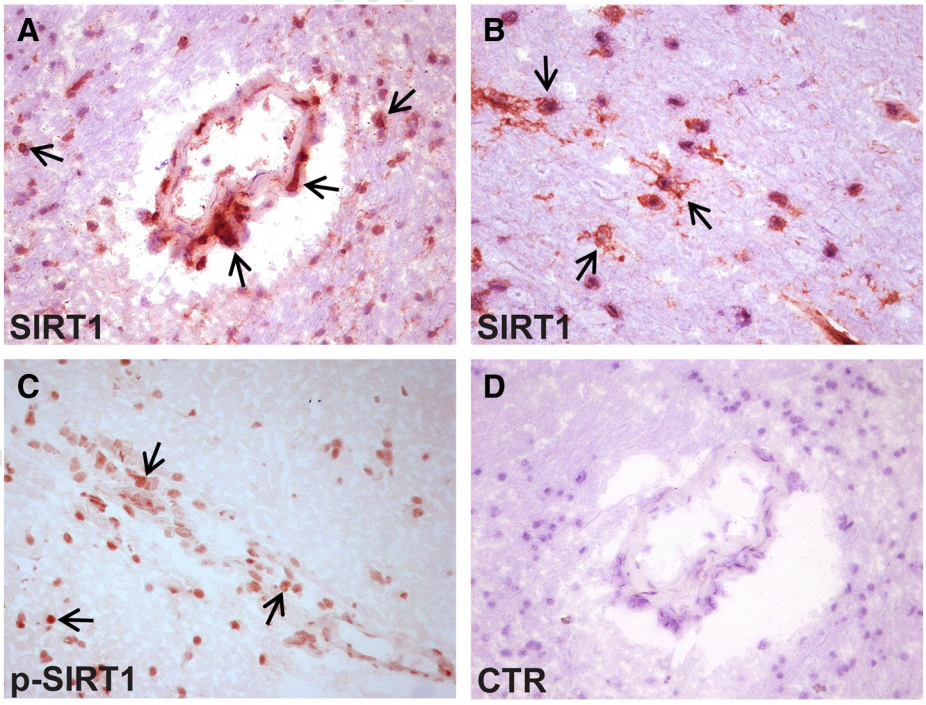

Fig. 1. Immunohistochemical staining for SIRT1 and phosphorylated SIRT1 (p-SIRT1) in MS brains. SIRT1 and p-SIRT1 were localized in MS brain by immunostaining. (A) Perivascular deposits of SIRT1 on inflammatory cells in MS plaque (arrows). (B) Parenchymal deposits of SIRT1 in normal adjacent gray matter; some positive cells have morphology suggestive of glial cells (arrows). (C) Perivascular deposits of p-SIRT1 on perivascular cells in an MS plaque. P-SIRT1 deposits are localized to the nucleus (arrows). Please note that hematoxylin was not used for counterstaining in this experiment. (D) Control for the immunoperoxidase reaction. A–D. Original magnification, ×400.

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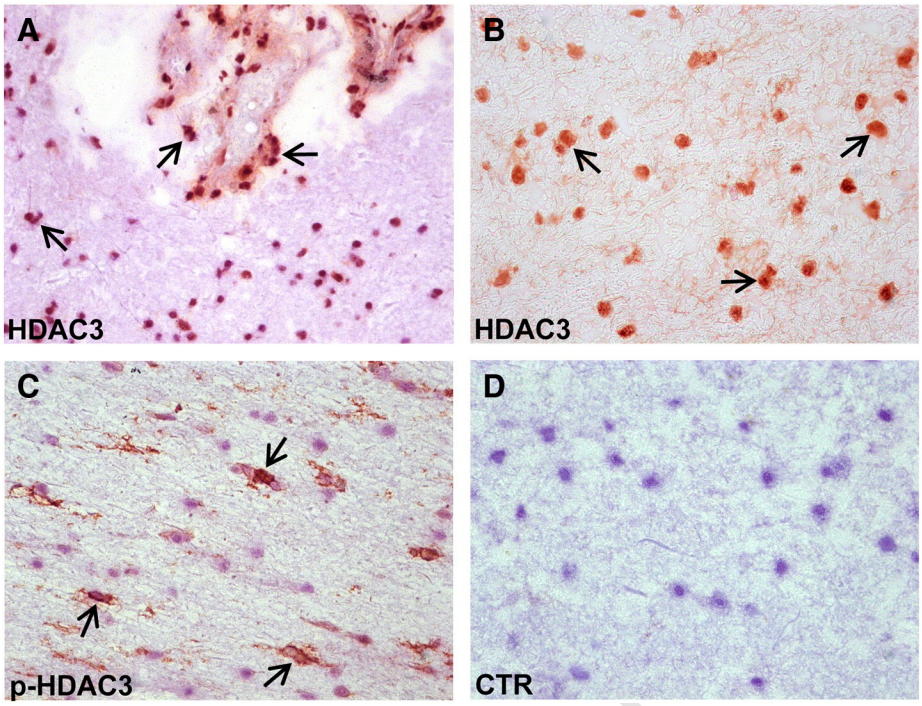

