

# Decreased FOXP3 Levels in Multiple Sclerosis Patients

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Autoimmune diseases such as multiple sclerosis (MS) may result from the failure of tolerance mechanisms to prevent expansion of pathogenic T cells. Our study is the first to establish that MS patients have abnormalities in FOXP3 message and protein expression levels in peripheral CD4<sup>+</sup>CD25<sup>+</sup> T cells (Tregs) that are quantitatively related to a reduction in functional suppression induced during suboptimal T-cell receptor (TCR) ligation. Of importance, this observation links a defect in functional peripheral immunoregulation to an established genetic marker that has been unequivocally shown to be involved in maintaining immune tolerance and preventing autoimmune diseases. Diminished FOXP3 levels thus indicate impaired immunoregulation by Tregs that may contribute to MS. Future studies will evaluate the effects of therapies known to influence Treg cell function and FOXP3 expression, including TCR peptide vaccination and supplemental estrogen.

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**Key words:** FOXP3; Treg cells; multiple sclerosis

Autoimmune diseases such as multiple sclerosis (MS) may result from the failure of tolerance mechanisms to prevent expansion of pathogenic T cells directed at myelin determinants or other self-tissue antigens. These tolerance mechanisms include CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs; Sakaguchi et al., 1995) that may have specificity for T-cell receptor (TCR) determinants (Buenafe et al., 2004; Kumar, 2004). CD4<sup>+</sup>CD25<sup>+</sup> Treg cells represent a unique lineage that maintains central tolerance in the thymus (Sakaguchi, 2000; Shevach, 2000). The Treg cells also exert their regulatory function in the periphery, where they constitute ~5–10% of circulating CD4<sup>+</sup> cells. However, peripheral Tregs may also be induced from CD4<sup>+</sup>CD25<sup>-</sup> precursors (Walker et al., 2003b) through

mechanisms involving CD28 costimulation (Tai et al., 2005) and estrogen (Polanczyk et al., 2004).

Although there is renewed interest in the role of Treg cells in preventing autoimmunity in animal models, very little has been published on the involvement of Treg cells in human autoimmune conditions. For diabetes, an initial study showed a decrease in circulating CD4<sup>+</sup>CD25<sup>+</sup> T cells but did not assess functional Treg suppression (Kukreja et al., 2002), whereas a second study showed no difference in the percentage of circulating CD4<sup>+</sup>CD25<sup>+</sup> T cells but a significant decrease in suppression by this T-cell fraction (Lindley et al., 2005). Functional suppression by CD4<sup>+</sup>CD25<sup>+</sup> T cells was also found to be defective in autoimmune polyglandular syndrome type II (Kriegel et al., 2004). For MS, initial studies indicated that

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CD4<sup>+</sup>CD25<sup>+</sup> T cells were increased (Putheti et al., 2003) or unchanged (Putheti et al., 2004). However, a recent study by Viglietta et al. (2004) demonstrated that CD4<sup>+</sup>CD25<sup>+</sup> blood mononuclear cells from MS patients had reduced functional suppression after suboptimal ligation of the TCR. None of the studies demonstrating reduced suppression by blood CD4<sup>+</sup>CD25<sup>+</sup> T cells established whether the decrease in functional suppression was related to changes in expression of the *FoxP3* gene that is an unequivocal marker for Treg function (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003; Walker et al., 2003a). However, such a link has been reported recently for thymocytes from patients with myasthenia gravis (MG; Balandina et al., 2004).

Treg cells provide a critical level of protection against autoimmunity, transplant rejection, and lymphoproliferative disease in several mouse models (Coffer and Burgering, 2004). The FOXP3 transcription factor is predominantly expressed by the Treg cell lineage and appears to act as a master regulator for cytokine production and cell–cell contact-dependent inhibition of T effector cell activation (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003; Ramsdell, 2003) that may involve membrane-bound perforin molecules (Grossman et al., 2004). Recessive X-linked mutations in the *FoxP3* gene in scurfy mice (Brunkow et al., 2001) and in humans with IPEX (immunodysregulation, polyendocrinopathy and enteropathy, X-linked; Bennett et al., 2001; Wildin et al., 2001; Gambineri et al., 2003) lead to a fatal lymphoproliferative autoimmune condition. A nearly identical disease developed in *Foxp3*<sup>-/-</sup> mice that coincidentally lacked a discrete population of suppressive CD4<sup>+</sup>CD25<sup>+</sup> T cells normally present in wild-type mice (Fontenot et al., 2003). FOXP3 expression is required for Treg development and was found to confer suppressive function on peripheral CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. Of importance is that transfer of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells can prevent autoimmune diseases (Shevach, 2000; Maloy and Powrie, 2001), immune pathology in scurfy mice (Fontenot et al., 2003), development of spontaneous insulin-dependent diabetes mellitus in NOD mice (Herman et al., 2004; Peng et al., 2004), and development of experimental autoimmune encephalomyelitis (EAE) in SJL/J mice (Kohm et al., 2002).

