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TITLE PAGE

The $CD4^+$ T-cell subset lacking expression of the CD28 costimulatory molecule is expanded and shows a higher activation state in multiple sclerosis

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ABSTRACT:

Multiple sclerosis (MS) is a chronic debilitating disease, in which T-cells are considered to play a pivotal role. CD28 is the quintessential costimulatory molecule on T-cells and its expression declines progressively with repeated stimulations, leading to the generation of $CD28^-$ T-cells. Our aim was to examine whether $CD4^+CD28^-$ T-cells were enriched in MS patients, and characterize the phenotype of this subset in MS patients and healthy controls (HC).

All these changes could provide these $CD4^+CD28^-$ T-cell characteristics that might be involved in the pathogenesis of MS, turning this T-cell subset into a potential target for future therapeutic strategies.

KEY WORDS: Multiple sclerosis, $CD4^+CD28^-$ T-cells, activation, NK cells markers, adhesion molecules, cytotoxic markers.

INTRODUCTION

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating and neurodegenerative disease of the central nervous system (CNS) caused, presumably, by an autoinmune process in which T cells are considered to play a pivotal role in orchestrating the self-reactive immune responses. Naive T lymphocytes require two independent signals to become fully activated. The first, an antigen-specific signal is sent via the T cell receptor (TCR). The second signal, delivered through the engagement of costimulatory receptors, is critical to allow full activation, sustain cell proliferation, prevent anergy and/or apoptosis, induce differentiation to effector and memory status, and allow cell-cell cooperation (Frauwirth and Thompson2002). CD28 is the main costimulatory molecule required to promote T cell activation, although memory T cells seem to be less dependent on CD28 for their reactivation than naive T cells (Chitnis and Khoury2003).

CD28 is expressed constitutively on $CD4^+$ T lymphocytes and, upon T cell activation and interaction with its ligands B7-1 (CD80) or B7-2 (CD86), it is transiently downmodulated in parallel with the upregulation of the cytotoxic T lymphocyte antigen 4 (CTLA-4), an inhibitory countereceptor that binds to the same ligands with higher affinity, and sends a negative signal that terminates T-cell activation (Linsley et al.1996).

CD28 expression declines progressively with repeated stimulation by the same antigens, leading invariably to generation of $CD28^-$ T cells. Thus, a progressive increase in the percentage of T cells lacking CD28 expression on their surface *in vivo* is common with advancing age in healthy individuals, presumably from exposure to common antigens throughout the life of a subject, so its expression is a good biological indicator of replicative senescence (Bryl et al.2001). Expansion of $CD4^+$ T cells lacking CD28

expression, disproportionate with patient age, has been reported in patients with inflammatory or autoimmune diseases and thus, has been considered as a marker of chronic inflammation (Thewissen et al.2005, Garcia de Tena et al.2004, Raffeiner et al.2005, Sun et al.2008, Lambers et al.2009).

These $CD4^+CD28^-$ T cells are functionally active, as the downregulation of CD28 expression in $CD4^+$ T cells has been related more with a gain rather than a loss in gene expression (Goronzy et al.2005). It has been postulated that dysregulation of costimulatory signals may be involved in the pathogenesis of MS (Oliveira et al.2003). In this sense, several authors have reported that autoreactive T cells in MS are less dependent on CD28-mediated costimulation: myelin basic protein (MBP)-reactive T cells from MS patients were able to be activated in the absence of CD28/B7 costimulatory pathway and blockade of CD28 failed to inhibit MBP-specific T-cell proliferation in MS patients, but not in healthy subjects (Lovett-Racke et al.1998, Markovic-Plese et al.2001).

Therefore our aim was to examine whether $CD4^+CD28^-$ T-cells would be enriched in patients with MS peripherally, and to further characterize the phenotype of the $CD4^+CD28^-$ T-cell subset in MS patients and healthy controls (HC).