Fig. 2. Immunohistochemical staining for HDAC3 and phosphorylated HDAC3 (p-HDAC3) in MS brains. HDAC3 and p-HDAC3 were localized in MS brain by immunostaining. (A) Perivascular deposits of HDAC3 on inflammatory cells in an MS plaque. (B) Parenchymal deposits of HDAC3, which are localized to the nucleus. Hematoxylin was not used for counterstaining in this experiment. (D) Perivascular deposits of p-HDAC3 on inflammatory cells in normal adjacent white matter. Some positive cells have morphology suggestive of glial cells (arrows) pHDAC3 was localized both in the cytoplasm and in the nucleus. (D) Control for the immunoperoxidase reaction. A–D. Original magnification, ×400.

at Ser 424 were also widely distributed in MS plaques as well as NAGM and NAWM (Fig. 1C, 2C) (Table 2). P-SIRT1 deposits were found only in the nucleus (Fig. 1C) whereas p-HDAC3 was found to be localized both in the cytoplasm and in the nucleus (Fig. 2C).

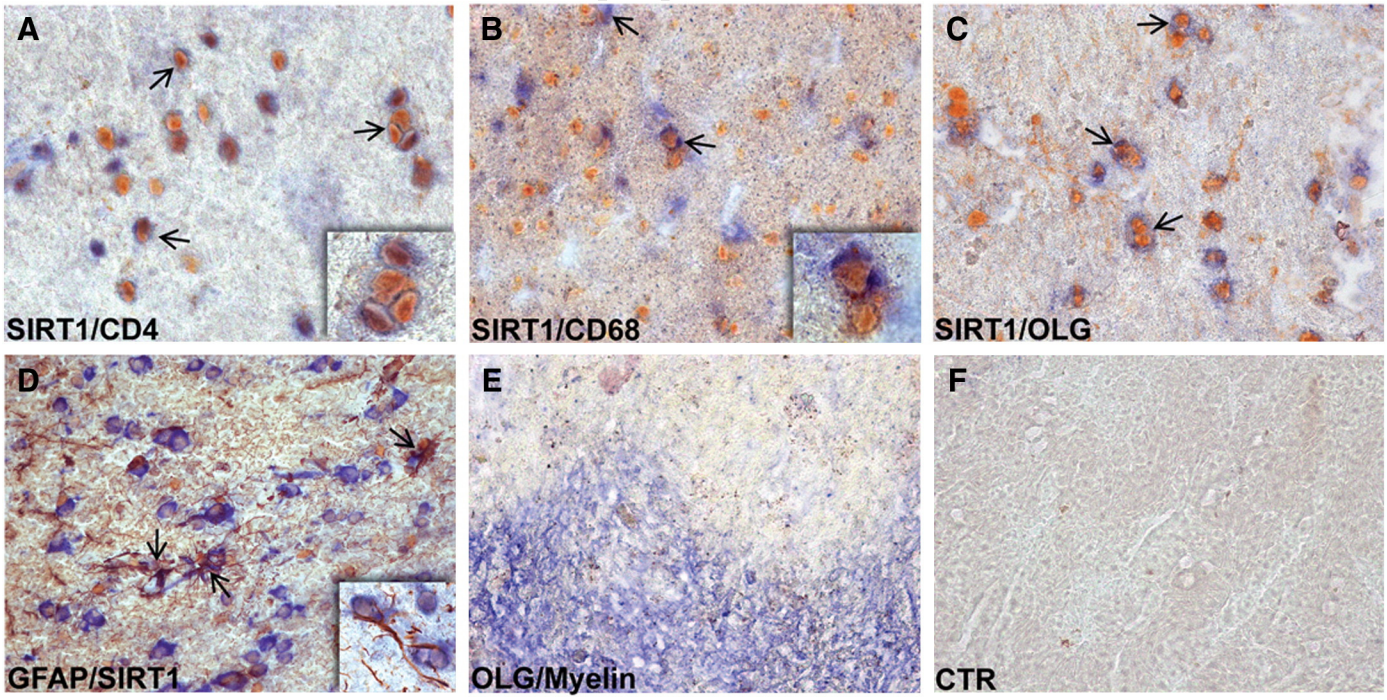

Fig. 3. Co-localization of SIRT1 with cells positive for CD4, CD68, GFAP, and MAB328 in MS brains. A, B. SIRT1-expressing cells were co-localized by double staining. (A) By double-staining, some of the SIRT1-positive cells (red deposits) are seen to be co-localized with CD4 (blue deposits) in an MS plaque (arrows and insert). Not all the SIRT1-positive cells in parenchymal areas express CD4. (B) SIRT1-positive cells (red deposits) are seen to be co-localized with CD68 (blue deposits) in an MS plaque (arrows). (C) SIRT1-positive cells (red deposits) are also co-localized with OLG (blue deposits) in an MS plaque (arrows). Almost all OLG have nuclear deposits of SIRT1. (D) SIRT1-positive cells (blue deposits) are co-localized with GFAP (red deposits) in an MS plaque (arrows). (E) Staining of the same area as in A–D for myelin/OLG using MAB328 shows significant demyelination in an MS plaque. (F) Control for the immunoperoxidase reaction. Original magnification: A–D (×400); E (×100); Inserts, (×1000). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Co-localization of SIRT1 with CD4-, CD68-, MAB328-, and GFAP-positive cells