In the current study, we evaluated expression of FOXP3 mRNA and protein as well as functional suppressive activity in CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from blood mononuclear cells from MS patients and age- and gender-matched healthy controls (HC). We found that MS patients had abnormalities in FOXP3 message and protein levels in peripheral CD4<sup>+</sup>CD25<sup>+</sup> T cells that were quantitatively related to a reduction in functional suppression induced with suboptimal TCR ligation.

## MATERIALS AND METHODS

### Subjects

Blood was obtained by venipuncture after informed consent was obtained from 19 HC donors (15 females and 4

males, age 22–61 years, mean age 40 years) and 19 MS patients (16 females and 3 males, age 23–61 years, mean age 47 years) with relapsing-remitting (n = 11), primary progressive (n = 1), or secondary progressive (n = 7) MS (disease duration 15.3 years) enrolled in an ongoing open-label clinical trial. The MS patients were not receiving any treatments at the time of sampling, having concluded a >3 month washout period from previous therapies.

### Isolation of T-Cell Subpopulations With Magnetic Beads

Blood was collected into heparinized tubes, and mononuclear cells were separated by Ficoll density centrifugation. The indicator (CD4<sup>+</sup>CD25<sup>-</sup>) and suppressor (CD4<sup>+</sup>CD25<sup>+</sup>) cells were isolated from 70 million peripheral blood mononuclear cells (PBMC) by using the Miltenyi magnetic bead separation protocol. These cells were first incubated with the Miltenyi CD4<sup>+</sup> No Touch T Cell Kit containing antibodies that remove non-CD4<sup>+</sup> cells, including CD8<sup>+</sup> and  $\gamma\delta$ <sup>+</sup> T cells, B cells, natural killer (NK) cells, monocytes, dendritic cells, granulocytes, platelets, and erythroid cells. The CD4<sup>+</sup> cells were then separated by using anti-CD25 mAb-conjugated magnetic beads into the CD25<sup>+</sup> suppressor T-cell fraction (>90% pure) and the remaining CD25<sup>-</sup> fraction that are used as indicator cells.

### FOXP3 Expression

**Real-time polymerase chain reaction.** T-cell subpopulations were analyzed for FOXP3 expression by using real-time polymerase chain reaction (PCR). Briefly, total RNA was isolated from frozen cell pellets with the Qiagen RNeasy Kit (Qiagen, Chatsworth, CA). RNA was Dnase treated with Turbo-DNA free (Ambion, Austin, TX), and cDNA was synthesized in a 20- $\mu$ l volume with Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD) and random primers (Invitrogen, La Jolla, CA) following the manufacturers' recommendations. FOXP3 message expression was determined by the TaqMan method of real-time PCR, with HPR1 as an endogenous control. TaqMan Universal PCR Master Mix, and both the FOXP3 primer/probe (part No. Hs00203958\_m1) and the HPR1 primer/probe sets (part No. 4333768F) were purchased directly from Applied Biosystems (Foster City, CA). HPR1 was chosen as an endogenous control after comparing several different housekeeping genes (18sRNA, PGK1, GAPDH, HPR1) with the goal of finding one which did not vary with the type of sorted cell population or the culture conditions used in this study (data not shown).

**Western blot analysis.** Sorted cells were lysed in lysis buffer [25 mM Tris-Cl, pH 8.8, 1 mM EDTA, and 2% sodium dodecyl sulfate (SDS)] and analyzed by Western blotting with 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels. Rabbit anti-human FOXP3 antibody (1:1,000; a gift from Dr. Ziegler, Benaroya Research Institute, Seattle, WA) and goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody (1:20,000; Pierce, Rockford, IL) and the enhanced ECL system (Amersham, Arlington Heights, IL) were used for the detection of FOXP3 protein. Actin was

subsequently detected with mouse antiactin antibody (1:1,000; Chemicon, Temecula, CA) and goat anti-mouse IgG HRP-conjugated antibody (1:20,000; Pierce) as an internal control.

