

## Activation-induced cell death in T lymphocytes from multiple sclerosis patients



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### ABSTRACT

Apoptosis is a major mechanism regulating immune tolerance by the elimination of autoreactive T lymphocytes. A failure of activation induced cell-death (AICD) has been described in T lymphocytes from patients with multiple sclerosis (MS). The aims of this study were to evaluate AICD in T lymphocytes from patients with MS and healthy controls, and to explore the molecular mechanisms underlying the deregulation observed in apoptosis induction. PHA-induced AICD was reduced in T lymphocytes from patients with relapsing–remitting MS compared with controls. This finding was associated with a diminished expression of Fas and a failure in caspase 3 activation.

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### 1. Introduction

Multiple sclerosis (MS) is an autoimmune disease affecting the central nervous system and a leading cause of neurological disability in young adults. Development of MS appears to be associated with activated T cells attacking myelin antigens, which results in demyelination and axonal damage (McFarland and Martin, 2007; Comabella and Khoury, 2012). A number of mechanisms have been described to maintain immune homeostasis and avoid the development of autoimmune diseases. One such mechanism is activation-induced cell death (AICD), a form of programmed cell death or apoptosis. In AICD, T cells die in a controlled manner by the activation of the T cell receptor and through the Fas signaling pathway (Pender, 1999; Zhang et al., 2004). Deregulation of this process has been linked to the development of autoimmune diseases including MS.

It has been reported that T cells from MS patients respond less strongly to apoptotic stimuli than those from healthy controls (HCs)

(Macchi et al., 1999; Comi et al., 2000), and T-cell apoptosis has also been associated with disease activity (Okuda et al., 2006). Little is known, however, about a potentially different apoptotic behavior of the different clinical forms of MS. The objectives of the present study were to evaluate spontaneous apoptosis and AICD in T cells from HC and MS patients with different clinical forms and activity phases of the disease; and to unravel the molecular mechanisms underlying the differences in apoptosis induction observed in T cells from MS patients.

### 2. Materials and methods

#### 2.1. Patients

Twelve HCs and 36 untreated patients who satisfied the 2010 McDonald's criteria for clinically definite MS (Polman et al., 2011) were included in the study. The MS group comprised 17 patients with relapsing–remitting MS (RRMS) in clinical remission, 6 RRMS patients in relapse and 13 patients with primary progressive MS (PPMS). In RRMS patients in relapse, blood samples were collected before treatment with corticosteroids. Neurological disability at the time of blood collection was evaluated by the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983). The study was approved by the Ethics Committee of Vall d'Hebron University Hospital and subjects gave written informed consent. Demographic and clinical characteristics of MS patients and healthy controls (HCs) are summarized in Table 1.

**Abbreviations:** AICD, activation-induced cell death; EDSS, Expanded Disability Status Scale; HC, healthy control; MS, multiple sclerosis; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PPMS, primary progressive multiple sclerosis; RRMS, relapsing–remitting multiple sclerosis.

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**Table 1**  
Demographic and clinical characteristics of MS patients and HC included in the study.

Characteristics	HC	MS <sup>a</sup>	PPMS	RRMS	Relapses
N	12	30	13	17	6
Female/male (% women)	8/4 (66.7)	20/10 (66.7)	8/5 (61.5)	12/5 (70.6)	5/1 (83.3)
Age (years)	41.4 (10.2)	44.3 (8.4)	50.0 (9.2)	40.4 (5.6)	41.8 (7.3)
Duration of disease (years)	–	11.6 (6.9)	13.1 (7.6)	11.0 (6.5)	12.5 (8.8)
EDSS	–	3.4 (2.7)	6.4 (1.7)	1.5 (1.0)	3.0 (1.4)
Number of relapses in the 2 previous years	–	0.5 (0.6)	–	0.5 (0.6)	0.7 (1.2)

Data are expressed as mean (standard deviation). EDSS: Expanded Disability Status Scale. HC: healthy control. RRMS: relapsing–remitting multiple sclerosis. PPMS: primary progressive multiple sclerosis. Relapses: RRMS patients during acute relapses.

