CD8<sup>+</sup>Foxp3<sup>+</sup> T cells in peripheral blood of relapsing-remitting multiple sclerosis patients

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ABSTRACT
A defect of CD4<sup>+</sup> regulatory T cells (Treg) seems to be involved in the pathogenesis of multiple sclerosis (MS). Besides Treg, CD8<sup>+</sup> T cells also can suppress the immune response. Forkhead box p3 (Forkp3) is known to program the acquisition of suppressive capacities in CD4<sup>+</sup> T cells and recent studies showed that in vitro antigen activation leads to Foxp3 expression in CD8<sup>+</sup> T cells, gaining of suppressive activity. By flow cytometry we found a lower percentage of circulating CD8<sup>+</sup>Foxp3<sup>+</sup> T cells in relapsing than in remitting patients with MS and in controls. No significant differences were observed in CD8<sup>+</sup>Foxp3<sup>+</sup> T cell percentage between healthy subjects and patients in remission. Our data suggest that peripheral CD8<sup>+</sup>Foxp3<sup>+</sup> T cells may play a role in the maintenance of tolerance in MS.

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1. Introduction
Regulatory T (Treg) cells suppress host immune responses against self- or non–self-antigens, thus playing a critical role in the prevention of autoimmune diseases by maintaining peripheral tolerance and in the modulation of immune responses to pathogens and alloantigens. Besides CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells also can differentiate into either cytotoxic effector cells or suppressor cells that suppress immune response by cell–to-cell contact-mediated suppression, anti-inflammatory cytokine secretion and cytotoxic activity [1]. Several types of adaptive CD8<sup>+</sup> Treg have been described in humans, such as Qa-1-dependent CD8 [2], CD8<sup>+</sup>CD28<sup>-</sup>Foxp3<sup>+</sup> [3], CD8<sup>+</sup>CD28<sup>-</sup>Foxp3<sup>+</sup> [4]CD8<sup>+</sup>CD25<sup>-</sup>CD28<sup>-</sup>Foxp3<sup>+</sup> [5–8], interleukin (IL)-10–producing CD8<sup>+</sup> [9]CD8<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>+</sup> lymphocyte activation gene-3 (LAG3)<sup>-</sup> [10], and human leukocyte antigen (HLA)-G–restricted CD8<sup>+</sup>Foxp3<sup>+</sup> [11] Treg subsets. The generation of all these subsets requires T-cell activation via T-cell receptor triggering and chronic antigenic stimulation [1]. In experimental murine models, CD8<sup>+</sup> Tregs can be induced by any antigen and can prevent as well as reverse already established autoimmunity [12]. Much less is known about the potential role that regulatory/suppressor CD8<sup>+</sup> T cells have in human autoimmune diseases.

Forkhead box p3 (Forkp3) is known to program the acquisition of suppressive capacities in CD4<sup>+</sup> Treg cells. Even if its role in CD8<sup>+</sup> T cells is still not well known, recent studies showed that in vitro antigen activation leads to Forkp3 expression in CD8<sup>+</sup> T cells, gaining of suppressive activity [13,14]. In mice, Forkp3 is a specific marker of Treg lineage, whereas in humans both CD4<sup>+</sup> and CD8<sup>+</sup> naive T cells transiently express Forkp3 upon in vitro T-cell receptor stimulation but do not necessarily acquire suppressive capabilities [15,16]. Transient expression of Forkp3 during activation might be a mechanism of limiting excessive immune activation and damage at the site of inflammation [17,18]. In activated T cells, Forkp3 expression is generally lower and more transient than in natural Tregs [15,16]. Otherwise both CD4<sup>+</sup>Forkp3<sup>+</sup> and CD8<sup>+</sup>Forkp3<sup>+</sup> Tregs deficiency and/or altered function have been associated with autoimmune diseases in humans, including inflammatory bowel disease and multiple sclerosis [19,20].