### Treg Suppression Assay With Bead-Sorted Cells

All suppression assays were performed in 96-well round-bottom plates (Becton Dickinson, Franklin Lakes, NJ) in a final volume of 200  $\mu$ l/well of 1% type AB human serum complete media (Bio Whittaker, Walkersville, MD). Prior to assay setup, 18 wells each in the 96-well plates were coated with 100  $\mu$ l of a final concentration of 2.0  $\mu$ g/ml anti-CD3 + anti-CD28 mAbs or 1.0, 0.5, and 0.1  $\mu$ g/ml anti-CD3 mAb only (Caltag, Burlingame, CA), and the plates were incubated overnight at 4°C. All wells were washed before assay setup. The CD4<sup>+</sup>CD25<sup>-</sup> cells were plated at  $2.0 \times 10^4$ /well alone or in combination with CD4<sup>+</sup>CD25<sup>+</sup> cells in triplicate at  $0.4 \times 10^4$ ,  $0.8 \times 10^4$ ,  $2.0 \times 10^4$ , and  $4.0 \times 10^4$ /well, and the CD4<sup>+</sup>CD25<sup>+</sup> cells were cultured alone at  $2.0 \times 10^4$ /well. Thus the cells were cocultured at the following ratios: 1:0, 1:0.2, 1:0.4, 1:1, 1:2, and 0:1. To the wells containing 1.0, 0.5, and 0.1  $\mu$ g/ml plate-bound anti-CD3 mAb, 5  $\mu$ g/ml anti-CD28 mAb and  $1.0 \times 10^4$  irradiated (2,500 rads) PBMC were added as antigen-presenting cells (APCs). On day 5, 0.5  $\mu$ Ci of <sup>3</sup>H-thymidine (NEN, Boston, MA) was added to each well for the final 16 hr of culture. The cells were then harvested on glass fiber filters and assessed for uptake of the labeled thymidine by liquid scintillation. Percentage suppression was determined at each mixed cell ratio compared with responses of CD4<sup>+</sup>CD25<sup>+</sup> (suppressor cells) and CD4<sup>+</sup>CD25<sup>-</sup> T cells (indicator T cells) alone, as follows: [mean cpm (indicator cells) – mean cpm (mixed cell culture)]/[mean cpm (indicator cells) – mean cpm (suppressor cells)]. The percentage suppression was plotted vs. increasing percentage of suppressor:indicator cells, and a regression line was calculated. I<sub>50</sub> values were determined as the ratio of suppressor:indicator cells that produced 50% suppression.

### Statistical Analyses

Spearman rank-order correlation was used to test the correlation between paired samples of FOXP3 message and FOXP3 protein, FOXP3 message and I<sub>50</sub>, and FOXP3 protein and I<sub>50</sub>. A *t*-test was used to test the significance of the difference between the mean FOXP3 protein in HC and MS, between FOXP3 message in HC and MS, and between average I<sub>50</sub> values in HC and MS for each concentration of anti-CD3.

## RESULTS

Although suppression assays have suggested lower Treg activity in PBMC from MS patients vs. HC donors, no studies have evaluated expression of FOXP3, considered an unequivocal marker of Treg cells. Therefore, we compared for the first time the expression of FOXP3 in the CD4<sup>+</sup>CD25<sup>+</sup> fraction of PBMC with both mRNA and protein detection assays in five MS patients enrolled sequentially in an open-label clinical trial vs. five age- and gender-matched HC donors. A comparison of FOXP3 mRNA expression by quantita-

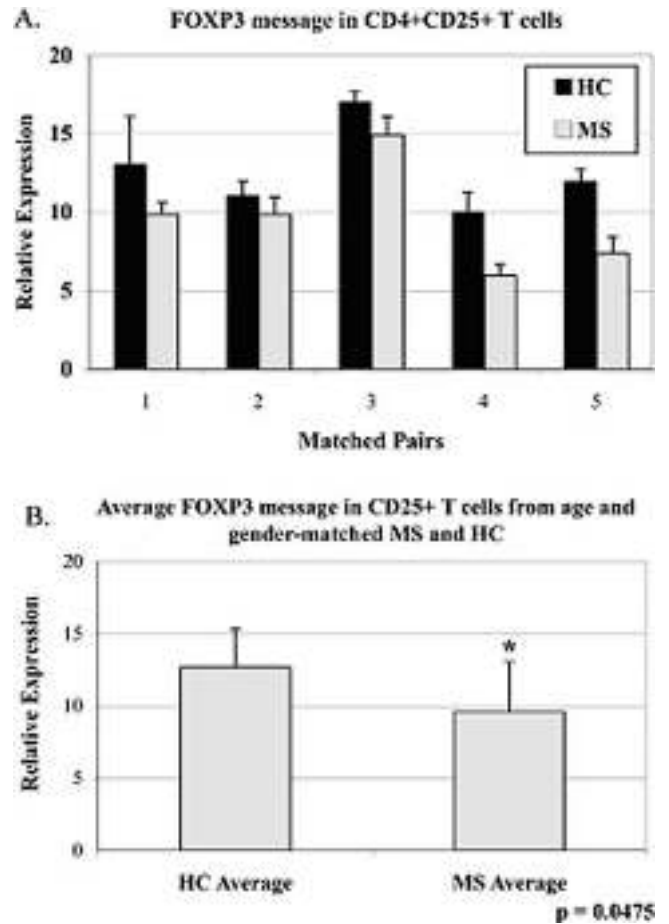


Fig. 1. FOXP3 message in CD4<sup>+</sup>CD25<sup>+</sup> T cells from MS patients and HC. **A:** Pairwise comparison of FOXP3 message in five age- and gender-matched sets of MS patients and HC subjects. FOXP3 message was assessed by real-time PCR on magnetic bead-sorted cells. **B:** Average message percentage  $\pm$  SD. MS patients have significantly less FOXP3 message than HC subjects ( $P = 0.0475$ , *t*-test).