MATERIALS AND METHODS

Subjects

A total of 78 Caucasian patients with clinically definite MS according to the McDonald criteria (McDonald et al.2001, Polman et al.2011), and naive to immunomodulatory therapy were recruited through the Multiple Sclerosis unit of the Carlos Haya Hospital, Málaga, Spain. All of them had a typical relapsing-remitting MS (RRMS). None of the patients had presented attacks or had received corticosteroids for at least 3 months before enrolment. As controls, 74 age- and sex-matched healthy unrelated subjects were obtained from the BioBank of our Hospital.

Demographic and clinical characteristics of the MS patients recorded were: sex, age, age at onset, clinical form at onset and at present, disease duration, expanded disability status scale (EDSS) score and progression index (current EDSS score /disease duration).

Written informed consent was obtained from patients and controls. The study was approved by the Institutional Research Ethics Committee (Comisión de Ética y de Investigación del Hospital Regional Universitario Carlos Haya).

Surface immunofluorescence

Fresh lithium heparinised blood was obtained by venipuncture from each MS patient and control. Peripheral blood mononuclear cells (PBMC) were purified using a Ficoll-Hypaque gradient, as described in the supplier's protocol (ICN Biomedicals Inc., OH, USA).

To evaluate the expression of surface markers, a total of 2×10^5 PBMC were incubated for 15 min, with several combinations of the following monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) or allophycocianin (APC): anti-CD45RA, anti-CD11a,

anti-CD54, anti-CD45RO, anti-CD11b (Caltag Laboratories); anti-CD28, anti-CD69, anti-CD57, anti-CD161, anti-NKB1, anti-CD134, anti-CTLA-4, anti-CD25, anti-CD154, anti-CD95, anti-CD4, anti-CD3 (BD Biosciences and BD Pharmingen) and anti-CD49d-FITC (Cytognos).

After incubation, cells were washed and resuspended in saline buffer. Twenty thousand events gated on the $CD3^+CD4^+$ lymphocyte subpopulation were acquired in FACSCalibur™ flow cytometer and analyzed with the Cell Quest Pro software (BD Biosciences). Isotype-matched controls were used to verify the staining specificity of the antibodies.

Results were expressed as percentages of expression of each marker to respectively gated $CD4^+CD28^+$ and $CD4^+CD28^-$ T lymphocytes.

Intracellular immunofluorescence: cytokine, perforin and Granzyme B measurements

Flow cytometric determination of cytokines and granzyme B in the cytoplasm of PBMC was performed as follows. Briefly, aliquots from each subject of 2×10^6 PBMC/ml in RPMI-1640 (BioWhittaker) supplemented with 2 mM L-glutamine (ICN Biomedicals), 5% heat-inactivated fetal calf serum FBS (BioWhittaker) and 0.032 mg/ml gentamicin (Normon), were stimulated and cultured for 4 h at 37°C with 5% CO_2 with a combination of 10 ng/ml of phorbol myristate acetate (PMA) and 1 µg/ml ionomycin in the presence of 10 µg/ml of brefeldin A for the last two hours (all from Sigma Chemical Co).

Spontaneous perforin production was determined in parallel in the absence of exogenous stimuli activation.

After stimulation, cells were washed, aliquoted and stained with combinations of the following monoclonal antibodies: PerCP-conjugated anti-CD3, PE-conjugated anti-

CD28, APC-conjugated anti-CD4 or anti-CD8 or FICT-conjugated anti-CD8 (BD Biosciences and BD Pharmingen) for 15 min at room temperature and fixed for 10 min with fixing solution (Fix and Perm, Caltag Laboratories) and washed. Subsequently, each aliquot of cell suspension was labeled with APC-conjugated anti-IL10 or with one of the following FITC-conjugated MoAb: anti-CD69, anti-IL2, anti-IFN-γ, anti-TNF-α, anti-Granzyme B, anti-Perforin (BD Biosciences and BD Pharmingen), anti-IL4 or anti-IL5 (Caltag Laboratories) in the presence of permeabilizing solution (Fix and Perm; Caltag) for 30 min at room temperature, in the dark, according to manufacturers' instructions.