The aim of this study was to evaluate the percentage and the mean expression of Forkp3 in CD8<sup>+</sup> T cells in peripheral blood of patients with untreated relapsing-remitting multiple sclerosis (RRMS) in different phases of disease (remission and relapse) and in healthy subjects.

2. Materials and methods
2.1. Patients
Forty patients with RRMS defined by McDonald’s criteria in different phases of disease, 20 in relapse (relapsing patients) and 20 in remission (remitting patients), and 25 age- and sex-matched healthy subjects were included in the study. Five patients were studied both in relapsing and in remitting phases of disease.

No patients had received immunosuppressive drugs or disease-modifying agents. No corticosteroid treatment was used for 6 months before inclusion in the study and patients in relapse were bled before...
starting high-dose corticosteroid treatment. Clinical examination and brain and spinal cord magnetic resonance imaging (MRI) were performed in all patients before starting the enrolment. Patients were considered in relapse (relapsing patients) when they showed an episode of new neurological disturbance lasting at least 24 hours and MRI activity (≥1 gadolinium-diethylenetriaminepentaacetic acid-enhancing lesion). Patients were considered in remission (remitting patients) when neither new neurological symptoms nor MRI activity was registered. Disability degree was assessed using the Expanded Disability Status Scale (EDSS).

MRI data were acquired using a high-resolution 1.5-Tesla system (slice thickness 5 mm). Scanning sessions included acquisition of proton-density (echo time [TE] 20, repetition time [TR] 2500), and T2-weighted (TE 80/TR 2500) and T1-weighted (TE 17/TR 600) images. The T1-weighted images were acquired before and 10 minutes after an intravenous injection of gadolinium-diethylenetriaminepentaacetic acid (0.1 mmol/kg). This study was approved by the ethics committee of the Catholic University (Rome, Italy), and all the participants gave written informed consent before enrolment.

2.2. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood by density gradient centrifugation (2,500g, 30 minutes) over a Ficoll-Hypaque density gradient (Pharmacia, Uppsala, Sweden). PBMCs were then harvested by pipetting cells from the Ficoll/serum interface and washed twice.

2.3. Flow cytometry

For the detection of Foxp3 expression, PBMCs were analyzed by three-color intracellular flow cytometry using anti-CD8- phycoerythrin-cyanine conjugate (Convergent, Miami, FL), anti-CD25- fluorescein isothiocyanate conjugate (Convergent, Miami), and anti-Foxp3–phycoerythrin conjugate monoclonal antibody (clone 236A/E7; eBioscience, San Diego, CA). In particular, isolated PBMCs were washed once in culture medium (Dulbecco) containing fetal calf serum and once in phosphate-buffered saline (PBS) and incubated with both anti-CD8- phycoerythrin-cyanine conjugate and anti-CD25- fluorescein isothiocyanate conjugate. After fixation, cells were permeabilized using a commercially available permeabilization/wash kit (BD Bioscience/PharMingen, Franklin Lakes, NJ). Upon permeabilization, 5 × 10^5 cells were resuspended in 100 μl of PBS and incubated for 30 minutes with the anti-Foxp3–phycoerythrin conjugate. Cells were washed again with cold PBS and resuspended in PBS for flow cytometry (EPICS XL; Convergent). Each analysis was performed using at least 50,000 cells that were gated in the region of the lymphocyte population, as determined by light scatter properties (forward scatter vs side scatter). To analyze the expression of transcription factors in lymphocytes (CD8+ T cells), cells were gated in both the lymphocyte and CD8+ regions. Quadrants of dot-plots were set using appropriate isotype controls for each intracellular and extracellular antibody. Appropriate fluorochrome-conjugated isotype-matched monoclonal antibodies (Convergent) were used as a control for background staining in each flow acquisition. In these assays, careful color compensation was performed before cell analysis.