tive real-time PCR revealed reduced message levels in CD4<sup>+</sup>CD25<sup>+</sup> T cells from each of the MS patients vs. paired HC (Fig. 1A) and a significant difference between the two groups ( $P < 0.0475$ ; Fig. 1B). Similarly, comparison of FOXP3 protein expression by Western blots of CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from the same five donor pairs demonstrated a consistent reduction in FOXP3 protein (range 31–53%; Fig. 2A) and a highly significant difference between the two groups ( $P < 0.01$ ; Fig. 2B).

To assess functional suppression from the paired MS and HC donors, we stimulated various ratios of CD4<sup>+</sup>CD25<sup>-</sup> indicator cells mixed with CD4<sup>+</sup>CD25<sup>+</sup> T cells (1:0, 1:2, 1:1, 1:0.4, 1:0.2, and 0:1) with superoptimal (2  $\mu$ g/ml), optimal (1 and 0.5  $\mu$ g/ml), and suboptimal (0.1  $\mu$ g/ml) concentrations of plate-bound anti-CD3 antibody + anti-CD28 antibody. Figure 3A shows *P* values for the differences in suppression mediated by CD4<sup>+</sup>CD25<sup>+</sup> T cells from MS patients vs. matched HC

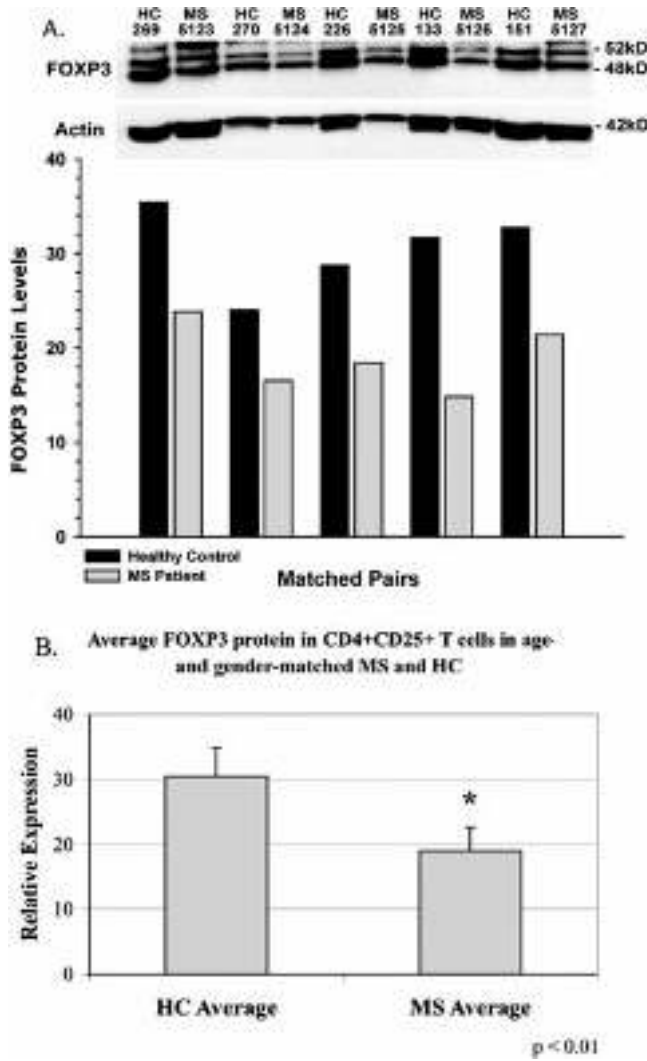


Fig. 2. FOXP3 protein in CD4<sup>+</sup>CD25<sup>+</sup> T cells from MS patients and HC. **A:** Upper panel shows Western blot results from age- and gender-matched MS and HC. FOXP3 protein stains as two bands of approximately 50 kD. Densitometry was used to give a numerical value to the FOXP3 bands for each subject, and these results are depicted in the lower panel. Each MS patient shows less FOXP3 protein than the adjacent age- and gender-matched HC. The matched pairs are shown in the same order as in Figure 1A. **B:** Mean FOXP3 protein expression percentage ± SD. There is a highly significant difference in the FOXP3 protein levels of HC vs. MS ( $P < 0.01$ , *t*-test).

at the different anti-CD3 concentrations and various cell ratios. It appears that, at both the super- and suboptimal anti-CD3 concentrations, CD4<sup>+</sup>CD25<sup>+</sup> T cells from MS patients consistently produced less suppression than those from matched HC. For each concentration of anti-CD3, we calculated an I<sub>50</sub> value (percentage of CD4<sup>+</sup>CD25<sup>+</sup> cells needed to cause a 50% suppression of proliferation response by the CD4<sup>+</sup>CD25<sup>-</sup> indicator cells) based on dose-dependent suppression observed at various cell ratios. Thus, higher I<sub>50</sub> values indicate less suppression.