To further concentrate our efforts on investigating whether CD4- and CD68-positive cells also express SIRT1, we carried out double-labeling experiments using specific antibodies. We discovered that some of the SIRT1 deposits co-localized with $CD4^+$ cells (Fig. 3A). We also found that some of the $CD68^+$ cells in MS plaques co-localized with SIRT1 (Fig. 3B). In addition, SIRT1 was present on CD68 $^+$ cells that had morphology suggestive of glial cells (data not shown). In addition to CD4- and CD68-positive cells, MAB328 and GFAP-positive cells also co-localized with SIRT1 (Fig. 3C, D), indicating that OLG and astrocytes in MS brains also express SIRT1. Immunoperoxidase reaction controls by replacing primary antibodies with PBS were negative (Fig. 3F).

Expression of SIRT1 and HDAC3 in PBMCs of RR MS patients and controls

We next examined the expression of SIRT1 and HDAC3 in unstimulated PBMCs in relation to disease activity. Expression of SIRT1, HDAC3, and L13 (a housekeeping gene) mRNA was measured by real-time PCR. MS samples were divided into those from stable periods in which no clinical activity was present and those from periods of relapse in which clinical activity was present and had been detected by a neurologist. A statistically significant decrease in SIRT1 mRNA was seen in patients with relapses when compared to controls ($p < 0.04$). Stable relapsing–remitting MS patients had levels of SIRT1 mRNA expression that were similar to those of control subjects (Fig. 4A). Also, HDAC3 mRNA levels were not significantly changed in MS patients when compared to controls (Fig. 4B). SIRT1 protein expression was determined by Western blotting, and levels of SIRT1 protein were also found to be significantly decreased in patients with relapses when compared to stable MS patients ($p < 0.01$) and controls ($p < 0.02$) (Fig. 3C). All these data indicate a significant decrease in SIRT1 levels during relapses.