The typical forward and side scatter gate for lymphocytes together with a $CD3^+CD4^+$ gate were set to exclude monocytes from the analysis. Percentages of cytokine, perforin or granzyme B-producing cells in each $CD4^+CD28^+$ or $CD4^+CD28^-$ T cell subset were analyzed as described for the surface immunofluorescence.

Statistical analysis

Descriptive statistics included means and standard deviations or medians and interquartile ranges (IR) for quantitative variables and relative frequency for qualitative variables.

Comparisons of demographic characteristics between controls and MS patients were performed by Pearson χ^2 test for the categorical variable of sex, and by Mann-Whitney test for the quantitative variable of age.

Expression of surface markers, cytokines and cytotoxic markers on $CD4^+CD28^+$ and $CD4^+CD28^-$ T cell subsets were tested for the distribution using the Kolmogorov-Smirnov and Shapiro-Wilks tests and expressed as medians and IR. As many of the variables did not follow a normal distribution, two group comparisons were assessed by the non-parametric Mann-Whitney U test.

For every comparison, p values < 0.05 were considered statistically significant.

RESULTS

1. Demographic and clinical characteristics of the patients.

The demographic and clinical characteristics of the 78 untreated RRMS patients included in the study are shown in Table 1. Sex and age distribution did not differ significantly between controls and non-treated MS patients.

2. Patients with MS showed increased frequencies of $CD4^+$ T cells that had lost CD28 expression.

The percentage of $CD4^+CD28^-$ T cells in peripheral blood varies dramatically in different individuals, though the non-treated MS patients showed an increased frequency of $CD28^-$ T cells in the CD4 compartment (mean: 5.39%; median: 3.72%; IR: 1.45-7.67) as compared to the control subjects (mean: 2.00%; median: 0.85%; IR: 0.36-2.38) ($p=3.42 \times 10^{-8}$), as shown in Figure 1.

As a loss of CD28 expression related to senescence has been described, we evaluated the correlation between the age of the subjects and the frequency of $CD4^+CD28^-$ T-cells. No correlation was found for HC ($r=0.09$, $p=0.43$) nor MS patients ($r=-0.06$, $p=0.67$).

The number of $CD4^+$ T cells lacking CD28 did not correlate with gender in either the patient or the control group, nor with the clinical form, disease duration, baseline EDSS, or progression index (data not shown).

As expected in the control group, analysis of CTLA-4, the CD28 counterreceptor, showed a higher expression in the $CD4^+CD28^-$ T-cell subset than in their $CD28^+$ counterparts (0.64% *vs.* 0.33%; $p=0.006$); however, no differences were found between the two subpopulations in the non-treated MS patient group (0.29% *vs.* 0.27%; p= n.s.).The expression of CTLA-4, within the $CD4^+CD28^-$ T-cell subset, was higher in controls than in untreated MS patients ($p=0.032$) (Figure 2).

3. $CD4^+CD28^-$ T cells presented a predominant memory/effector phenotype

The differentiation stage of human $CD4^+$ T cells can be classified according to their surface expression of CD45 isoforms, with antigen-naive T cells being $CD45RA^+$ and $CD45RO^-$ and antigen-experienced T cells $CD45RA^-$ and $CD45RO^+$. The proportion of the memory/effector phenotype cells ($CD45RA^-CD45RO^+$) in the $CD28^-$ population was significantly higher than that in the $CD28^+$ counterpart, both in MS patients (0.00007) and HC (0.006), as shown in Figure 3.

4. Differences in the activation state and expression of surface molecules, cytokines and cytotoxic markers in $CD4^+CD28^-$ versus $CD4^+CD28^+$ T cell subpopulations, within each group of subjects.

4.1. Activation state

Activation of T cells, assessed by the surface expression of CD69, was significantly higher in $CD4^+CD28^-$ T-cell subset in both groups of subjects, as shown in Table 2.