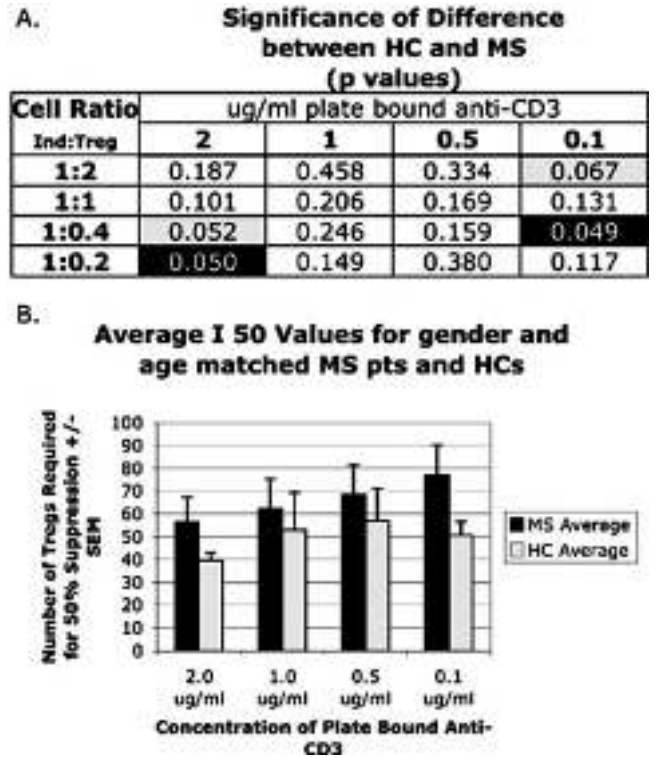


Fig. 3. Suppression assay results in MS patients and age- and gender-matched HC. **A:** Results of *t*-test comparison of percentage suppression in MS patients and HC under different conditions and cell ratios of Ind (indicator):Treg cells. In the suppression assay, different concentrations of anti-CD3 were used to stimulate the CD25<sup>-</sup> indicator cells, along with different ratios of indicator cells to regulatory CD25<sup>+</sup> cells. Suppression data were not available from one MS donor at the lowest 3 concentrations of anti-CD3. Thus, matched values were compared from 4MS and 4HC donors at these anti-CD3 concentrations. There was a significant difference in the suppressive ability of CD25<sup>+</sup> T cells in HC and MS under two of the sixteen conditions ( $P$  values shown in black boxes). At a superoptimal (2 μg/ml) and suboptimal (0.1 μg/ml) concentrations of anti-CD3, there was a marked difference in the suppressive ability of MS and HC, which was either close to being significant ( $P$  values shown in gray boxes) or was significant (black boxes). **B:** Average I<sub>50</sub> values for MS and HC at four concentrations of anti-CD3. The I<sub>50</sub> value takes into account the suppression seen at all four different cell ratios used, and higher I<sub>50</sub> values indicate less suppression. MS patients showed less suppression (higher I<sub>50</sub>) than HC at all concentrations of anti-CD3, and this difference was greatest at the suboptimal anti-CD3 concentration.

As is shown in Figure 3B, MS patients had higher I<sub>50</sub> values (less suppression) vs. matched HC at all four concentrations of anti-CD3 antibody, with the lowest concentration (0.1 μg/ml) showing the greatest difference between groups (78 ± 12 vs. 51 ± 7).

Reduced expression of FOXP3 and less suppression in MS might be explained by a decreased percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells in PBMC. However, this was not the case; the two groups had essentially identical levels of CD4<sup>+</sup>CD25<sup>+</sup> T cells (4.6% ± 1.5% for HC vs. 4.7% ± 1.3% for MS). Because the CD4<sup>+</sup>CD25<sup>+</sup> compartment contains a mixture of both activated effector T cells and