<sup>a</sup> MS: refers to the whole MS group without including RRMS patients in relapse.

## 2.2. Cell collection and induction of apoptosis

Peripheral blood was collected in cell preparation tubes (CPTs) (Beckton Dickinson, BD, New Jersey, USA). PBMC were obtained with the Ficol–Hypaque density gradient method within 2 h from sampling. Cells were resuspended in a culture medium composed of RPMI 1640 supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 25 mM HEPES buffer, 50 U/ml penicillin, and 50 µg/ml streptomycin (GIBCO–Life technologies, Carlsbad, CA), counted and cultured at a density of 2 million cells/ml in 24-well plates (1 ml per well). To induce AICD, cells were stimulated with phytohemagglutinin (PHA; Sigma–Aldrich, St. Louis, MO) at a concentration of 10 µg/ml for 20 h. This concentration and time point were considered optimal after AICD optimization experiments carried out by the group.

## 2.3. Determination of Fas expression by flow cytometry

Freshly isolated PBMC were washed twice with PBS and blocked for 10 min at room temperature in blocking solution (PBS with 10% of fetal bovine serum). Cells were then incubated with specific antibodies for 25 min at room temperature with shaking. The following antibodies were used: phycoerythrin (PE)-conjugated anti-human Fas or PE-isotype control, fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 or FITC-isotype control (BD). Cells were then washed twice with PBS and analyzed on a dual laser FACSCanto (BD) flow cytometer equipped with FACSDiva software. Lymphocytes were gated based on forward and side scatters and CD3 was used as a marker of T-lymphocytes. Gating strategy is shown in Supplementary Fig. 1.

## 2.4. Evaluation of apoptosis by flow cytometry

After 20 h of culture, cells were collected, washed twice with PBS, blocked for 10 min at room temperature (PBS with 10% fetal bovine serum) and incubated with antibodies for 25 min at room temperature with shaking. PE-conjugated anti-human CD3 (BD), APC-conjugated anti-human CD4 (eBioscience, San Diego, CA) and CD8 (Biolegend, San Diego, CA) or their PE-isotype control (BD) and APC-isotype control (eBioscience) antibodies were used. Subsequently, cells were washed with annexin V binding buffer (BD) and FITC-conjugated annexin V (BD) was added and incubated for 15 min at room temperature with shaking. Cells were then washed twice with binding buffer and analyzed with the FACSCanto flow cytometer. Annexin V positive cells were considered apoptotic cells. Finally, 7-aminoactinomycin D (7-AAD) was added to the cells. Gating strategy is shown in Supplementary Fig. 1. Activation-induced cell death was determined by subtraction of the percentage of T lymphocytes undergoing spontaneous apoptosis from the total percentage of T lymphocytes in apoptosis after PHA stimulation.

## 2.5. Protein extraction and caspase 3 western blot analysis

After 20 h of culture, the cells were collected and washed twice with PBS. Whole cell extracts were obtained in RIPA lysis buffer containing

protease inhibitors. Ten micrograms of protein was loaded onto 15% SDS acrylamide gels, electrophoresis was carried out, and proteins were blotted onto nitrocellulose membranes. Membranes were blocked for 1 h in TBST buffer with 5% nonfat milk, and incubated with a specific antibody recognizing both the full and cleaved forms of caspase 3 (Cell Signaling, Beverly, MA) at a 1:1000 dilution in TBST buffer with 3% milk overnight at 4 °C with shaking. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Proteins were detected by enhanced chemiluminescence reagent (Millipore, Billerica, MA). For analysis of β-actin expression, membranes were stripped with a commercial stripping buffer (Millipore) and stained with a β-actin antibody (Sigma–Aldrich) using the same conditions as caspase 3 staining. Quantification of protein expression was carried out using the Science Lab Image Gauge software (Fujifilm, Tokyo, Japan). Nine samples were analyzed for each group.