4.2. Surface markers.

The natural killer cell receptors CD57 and NKB1 as well as functionally important surface adhesion molecules such as ICAM-1 (CD54), VLA-4 (CD49d), LFA-1 (CD11a) and macrophage-1 antigen (Mac-1, CD11b/CD18) were always preferentially expressed on $CD4^+CD28^-$ T cells. Expression of another natural killer receptor, CD161, was also slightly higher in the $CD4^+CD28^-$ T subset in untreated MS patients, and that of the costimulatory molecule CD40L (CD154) was higher in this subset in controls, as shown in Table 2.

On the contrary, Fas death receptor expression was always significantly higher in the $CD4^+CD28^+$ T-cell subpopulation, as was the expression of $CD25^{bright}$ regulatory

$CD4^+$ T cells. Expression of the costimulatory molecule OX40 (CD134) was slightly higher in this subset in the untreated MS patients, as can be seen in Table 2.

A representative example of the expression of surface molecules in both $CD4^+$ T-cell subsets is shown in Figure 4.

4.3. Cytokine and cytotoxic marker production.

Within each group of subjects, the frequency of IFN-γ and TNF-α -producing cells was significantly higher in the $CD4^+CD28^-$ T-cell subset. Additionally, granzyme B and perforin were almost exclusively produced by this $CD28^-$ T-cell subset.

Conversely, IL2 production was increased in their $CD28^+$ counterpart in both groups of subjects. Additionally, IL-4 production was significantly higher in the $CD28^+$ subset in untreated MS patients, and IL-5 in healthy controls as shown in Table 3.

5. Differences between treatment-naive MS patients and controls in the expression of surface molecules, cytokines and cytotoxic markers within each $CD4^+$ T-cell subpopulation.

5.1. Activation state.

The activation of $CD4^+CD28^-$ T cells was significantly higher in non-treated MS patients than in controls (13.99% vs. 7.12%; p=0.016), as can be observed in Figure 5.

5.2. Natural killer cell receptors expression.

Within the $CD4^+CD28^-$ T cells, CD57 expression was significantly higher in untreated MS patients than in controls (p=0.010), but CD161 and NKB1 expression did not show significant differences.

Expression of NK cell receptors did not differ between untreated MS patients and controls in the $CD4^+CD28^+$ T subset (Figure 6).

5.3. Surface adhesion molecules expression.

In the $CD4^+$ T-cell subpopulation lacking CD28, treatment-naive patients showed a higher expression of VLA-4 (p=0.005) and LFA-1 (p=0.03) than controls. Conversely, ICAM-1 expression in this $CD4^+$ subset was higher in controls than in untreated MS patients (p=0.010) and MAC-1 expression did not differ between them. In the $CD4^+CD28^+$ T-cell subset, untreated patients showed a higher expression of VLA-4 when compared to controls (p=0.001), as shown in Figure 7.

5.4. Fas expression.

In both $CD4^+$ T-cell subpopulations, the expression of the cell death receptor Fas was always higher in the control group (p=0.006 for $CD28^-$ cells and p=0.011 for $CD28^+$cells), as can be observed in Figure 8a.

5.5. Expression of accessory molecules.

CD40L expression did not show significant differences between both groups of subjects in the $CD4^+CD28^-$ T-cell subpopulation, but was higher in non-treated MS patients when compared to controls (p=0.005) in their $CD28^+$ counterparts, as can be seen in Figure 8b. Conversely, a higher expression was noted of OX40 in controls than in untreated MS patients in both $CD4^+$ T cell subpopulations (p=0.007 for $CD28^-$ T-cells and p= 0.010 for $CD28^+$ T-cells) (Figure 8c).

5.6. $CD25^{bright}$ $CD4^+$ T lymphocyte expression.

No significant differences were found in $CD25^{bright}$ expression in the $CD4^+CD28^-$ T cell subpopulation between the different groups of subjects, but $CD25^{bright}$ expression on $CD4^+CD28^+$ T cells was significantly higher in untreated MS patients when compared to controls (p=0.010) (Figure 8d).