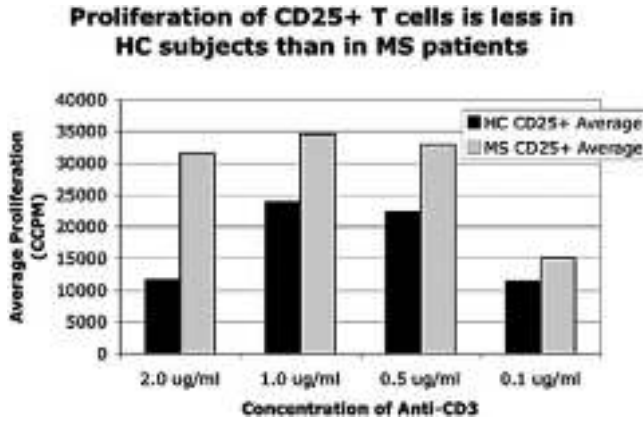


Fig. 4. CD4<sup>+</sup>CD25<sup>+</sup> T cells from HC proliferate less than CD4<sup>+</sup>CD25<sup>+</sup> T cells from MS patients. Equal numbers of bead-sorted CD4<sup>+</sup>CD25<sup>+</sup> cells were added to wells containing various concentrations of plate-bound anti-CD3, and the amount of proliferation was determined by tritiated thymidine uptake. HC showed less proliferation than MS at all concentrations of anti-CD3, but this difference was not significant. HC cells proliferated best at 1 and 0.5  $\mu\text{g}/\text{ml}$  anti-CD3, and proliferation declined at “superoptimal” 2  $\mu\text{g}/\text{ml}$  and “suboptimal” 0.1  $\mu\text{g}/\text{ml}$  anti-CD3.

Treg cells, it is possible that reduced Treg function might be explained by a reduced percentage of Treg cells. Activation of purified CD4<sup>+</sup>CD25<sup>+</sup> Treg cells alone with anti-CD3 antibody typically produces lower responses than the CD4<sup>+</sup>CD25<sup>-</sup> indicator cells resulting from the anergic nature of the Treg suppressor cells. Moreover, the better the Treg enrichment within the CD4<sup>+</sup>CD25<sup>+</sup> compartment, the lower the expected proliferation response. We therefore compared responses of the purified CD4<sup>+</sup>CD25<sup>+</sup> cells from MS vs. HC donors without any indicator cells present (the 1:0 ratio). As is shown in Figure 4, purified CD4<sup>+</sup>CD25<sup>+</sup> T cells from MS patients consistently had more proliferation than the same number of CD4<sup>+</sup>CD25<sup>+</sup> T cells from HC donors, particularly at the 2  $\mu\text{g}/\text{ml}$  anti-CD3 concentration. These findings suggest reduced Treg cell activity in MS.

An additional question of interest is whether a correlation exists between functional suppression as determined by  $I_{50}$  values and expression of FOXP3 message and FOXP3 protein. As shown in Figure 5A, there was a significant correlation between FOXP3 message and protein levels when evaluated for all MS and HC donors ( $P < 0.05$ ). Moreover, there was a highly significant negative correlation between FOXP3 mRNA levels and  $I_{50}$  suppression values determined at the 0.1  $\mu\text{g}/\text{ml}$  concentration of anti-CD3 mAb ( $P < 0.01$ ; Fig. 5B) and between FOXP3 protein levels and  $I_{50}$  suppression values determined at the 0.1  $\mu\text{g}/\text{ml}$  concentration of anti-CD3 mAb ( $P < 0.01$ ; Fig. 5C). These statistically significant correlations validate FOXP3 expression levels as an indicator of ex vivo suppression assessed during suboptimal activation with anti-CD3.

To compare FOXP3 expression further in a larger sampling of MS patients and HC, we assessed mRNA