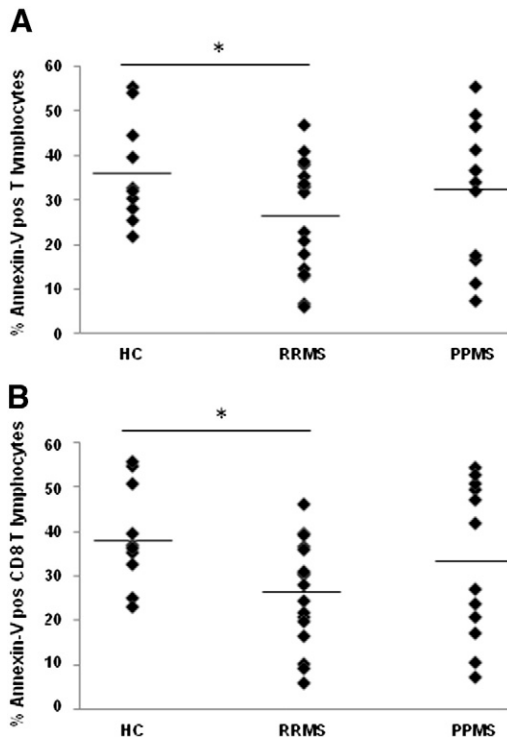
## 2.6. Statistical analysis

Statistical analysis was performed with the SPSS 17.0 package (SPSS Inc, Chicago, IL). Student's t-test was used to compare variables between groups. Linear associations between apoptosis or Fas expression and clinical variables were assessed by the Pearson correlation coefficient. Results were considered statistically significant when  $p < 0.05$ .

## 3. Results

### 3.1. AICD is decreased in T cells from RRMS patients

To evaluate AICD in T cells, PBMC were cultured in the presence or absence of PHA and annexin V was used as a marker of apoptosis. In the presence of PHA, AICD was decreased in the whole MS group when compared with the HC group, as shown by the lower percentage of annexin V positive T cells (mean values: 28.9% and 36.7% in MS patients and HC respectively; Fig. 1A); however, differences did not reach statistical significance ( $p = 0.122$ ). Further stratification of the MS group into different clinical forms revealed a statistically significant decrease in AICD in RRMS patients in remission compared with HC (mean values: 36.7% in HC and 26.5% in RRMS in remission;  $p = 0.04$ ) (Fig. 1A). A representative flow cytometry staining of a HC and a patient with RRMS is shown in Supplementary Fig. 1. The percentage of annexin V positive T cells did not significantly differ between PPMS patients and RRMS patients in remission and HC (32.3, 26.5 and 36.7% respectively). Likewise, AICD was comparable between RRMS patients in clinical remission and RRMS patients whose blood was extracted at the time of acute exacerbations (26.5 and 33.2% respectively). In the absence of PHA, spontaneous apoptosis was similar between the whole MS group or different clinical forms of the disease and HC and between RRMS patients in clinical remission and RRMS patients in relapse (15.5 for HC, 18.0 for the whole MS group, 17.8 for RRMS patients in remission, 18.3 for PPMS patients and 13.6% for RRMS patients in relapse). Both spontaneous apoptosis and AICD were also evaluated in the CD4 and CD8 subsets of T cells. Interestingly, AICD was significantly reduced in