2.4. Statistical analysis

Differences in variables between groups were tested by analysis of variance (ANOVA). Post hoc tests were performed using Fisher’s protected least significant difference. Results are expressed as mean ± standard deviation. A p level < 0.05 was considered statistically significant. Correlation was estimated by Spearman rank correlation. Correlation was considered at p < 0.05.

3. Results

There was no significant difference in age, sex, disease duration, or annualized relapse rate between remitting and relapsing patients. The EDSS score was similar in remitting and relapsing patients before the last relapse, while the EDSS score was higher in relapsing patients during relapse than in remitting patients. Moreover, the time since last relapse was significantly higher in patients with RRMS during remission than relapse phase (Table 1).

Circulating Foxp3+ CD8+ T cells were present both in patients with RRMS and in controls. No difference in Foxp3+ mean fluorescence intensity (MFI, Fig. 1A) and in the percentage of CD8+ Foxp3+ T cells (Fig. 1C) was observed between patients with RRMS and in controls. Foxp3+ MFI was lower in circulating CD8+ T cells from relapsing patients than in ones from remitting patients (p = 0.0294) (Fig. 1B). A lower percentage of circulating CD8+ Foxp3+ T cells was observed in relapsing than in remitting RRMS patients (p = 0.0255) and in controls (p = 0.0362) (Fig. 1D). Most of the CD8+ Foxp3+ T cells in peripheral blood of relapsing RRMS patients expressed on their surface CD25, while CD25 expression was significantly lower in CD8+ Foxp3+ T cells obtained from healthy controls (Fig. 1E). No significant differences were observed in Foxp3+ MFI in CD8+ T cell and in CD8+ Foxp3+ T-cell percentages between healthy subjects and remitting patients (Fig. 1B–D). There were no differences in the percentages of CD8+ Foxp3+ T cells expressing CD25 between remitting and relapsing patients with multiple sclerosis (MS) and between remitting MS patients and controls (Fig. 1F).

Also, the five patients studied longitudinally showed a lower percentage of CD8+ Foxp3+ T cells in relapsing than in remitting phase of the disease, whereas there was no modification in the percentage of CD8+ Foxp3+ T cells in the five healthy subjects studied at baseline and after 6 months (Fig. 2).

We did not find any correlation between the percentage of CD8+ Foxp3+ T cells and patient age, sex, disease duration, and disease severity.

4. Discussion

The transcription factor Foxp3 is both necessary and sufficient to confer suppressive activity to T cells and induces a Treg cell phenotype in conventional CD4+ CD25+ T cells [21]. CD4+ T cells that lose Foxp3 function become unable to suppress immune responses [22]. Recently, Cosmi et al. demonstrated the existence of a subset of human CD8+ CD25+ thymocytes, which expressed high levels of Foxp3 and shared phenotype, functional features and mechanism of action with CD4+ CD25+ Treg cells [23]. Adaptive CD8+ CD25+ Foxp3+ T cells that express markers associated with a regulatory phenotype and cell-cell contact suppressive activity can be induced from CD8+ CD25+ T cells by continuous antigen stimulation [24]. Besides CD4+ Treg, CD8+ Treg expressing Foxp3 also

### Table 1

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<th>Demographic and clinical feature of RRMS patients and controls</th>
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Demographic and clinical features of RRMS patients and healthy subjects included in the study. All data are mean ± SD.

EDSS = Expanded Disability Status Scale; NA = not applicable. Group comparisons were performed by the Student’s t-test, and p < 0.05 (*) was taken as significant.
seem to contribute to the suppression of antiviral and antitumoral immune response. Peripheral blood CD8^+CD25^+Foxp3^+ T cells that share phenotypic and functional features with CD4^+CD25^+Foxp3^+ T cells [5,6,25] and increase with age [25] have been identified in low amount ex vivo in humans but they may be recruited to the cancer tissue or to the sites of inflammation where they suppress antigen-specific immune response [5,6]. Increased percentage of CD8^+CD25^+Foxp3^+ T cells was significantly higher in relapsing MS patients than controls. Box plots express the first (Q1) and third (Q3) quartiles within a given dataset by the upper and lower horizontal lines in a rectangular box, in which there is a horizontal line showing the median. The whiskers extend upwards and downwards to the highest or lowest observation within the upper (Q3 +1.5 x interquartile range) and lower (Q1 – 1.5 x interquartile range) limits. P values indicate statistical significances (<0.05) between the different groups. MFI: mean fluorescence intensity.