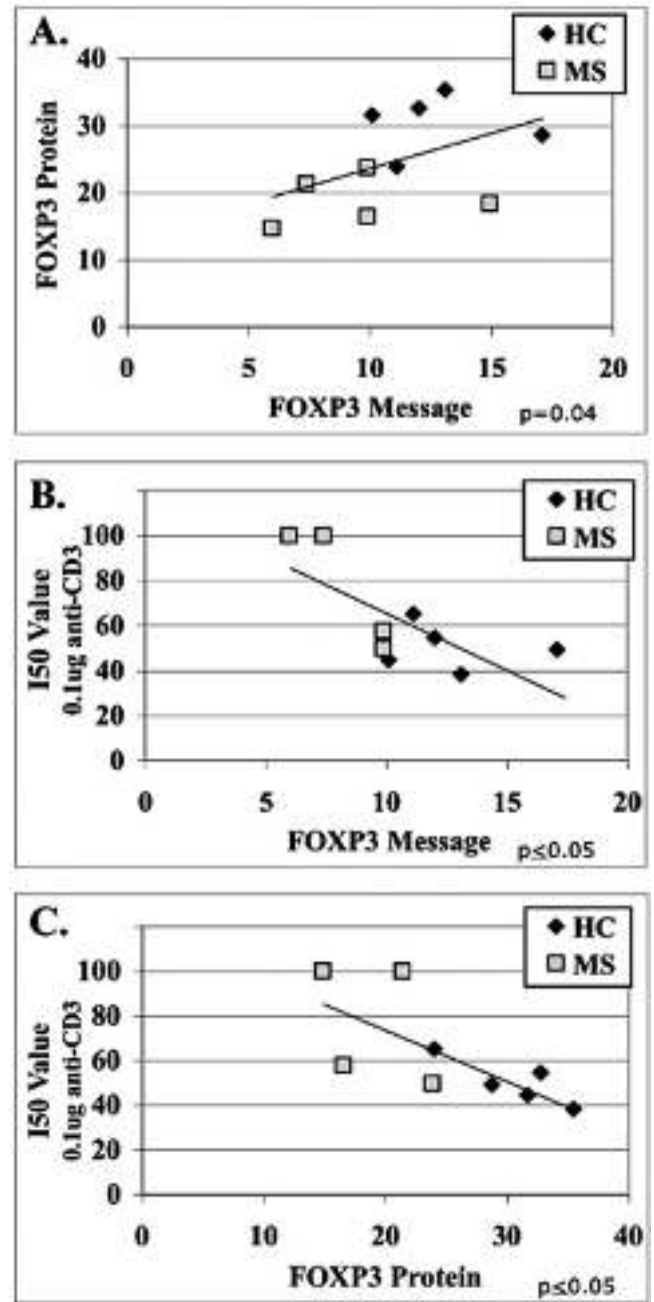


Fig. 5. Correlation between FOXP3 message, FOXP3 protein, and  $I_{50}$  values. **A:** Correlation between FOXP3 message and FOXP3 protein in MS patients and HC. FOXP3 message in CD4<sup>+</sup>CD25<sup>+</sup> T cells was determined by real-time PCR, and FOXP3 protein by Western blot followed by densitometry. There is a significant positive correlation between FOXP3 message and FOXP3 protein in CD4<sup>+</sup>CD25<sup>+</sup> T cells ( $P = 0.04$ , Spearman rank-order test). **B:** Highly significant correlation between FOXP3 message and  $I_{50}$  values ( $P < 0.01$ , Spearman rank order test). Lower  $I_{50}$  values, which indicate better suppression, are correlated with higher levels of FOXP3 message in CD4<sup>+</sup>CD25<sup>+</sup> T cells. **C:** Highly significant correlation between  $I_{50}$  values and FOXP3 protein in CD4<sup>+</sup>CD25<sup>+</sup> T cells ( $P < 0.01$ , Spearman rank-order test). Individuals with higher levels of FOXP3 protein in CD4<sup>+</sup>CD25<sup>+</sup> T cells show better suppression (lower  $I_{50}$  value). For B and C, the  $I_{50}$  value was missing from 1 MS patient.

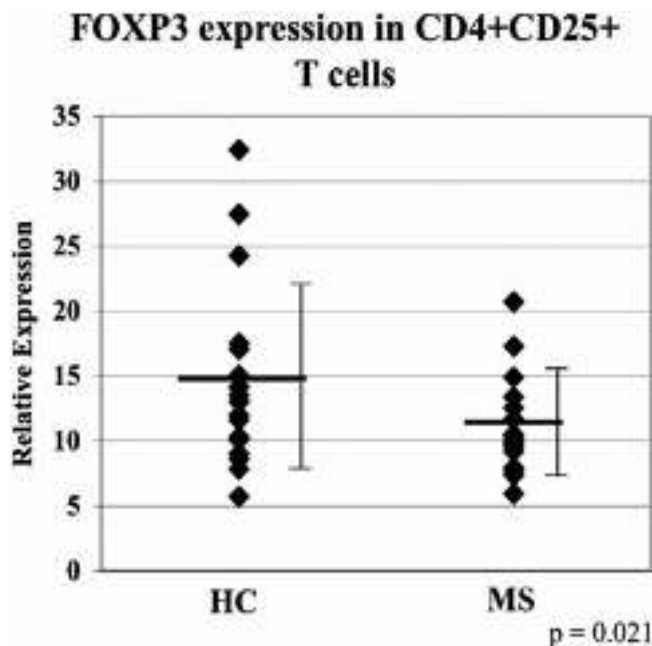


Fig. 6. FOXP3 expression is higher in HC subjects than in MS patients. Comparison of FOXP3 message in  $CD4^+CD25^+$  T cells from 19 HC subjects and 19 MS patients. FOXP3 message, as determined by real-time PCR, was significantly lower in MS patients than in HC subjects ( $P = 0.021$ , *t*-test).

expression by real-time PCR in a total of 19 MS patients enrolled in the open-label study (including the five patients described above) vs. a total of 19 HC controls (including the five described above). As is shown in Figure 6 for all donors, FOXP3 expression was significantly reduced in the MS patients vs. HC donors ( $P = 0.021$ ). No significant differences were found in FOXP3 expression in RRMS vs SPMS patients, although there was a trend toward lower FOXP3 levels in RRMS patients.