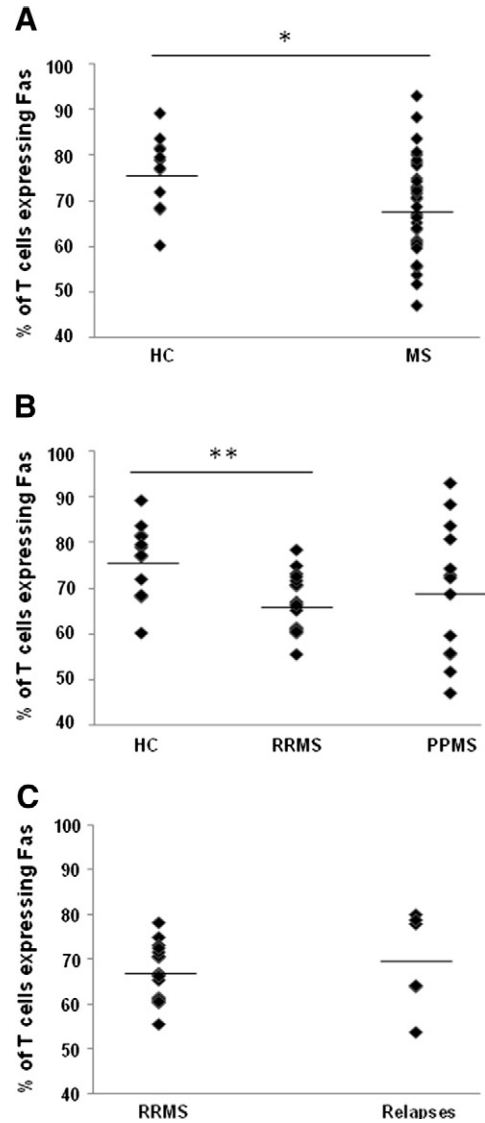
**Fig. 1.** Dot plots showing AICD in T cells (A) and CD8 T cells (B) from MS patients and HC. AICD results from the subtraction of the percentage of T lymphocytes undergoing spontaneous apoptosis from the total percentage of T lymphocytes in apoptosis after PHA stimulation. \**p* < 0.05 in RRMS patients in respect to HC. HC: healthy control. RRMS: relapsing–remitting MS patients in clinical remission. PPMS: primary progressive multiple sclerosis.

CD8 but not in CD4 T cells from RRMS patients (39.3% in HC and 26.5% in RRMS in remission; *p* = 0.01) (Fig. 1B and Table 2).

There were no significant correlations between AICD or spontaneous apoptosis and clinical variables such as EDSS, number of relapses in the two previous years, and disease duration (data not shown).

### 3.2. Fas expression is reduced in T cells from MS patients

Insomuch as Fas is the main death receptor mediating AICD, we first investigated Fas receptor expression in T cells from MS patients and HC. As shown in Fig. 2A, comparisons between the whole MS group and the HC group revealed a statistically significant reduction of Fas expression in T cells from MS patients compared with controls (mean values: 76.0% in HC versus 68.4% in MS patients, *p* = 0.02). In MS patients classified according to their clinical forms, Fas expression was significantly lower in T cells from RRMS patients in remission compared with HC



**Fig. 2.** Dot plots showing Fas expression in freshly isolated T cells from HC and the whole MS group (A), different clinical forms of MS (B), RRMS patients in clinical remission and RRMS patients in relapse (C). \**p* = 0.02 in the whole MS group versus HC. \*\**p* = 0.001 in RRMS patients in clinical remission versus HC. MS: whole group of MS patients excluding RRMS patients in relapse. HC: healthy control. RRMS: relapsing–remitting MS patients in clinical remission. PPMS: primary progressive multiple sclerosis. Relapses: RRMS patients during acute relapses.

(76.0% in HC and 67.0% in RRMS patients; *p* = 0.001) (Fig. 2B). A representative flow cytometry staining of Fas in HC and patients with RRMS is shown in Supplementary Fig. 1. Finally, the percentage of T cells expressing Fas was similar between RRMS patients in clinical remission and RRMS patients in relapse (Fig. 2C).

**Table 2**

Spontaneous apoptosis and AICD in CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets in different clinical forms of MS and healthy controls.