Patients affected by HIV showed higher percentage of circulating CD8^+CD25^+Foxp3^+ T cells than healthy subjects [27] while a parallel expansion of human HCV- and influenza virus–specific CD8^+Foxp3^+ effector memory and de novo–generated CD8^+Foxp3^+ Tregs upon antigen recognition in vitro in an IL-2–dependent manner has been reported in HCV patients and controls [6].

We detected in peripheral blood of patients with MS and in controls CD8^+ T cells expressing high levels of Foxp3. Unlike CD4^+ T cells analyzed in the same subjects in a previous study [28] where almost all CD4^+Foxp3^+ T cells expressed CD25 both in relapsing and remitting patients and in controls, only a fraction of CD8^+Foxp3^+ T cells expressed CD25 in all three groups of subjects. Several studies showed that, unlike Foxp3, CD25 represents a general T-cell activation marker rather than being truly Treg-specific.

Fig. 1. Foxp3 expression in CD8^+ T cells and percentage of CD8^+Foxp3^+ T cells in peripheral blood of MS patients and controls A,C,E: No difference in Foxp3 expression in CD8^+ T cells, percentage of CD8^+Foxp3^+ T cells and CD25 expression in CD8^+Foxp3^+ T cells was observed between MS patients and controls. B: Foxp3 MFI was significantly higher in Remitting than Relapsing MS patients. D: CD8^+Foxp3^+ percentage was significantly lower in relapsing than in remitting MS patients and controls. F: Percentage of CD25^+ cells was significantly higher in relapsing MS patients than controls. Box plots express the first (Q1) and third (Q3) quartiles within a given dataset by the upper and lower horizontal lines in a rectangular box, in which there is a horizontal line showing the median. The whiskers extend upwards and downwards to the highest or lowest observation within the upper (Q3 +1.5 x interquartile range) and lower (Q1 – 1.5 x interquartile range) limits. P values indicate statistical significances (<0.05) between the different groups. MFI: mean fluorescence intensity.
In agreement with this view we found a higher percentage of CD8\(^+\)/Foxp3\(^+\) T cells expressing CD25 in relapsing than in remitting patients. Moreover, CD8\(^+\)/Foxp3\(^+\)/CD25\(^+\)/Foxp3\(^+\)/CD25\(^+\) T cells that expressed CD25 after polyclonal activation and were able to suppress the proliferation of CD4\(^+\)/CD25\(^+\)/FOXp3\(^+\)/CD25\(^+\)/Foxp3\(^+\)/CD25\(^+\) T cells in coculture have been described in peripheral blood and in tonsils of healthy subjects [29]. Both patients with MS and controls showed a lower percentage of circulating CD8\(^+\)/Foxp3\(^+\) T cells compared with the percentage of circulating CD4\(^+\)/CD25\(^+\)/Foxp3\(^+\)/CD25\(^+\)/Foxp3\(^+\)/CD25\(^+\) T cells [28].