Expression profile of histone H3K9ac and H3K9me2 in MS patients

Acetylation and methylation of histone H3 at lysine 9 were assessed by Western blotting using specific antibodies. Statistically significant higher levels of H3K9ac were found during relapses ($p < 0.05$). A reduction in H3K9 me2 was observed in the PBMCs of stable MS patients

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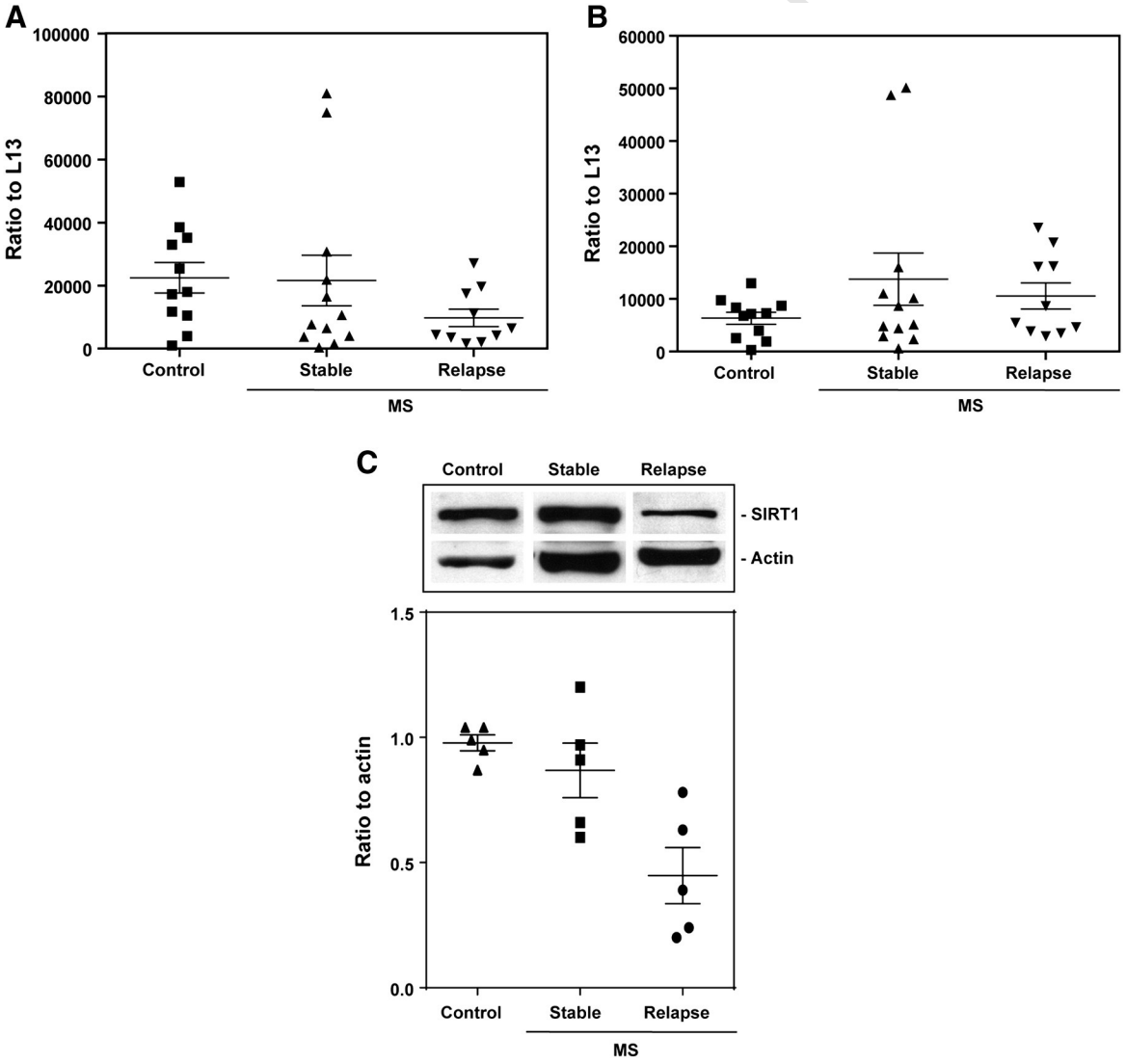

Fig. 4. Expression of SIRT1 and HDAC3 in MS patients and controls. The expression of SIRT1 and HDAC3 mRNA was measured by real-time PCR and expressed as a ratio to L13. SIRT1 protein was measured by Western blotting. (A) A statistically significant decrease in SIRT1 was found in patients with relapses when compared to controls ($p < 0.04$). (B) Levels of HDAC3 mRNA in MS patients were similar to those in controls. (C) SIRT1 protein expression was significantly decreased in the MS patients with relapses when compared to both stable MS patients and controls.