## DISCUSSION

Our data demonstrate for the first time a significant correlation between FOXP3 expression and functional suppression in human  $CD4^+CD25^+$  PBMC and a significant reduction in FOXP3 expression in a cohort of relapsing and secondary progressive MS patients enrolled in our open-label TCR peptide vaccine study. Although previous studies have clearly implicated FOXP3 expression as being necessary and sufficient to observe suppression in vitro (Fontenot et al., 2003; Walker et al., 2003b), ours is the first study to correlate quantitative differences in human FOXP3 expression and functional suppression in the peripheral  $CD4^+CD25^+$  T-cell compartment. This is an important advance because of the difficulties in obtaining reliable suppression data from fluorescence-activated cell sorting (FACS) or magnetic bead-enriched  $CD4^+CD25^+$  Treg cell populations from fresh or frozen PBMC that also contain variable levels of activated T effector cells that are not suppressive.

Unlike children with IPEX who completely lack a functional *Foxp3* gene (Gambineri et al., 2003), MS patients express FOXP3, but at variably reduced levels. Reduced rather than absent levels of FOXP3 have also been reported in thymocytes from patients with MG (Balandina et al., 2004). Correlation of functional suppression with FOXP3 expression indicates that the defect in MS is quantitative rather than qualitative. However, it remains to be determined whether there are fewer FOXP3<sup>+</sup> Treg cells present in MS or whether there is less FOXP3 expressed per cell. In summary, quantifying FOXP3 message and protein should provide a reliable indication of the degree of functional suppression by  $CD4^+CD25^+$  Treg cells. Measurement of gene expression levels is preferred over ex vivo functional assays for assessing Treg function in MS for two important reasons. The functional assay may also detect suppression mediated by other types of regulatory  $CD4^+$  T cells, such as Th2, Th3, and Tr1 cells. Even more important is that the  $CD4^+CD25^+$  T cells used in functional assays contain variable mixtures of Treg cells and nonsuppressive T effector cells that tend to be highly proliferative.

Our data confirm in part the results of Viglietta et al. (2004) demonstrating that the defect in functional Treg suppressive activity in MS can best be detected with suboptimal stimulation through the TCR. Comparison of activation levels with a range of anti-CD3 concentrations clearly demonstrated reduced proliferation of both  $CD4^+CD25^+$  T cells (including Treg cells) and  $CD4^+CD25^-$  indicator cells at the suboptimal 0.1  $\mu$ g dose of plate-bound anti-CD3 mAb (not shown). One can speculate that the low concentration of anti-CD3 might only be suboptimal for induction of proliferation responses and secreted interleukin-2 by cocultured  $CD4^+CD25^+$  effector T cells, whereas it remains optimal for inducing Treg suppression. Thus, as we observed, HC donors showed less proliferation but more suppression at the 0.1  $\mu$ g than at the 0.5  $\mu$ g anti-CD3 concentration, whereas MS patients showed less proliferation and less suppression under these conditions. Moreover, Treg cells might be more efficient at inhibiting suboptimal activation of  $CD4^+CD25^-$  indicator cells in the mixed cultures. These considerations would seem to be relevant to the in vivo situation that likely involves suboptimal activation of both Treg and T effector cells as a result of limiting concentrations of neuroantigens.

Although alterations in the functional suppression of enriched populations of  $CD4^+CD25^+$  T cells have been reported previously for MS (Viglietta et al., 2004), type I diabetes (Lindley et al., 2005), and autoimmune polyglandular syndrome type II (Kriegel et al., 2004), the deficiencies were not clearly linked to reduced expression of FOXP3. The only other report clearly linking reduced functional suppression with lower FOXP3 expression focused on thymocytes obtained from patients with MG (Balandina et al., 2004). In these patients, there were equivalent levels of  $CD4^+CD25^+$  thymocytes compared with controls but essentially no detectable suppression coupled with significantly lower

but not absent levels of expressed FOXP3 message. It remains to be determined whether the lack of suppression in the presence of detectable FOXP3 message can be accounted for by lack of FOXP3 protein or whether there is a functional defect in the FOXP3 regulatory mechanism. Moreover, no information was presented concerning FOXP3 expression or functional suppression by peripheral CD4<sup>+</sup>CD25<sup>+</sup> T cells from MG patients. This comparison will be of particular interest because of suggested differences in Treg cells in the thymus vs. the periphery (Bluestone and Abbas, 2003; Walker et al., 2003b; Curotto de Lafaille et al., 2004; Grossman et al., 2004).

In conclusion, our study clearly established that MS patients have reduced levels of FOXP3 expression in peripheral CD4<sup>+</sup>CD25<sup>+</sup> T cells that is quantitatively related to a reduction in functional suppression induced with suboptimal TCR ligation. This observation is the first to link a defect in functional immunoregulation to an established genetic marker that has been unequivocally shown to be involved in maintaining peripheral immune tolerance and preventing autoimmune diseases. How FOXP3 proteins are regulated in the Treg cells is unknown, but it is possible that regulatory mechanisms are altered during the course of MS. Future studies will further evaluate differences in FOXP3 expression and Treg cell function in relapsing vs. progressive MS, and the effects of therapies known to influence Treg cell function and FOXP3 expression, including TCR peptide vaccination and supplemental estrogen.

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