We observed higher Foxp3 expression at the single cell level in CD8\(^+\)/Foxp3\(^+\) T cells than in CD4\(^+\)/CD25\(^+\) T cells in all three groups of subjects [28]. In addition, CD8\(^+\)/Foxp3\(^+\)/CD25\(^+\)/Foxp3\(^+\)/CD25\(^+\) T cells showed higher Foxp3 expression in RRMS patients in remission than in relapse. All these data indicate that Foxp3 was not transiently expressed in CD8\(^+\)/Foxp3\(^+\) T cells upon activation [15,16]. There was no difference in the percentages of circulating CD8\(^+\)/Foxp3\(^+\) T cells between patients with RRMS and controls but remitting patients showed a higher percentage of these cells and higher expression of Foxp3 in CD8\(^+\)/Foxp3\(^+\) T cells than relapsing patients, suggesting that CD8\(^+\)/Foxp3\(^+\) T cells are involved in the maintenance of peripheral tolerance in MS.

Recent studies demonstrated that CD8\(^+\) T reg may play a role in the immunopathogenesis of MS. Untreated MS patients showed overall deficit in CD8\(^+\) T cell–mediated suppression compared with healthy subjects and this suppressive ability was significantly enhanced by glatiramer acetate (GA) therapy [20]. CD8\(^+\) T cells from GA-treated patients and healthy subjects exhibited potent, HLA-E–restricted GA-specific cytotoxicity [20]. Moreover, HLA-E–restricted CD8\(^+\) T-cell clones able to kill myelin-specific CD4\(^+\) T cells were isolated less frequently from blood and cerebrospinal fluid from MS patients in relapse than in remission or controls [30].

A subpopulation of CD4\(^+\) and CD8\(^+\)/CD25\(^+\) and Foxp3\(^+\) T cells expressing the immune-tolerizing molecule HLA-G with potent suppressive activity have recently been described in the inflamed cerebrospinal fluid of MS patients and in inflammatory demyelinating lesions of MS brain specimens [11]. Aristimuñoz et al. found an inverse correlation between regulatory CD8\(^+\)/CD25\(^+\)/Foxp3\(^+\) T lymphocytes and activated CD4\(^+\) and CD8\(^+\) T lymphocytes at MS relapse [8].

The role of CD8\(^+\)/Foxp3\(^+\) T cells in autoimmunity is still largely unknown but this subset of CD8\(^+\) T cells might be involved not only in viral persistence and cancer progression but also in the development of autoimmune diseases such as MS. CD8\(^+\)/Foxp3\(^+\) T cells might kill CD4\(^+\)/myelin-reactive T cells by perforin or inhibit their proliferation and proinflammatory cytokine production by cell-cell contact and by producing anti-inflammatory cytokines such as IL-10 and transforming growth factor-\(\beta\) or they might inhibit antigen-presenting cells functions. Moreover, PD1 intracellular expression might enhance suppressive ability of CD8\(^+\)/Foxp3\(^+\) T cells as demonstrated for CD4\(^+\)/CD25\(^+\)/Foxp3\(^+\) T cells in MS [31].

**Fig. 2.** Longitudinal evaluation of Foxp3\(^+\)/CD8\(^+\) T cells percentage from RRMS patients in relapse and in remission phase. A: The percentage of Foxp3\(^+\)/CD8\(^+\) T cells increased in all 5 relapsing MS patients during remission phase. B: Representative two-parameter plots showing only cells gated on CD8 T cells from patient 4 during relapse and remission. The y-axis of each histogram represents specific fluorescence of Foxp3 PE; the x-axis represents specific fluorescence of extracellular CD8 PE-Cy5 on fourdecade logarithmic scales. These representative two-parameter plots are obtained from the same patient during relapse and remission. Quadrants were set using appropriate isotype controls for each intra- and extracellular antibody.
Our data, in addition to our previous observation of a decreased percentage of circulating CD4⁺ CD25⁺ Foxp3⁺ T cells in RRMS patients during relapse [28] suggest that besides CD4⁺ CD25⁺ Foxp3⁺, CD8⁺ Foxp3⁺ T cells may play a role in the maintenance of peripheral tolerance in MS. Further studies are necessary to better clarify the role of CD8⁺ Foxp3⁺ T cells in MS.

References