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when compared to controls, but this difference was not statistically significant. SIRT1 protein levels were positively correlated with expression of H3K9me2 ($R = 0.716$, $p = 0.0008$) and of H3K9ac ($R = 0.544$, $p = 0.0196$) (Fig. 5). These changes are in agreement with the described role of SIRT1 in H3K9 acetylation, with increased acetylation a consequence of the reduced levels of SIRT1 during relapses. SIRT1 is a known regulator of H3K9 methylation and can induce both an increase in methylation and gene silencing (Vaquero et al., 2004, 2007).

Effect of RGC-32 silencing on SIRT1 expression

We have previously shown that SIRT1 expression is regulated by RGC-32 in the SW480 tumor cell line (Vlaicu et al., 2010). To investigate whether RGC-32 is also required for the expression of SIRT1 mRNA in PBMCs, we used shRNAs targeting RGC-32 to silence its expression. PBMCs were transfected with RGC-32 shRNA lentivirus and then tested by real time-PCR to verify their effectiveness in blocking the endogenous expression of RGC-32 mRNA. We confirmed that RGC-32 shRNA effectively decreased mRNA RGC-32 expression (by 72%) when compared to cells transfected with control shRNA (shCTR) (Fig. 6A). We then examined the ability of RGC-32 shRNA to block SIRT1 mRNA expression. As shown in Fig. 6 B, SIRT1 expression was reduced by 50% after RGC-32 silencing, indicating an important role for RGC-32 in SIRT1 expression. These data are in agreement with the reported low levels of RGC-32 in MS patients with relapses (Tegla et al., 2013).

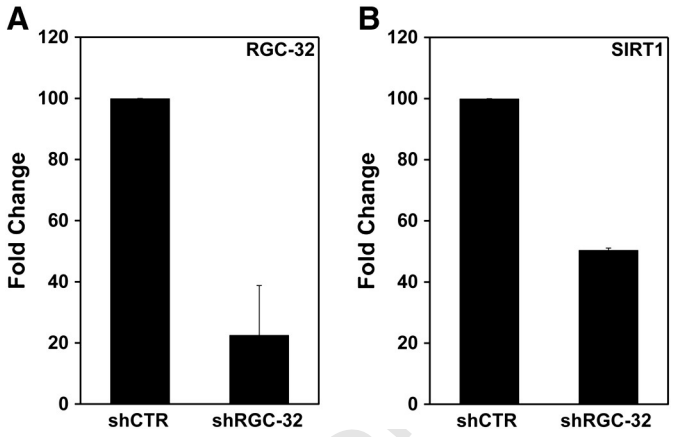

Fig. 6. SIRT1 expression is mediated through RGC-32. PBMCs from patients with stable MS were transfected with lentivirus RGC-32 shRNA or shCTR for 48 h. The expression of RGC-32 and SIRT1 mRNA was then determined using real-time PCR. (A.) RGC-32 shRNA treatment effectively decreased mRNA RGC-32 expression by 78% when compared to shRNA control (shCTR)-transfected cells. (B) SIRT1 mRNA expression was reduced by 50% ($p = 0.01$), indicating an important role for RGC-32 in mediating SIRT1 mRNA expression.

Effect of SIRT1 inhibition on apoptosis and FasL expression

We next investigated the effect of sirtinol on apoptosis in the Jurkat cell line, $CD4^+$, and $CD8^+$ cells. We found that sirtinol (200 μM)