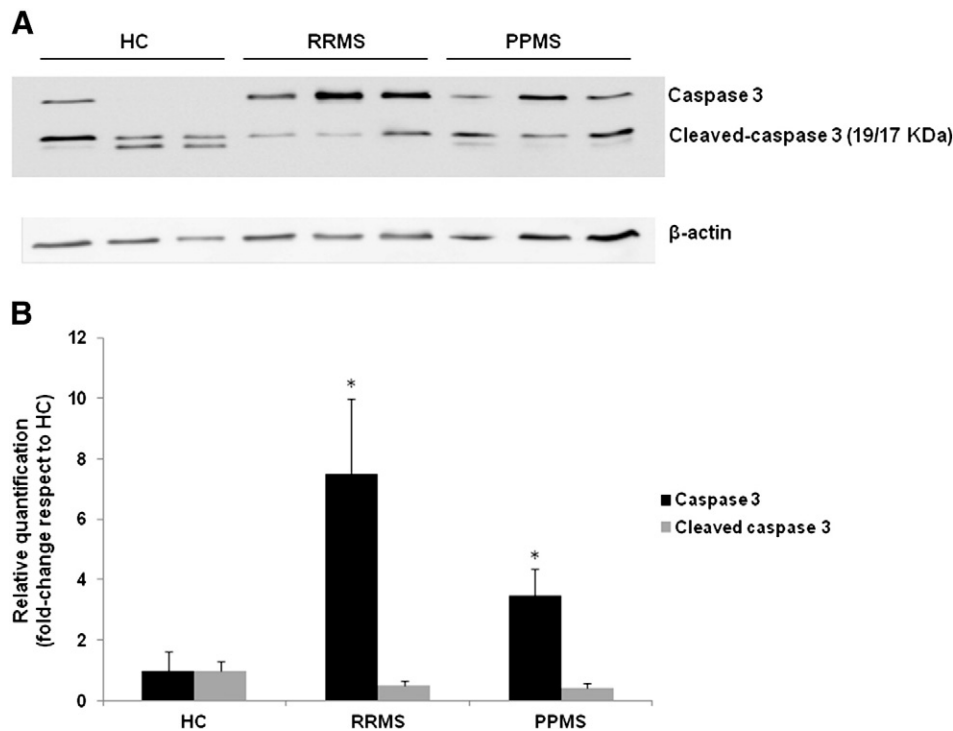
	HC	RRMS	PPMS	Relapses
<i>Spontaneous apoptosis</i>				
CD4 <sup>+</sup> T cells	11.1 (4.5)	13.8 (5.2)	17.2 (10.4)	15.1 (7.2)
CD8 <sup>+</sup> T cells	20.7 (8.3)	24.3 (10.1)	27.2 (13.7)	20.3 (8.0)
<i>AICD</i>				
CD4 <sup>+</sup> T cells	29.9 (12.2)	25.4 (14.0)	27.5 (16.6)	30.2 (10.0)
CD8 <sup>+</sup> T cells	39.3 (11.5)	26.5 (11.6)*	33.8 (17.5)	35.9 (15.4)

Data are presented as mean (SD). HC: healthy control. RRMS: patients with relapsing–remitting multiple sclerosis in clinical remission. PPMS: patients with primary progressive multiple sclerosis. Relapses: patients with relapsing–remitting multiple sclerosis in relapse.

\* *p* < 0.05 compared to HC.

### 3.3. Caspase 3 activation is defective in MS patients

To unravel the molecular mechanisms implicated in the impaired response to PHA-induced apoptosis, caspase 3 expression and activation were next evaluated in PBMC from HC and MS patients following PHA stimulation. As shown in Fig. 3, the full or inactive form of caspase 3 was clearly more expressed in MS patients compared to HC, while expression of the cleaved or active form was diminished in MS patients, particularly in the RRMS group. This observation implies an impairment in caspase 3 activation in patients with RRMS.



**Fig. 3.** Caspase 3 expression and activation in HC and MS patients after PHA stimulation. (A) Western blot represents the expression of the full-length caspase 3 (inactive form or pro-form) and the cleaved caspase 3 (active form) in 3 individuals per group. The figure is representative of a total of nine individuals per group.  $\beta$ -Actin was used as a housekeeping protein. (B) Quantification of the full-length caspase 3 and the 19 kDa cleaved form in the different groups normalized by  $\beta$ -actin expression. Error bars represent standard error of the mean. \* $p < 0.05$  in respect to HC. HC: healthy control. RRMS: relapsing–remitting multiple sclerosis in clinical remission. PPMS: primary progressive multiple sclerosis.

#### 4. Discussion

Activation-induced cell death is one of the main mechanisms regulating immune homeostasis, which is known to be altered in different autoimmune diseases including MS (Zhang et al., 2004). The fact that some MS therapies known to have beneficial effects in the disease act through an increase of apoptosis in T lymphocytes reinforces the relevance of this process in MS pathogenesis (Rieks et al., 2003; Chan et al., 2005; Ruggieri et al., 2006).

In the present work, we provide new insight into the apoptotic process of T lymphocytes in MS patients. We investigated spontaneous apoptosis and AICD in T cells from different clinical forms and activity phases of the disease. The results of our work are in agreement with previous reports indicating an impaired induction of apoptosis in peripheral T lymphocytes from MS patients (Sharief, 2000; Petcu et al., 2006; Julia et al., 2009). In addition to this observation, we found that AICD was mainly altered in patients with RRMS in clinical remission and it was not affected in patients with PPMS or RRMS patients during relapses, pointing to different pathogenic mechanisms underlying the different clinical forms of MS.

Regarding spontaneous apoptosis, previous studies have reported significantly increased spontaneous ex vivo apoptosis in MS patients compared to HC (Prieto et al., 2006; Garcia-Merino et al., 2009). However, in our study the percentage of T lymphocytes undergoing spontaneous apoptosis did not differ between groups. The discrepancy might be due to differences in the clinical activity of MS patients included in the studies. Thus, whereas in previous studies the increase in spontaneous apoptosis was more evident in patients with aggressive phenotypes (more than 2 relapses in the two previous years), in our study RRMS patients had a more benign disease course as revealed by the low number of relapses in the two years preceding blood extraction (0.4).

When exploring further the mechanisms underlying the impairment of AICD observed in RRMS patients, we found that Fas expression, a death receptor known to be important in AICD (Ju et al., 1995), was significantly reduced in T cells from MS patients, differences that were

primarily driven by the RRMS group in clinical remission. Fas function has been reported to be deficient in T cells from MS patients (Comi et al., 2000). Previous studies evaluating Fas expression in T lymphocytes from MS patients revealed higher expression in MS patients compared to controls and increased expression in MS patients during relapses and in patients with the secondary progressive form of MS (Ichikawa et al., 1996; Bilinska et al., 2001; Bilinska et al., 2003.) Another study comparing Fas expression in T cells from RRMS patients in remission and HC resulted in no significant differences between these two groups (Bilinska et al., 2001), finding that is in disagreement with our study and may also be explained by differences in the clinical characteristics of patients. RRMS patients in remission included in the study by Bilinska et al. had more aggressive clinical phenotypes compared with the more benign disease course observed for this group of patients in our study.

Another mechanism that might be involved in the failure of AICD observed in RRMS patients is the activation of caspases. Caspases are cysteine-aspartic acid proteases mediating the cellular events that finally lead to apoptosis. Caspase 3 is an executioner caspase activated by cleavage at the end of the apoptotic signaling cascade. Following PHA stimulation, we observed that in control individuals, caspase 3 was normally activated whereas in patients with MS caspase 3 activation was clearly deficient. This finding was associated with a significant increase in the expression of the full length or inactive form of caspase 3, which may be interpreted as a result of an impaired cleavage which leads to accumulation of the full-length caspase 3 or as an unsuccessful attempt to restore normal caspase 3 function by incrementing its expression. This failure in caspase 3 activation may in part be responsible for the impairment of AICD observed in RRMS patients.

In conclusion, our results point to a differential apoptotic behavior of the clinical forms of MS. T lymphocytes from patients with the RRMS form of the disease seem to be more resistant to the AICD process compared with control individuals. In contrast, AICD was not impaired in T lymphocytes from patients with the PPMS form of the disease or patients with RRMS whose blood was collected at the time of acute

exacerbations. These findings may possibly reflect differences in the pathogenic mechanisms underlying the various clinical forms and activity phases of the disease. In patients with RRMS, both a reduced expression of Fas and defective activation of caspase 3 are potential molecular mechanisms associated with the failure of AICD observed in this group of MS patients.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jneuroim.2014.04.007>.

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