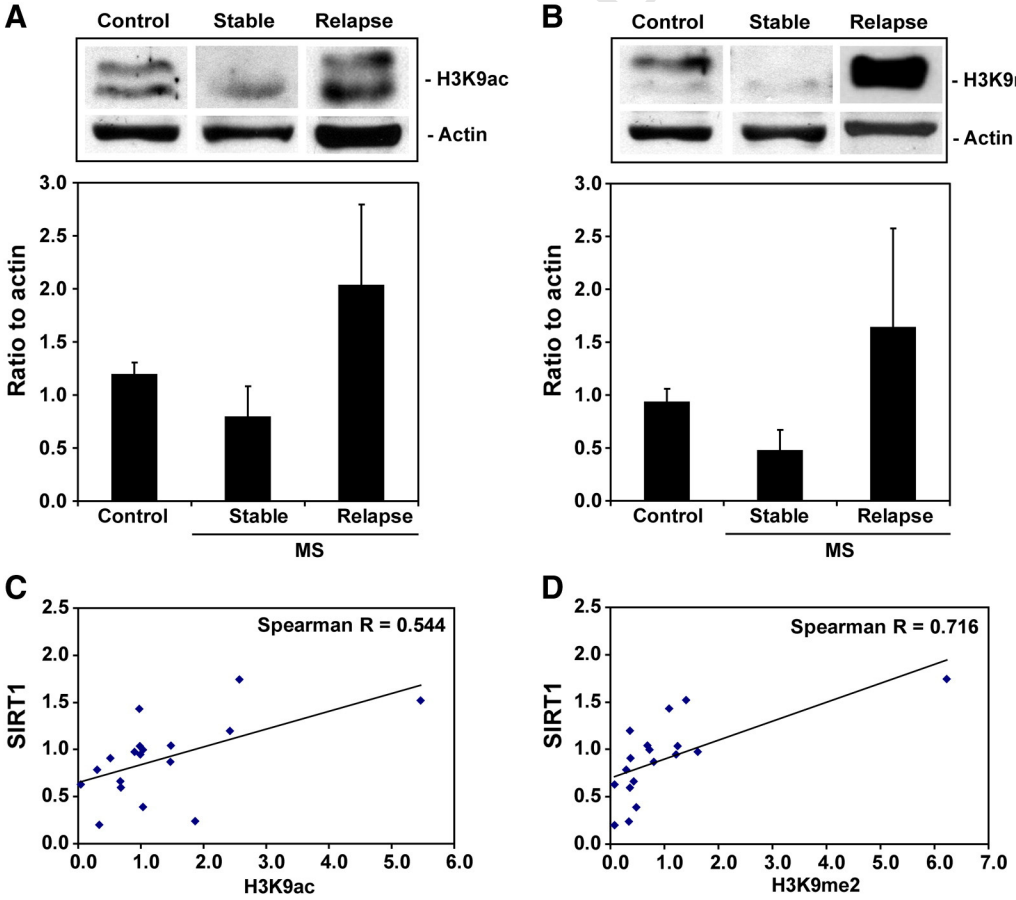

Fig. 5. Expression of histone H3K9ac and H3K9me2 in MS patients and controls. Acetylation and methylation of histone H3 at lysine 9 was assessed by Western blotting using specific antibodies against H3K9ac and H3K9me2. (A). An example of a Western blot relevant to the expression of histone H3K9ac (upper panel). Higher levels of H3K9ac were found during relapses ($p < 0.05$). Results are expressed as ratios to beta-actin (lower panel). (B) An example of a Western blot relevant to the expression of histone H3K9me2 (upper panel). Levels of H3K9me2 in MS patients did not differ significantly from those in controls. Results are expressed as ratios to beta-actin (lower panel). (C, D) Correlation of SIRT1 with histone H3K9ac and H3K9me2 expression by Spearman's correlation coefficient. SIRT1 protein levels were positively correlated with levels of H3K9ac ($R = 0.544$, $p = 0.0196$) (C) and H3K9me2 ($R = 0.716$, $p = 0.0008$) (D).

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induced binding of Annexin V in 65% of the Jurkat cells (as compared to 6% of the control cells exposed to vehicle [DMSO]). Similarly increased apoptosis as seen when CD4 cells (6% in control cells vs 55% in the MS patient) and CD8 cells (22% in control cells vs 81% in the MS patient) were exposed to sirtinol for 24 h (Fig. 7). In order to investigate the effect of SIRT1 inhibition on FasL expression, we used the PBMCs from stable MS patients. We found that sirtinol (200 μM) significantly inhibited the expression of SIRT1 mRNA (by 50%, data not shown) and significantly increased FasL expression (Fig. 8), suggesting a role for SIRT1 in the expression of FasL.

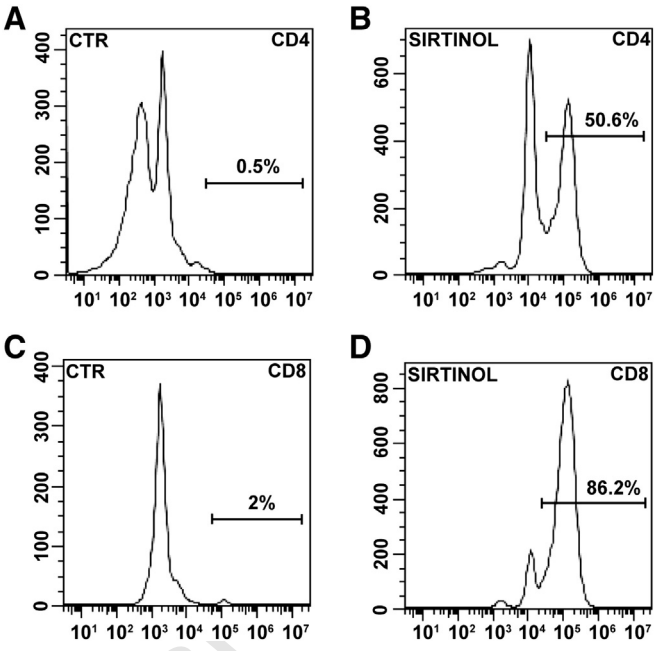

Fig. 8. Effect of SIRT 1 inhibition on FasL expression. PBMCs from a stable MS patient (A, C) were pretreated with the SIRT1 inhibitor sirtinol (200 μM) for 24 h (B, D), and expression of FasL was determined by FACS analysis. A significant increase in FasL expression in both $CD4^+$ (B) and $CD8^+$ (D) cells is seen after treatment with the SIRT1 inhibitor.

Discussion

The aim of our study was to evaluate the expression of SIRT1 and the role it plays in MS. Using immunohistochemical staining, we were able to show that inflammatory cells (T cells and macrophages), OLG, and astrocytes all express SIRT1 in the MS brain. The expression of SIRT1 was not confined to the MS plaques but was also present in NAWM and NAGM areas, indicating a widespread distribution of cells expressing SIRT1. It is important to note that most of the OLGs in the MS brain expressed SIRT1, including those that survived in areas with significant demyelination (Fig. 3). These data clearly suggest that SIRT1 is important for OLG survival in an inflammatory milieu and are in agreement with previous reports of increased OLG survival (Nimmagadda et al., 2013). Recent EAE studies in a SIRT1 transgenic mouse model have provided further evidence supporting the beneficial effects of SIRT1 on myelin-forming OLG in the EAE spinal cord. The enhanced MBP expression and greater myelin staining by LFB in the spinal cords of SIRT1 EAE mice when compared to wild-type EAE mice suggest that SIRT1 protects OLG from undergoing cell death (Nimmagadda et al., 2013).

To gain more insight into the role played by SIRT1 in inflammatory cells, we then examined the expression of SIRT1 mRNA in PBMCs, in relation to disease activity in MS patients. We performed this analysis in unstimulated PBMCs in order to mimic the in vivo situation as closely as possible (Lopatinskaya et al., 2003). We have now demonstrated that the levels of SIRT1 are significantly decreased in the PBMCs of MS patients with relapses when compared to those of healthy controls. The changes in SIRT1 mRNA expression levels that occurred in the PBMCs from MS patients during relapses were positively correlated with those of histone H3K9ac and histone H3K9me2. In addition, histone H3K9ac was found to be increased in the PBMCs of patients with relapses, consistent with their low levels of SIRT1. These data suggest that low levels of SIRT1 can be used a possible biomarker of disease activity in MS patients. In addition, the low levels of SIRT1 and loss of SIRT1 function in experimental models have been found to result in abnormally increased T-cell activation and a breakdown of $CD4^+$ T-cell tolerance (Zhang et al., 2009). SIRT1-deficient mice are also unable to maintain T-cell tolerance and develop severe EAE as well as spontaneous autoimmunity (Zhang et al., 2009).

To further investigate the factors that might regulate SIRT1 expression, we asked whether RGC-32 might be involved in SIRT1 expression. We found that silencing of RGC-32 in PBMCs led to a significant decrease in SIRT1 expression. These data suggest that the expression of SIRT1 is regulated by RGC-32, and they confirm our previous observations in cancer cell lines (Vlaicu et al., 2010, 2013). This finding is important because RGC-32 was found to promote cell cycle activation and survival through the activation of the cdc2/cyclinB1 complex (Badea et al., 1998, 2002), which also phosphorylates and activates SIRT1 (Sasaki et al., 2008). These new data suggest that regulation of SIRT1 expression is an additional mechanism by which RGC-32 promotes survival. It is also entirely possible that the low levels of RGC-32 expression seen in the PBMCs of MS patients with relapses are responsible for the reduction in SIRT1 expression seen during the active phase of the disease.

To further investigate the relationship between SIRT1 and T-cell survival, we examined the effect of the SIRT1 inhibitor sirtinol on apoptosis and FasL expression. We found that the expression of FasL was

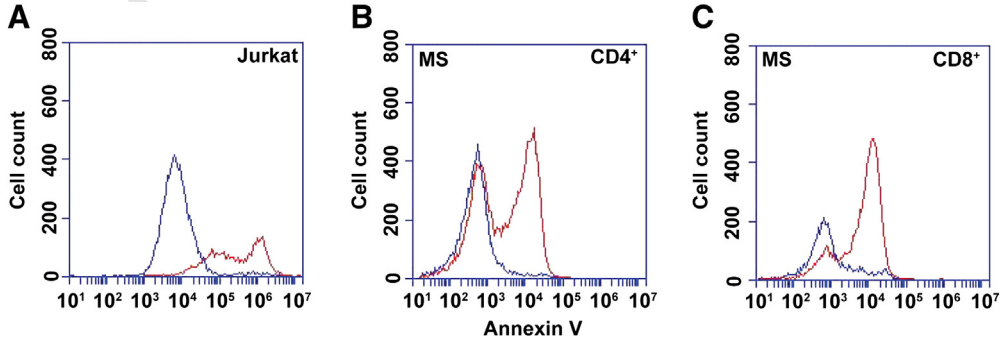

Fig. 7. Effect of SIRT 1 inhibition on annexin V binding. Jurkat cells (A) and $CD4^+$ (B) and $CD8^+$ (C) cells from an MS patient were pretreated with the SIRT1 inhibitor sirtuin (200 μM) for 24 h, and Annexin V FITC binding was determined by FACS analysis. Overlay histograms are shown for untreated control cells (blue line) and sirtinol treated cells (red line). As expected, cells that were treated with the vehicle (DMSO) were primarily Annexin V negative. The small number of Annexin V negative cells in control cells represents the basal level of apoptosis. A significant increase in Annexin V binding was found in all studied cells after 24 h of incubation with sirtinol for 24 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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significantly increased, indicating that FasL expression is, at least in part, SIRT1-dependent. In addition, inhibition of SIRT1 leads to significant apoptosis in Jurkat cells and in $CD4^+$ and $CD8^+$ from patients with MS. Our results showing a decreased level of SIRT1 in MS patients with relapses are in agreement with recent data obtained in EAE, an animal model of MS. These experiments showed that administration of resveratrol, a SIRT1 activator, ameliorates the disease course in EAE (Nimmagadda et al., 2013). In addition, resveratrol was found to trigger apoptosis in activated T cells and also to induce a decrease in spinal cord inflammation during EAE (Singh et al., 2007). In addition, resveratrol was found to be neuroprotective (Shindler et al., 2010), and the mechanism for its immunomodulatory and neuroprotective effects appears to involve the activation of SIRT1 (Singh et al., 2007).

We should mention that we did not find a significant difference in the expression of HDAC3 between patients with MS and controls. This finding is in contrast to previous published data that had showed an increase in HDAC3 expression in MS patients (Zhang et al., 2011). This difference in the results obtained can probably be explained by differences in the way MS patients were selected for these two studies. We have studied only patients with relapsing-remitting MS. In the previously published study, patients with both relapsing–remitting and secondary progressive MS were included (Zhang et al., 2011). In addition, we have separated relapsing–remitting MS patients into two groups on the basis of disease activity.

Finally, we can conclude that SIRT1 represents a possible biomarker of relapses and a potential new target for therapeutic intervention in MS. The use of resveratrol or other activators of SIRT1 should be considered in future clinical trials in order to investigate their potential for preventing relapses and promoting OLG survival.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yexmp.2013.12.010.

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