5.7. Cytokine production.

In the $CD4^+CD28^-$ T-cell subpopulation, there were no differences in the production of IL2, IL10 and IL5 between untreated MS patients and controls (Figure 9).

Untreated MS patients showed higher percentages of IFN-γ and TNF-α-producing cells than controls (p=0.015 and p=0.023, respectively) and lower percentages of IL4-producing $CD4^+CD28^-$ T cells (p=0.035).

In the $CD4^+CD28^+$ T cells, IL2, IL10 and IFN-γ production was higher in the untreated MS patients than in controls (p=0.004, p=0.001 and p=0.001, respectively), as can be observed in Figure 9. No differences for TNF-α, IL4 and IL5 production were detected between MS patients and controls within this cell subset.

5.8. Intracellular expression of cytotoxic markers.

In the $CD4^+CD28^-$ T-cell subpopulation no significant differences were found in perforin and Granzyme B production between MS patients and controls, as shown in Figure 9.

On the other hand, the granzyme B-producing $CD4^+CD28^+$ T cells were less frequent in controls than in untreated patients (p=0.006), differences that were not observed for the perforin-producing cells.

DISCUSSION

Many authors have shown the expansion of $CD4^+CD28^-$ T cells in peripheral blood from patients with inflammatory or autoimmune diseases (Thewissen et al.2005, Garcia de Tena et al.2004, Raffeiner et al.2005, Sun et al.2008, Lambers et al.2009). In this study, we confirmed the expansion of these $CD4^+CD28^-$ T cells in peripheral blood from MS patients, with widely variable interindividual frequencies of expression, as previously described (Markovic-Plese et al.2001, Thewissen et al.2007, Miyazaki et al.2008). The original of this study is the assessment of the expression of surface receptors, cytokines and cytotoxic markers *ex vivo* in the $CD4^+CD28^+$ and $CD4^+CD28^-$ T cell subsets obtained from healthy controls and untreated MS patients, and not *in vitro* in T cell clones, as in previous studies.

No correlation between the frequency of $CD4^+CD28^-$ T cells and the age of the subjects was found, a fact corroborated by others (Thewissen et al.2005), probably because the mean age of our patients and, therefore, of our matched controls, was lower than in other studies where a correlation was described (Vallejo et al.1998, Miyazaki et al.2008).

In general, loss of CD28 expression is due to T cell activation by occupation of the T cell receptor, and is inexorably accompanied by a transient increase in CTLA-4 expression. In MS patients, this loss might be due to a chronic antigen stimulation of T cells that leads to a progressive and permanent lack of CD28 expression that is not associated with an upregulation in CTLA-4 expression. This loss is accompanied by phenotypic and functional changes that distinguish these T cells from the other $CD4^+$ T cells, allowing them to use alternative costimulatory pathways (Park et al.1997).

The predominance of a memory/effector phenotype within the $CD4^+CD28^-$ T-cell subset in MS patients, also reported in other autoimmune diseases (McRae et

al.1998, Kobayashi et al.2007), could be the logical result of a chronic exposure to antigen stimuli. In this study we found that the proportion of the memory/effector phenotype cells in the $CD28^-$ population was significantly higher than that in the $CD28^+$ counterpart, and that $CD4^+CD28^-$ T-cells showed a higher degree of activation in non-treated MS patients than in healthy controls. These data suggest that $CD4^+CD28^-$ T-cells are more likely to have been activated *in vivo* and/or have more extensively differentiated into memory effector T cells in MS patients compared to controls, indicating that these cells may be participating in the pathogenesis of MS.

To assess whether this higher *in vivo* activation state in the $CD4^+CD28^-$ T-cell subset was accompanied of functional changes in these cells, we evaluated the acquisition of NK cells markers, adhesion molecules, death receptor, and accessory molecules that could act as specific costimulatory molecules, as well as of cytokines and cytotoxic markers.

In humans, expression of prototypic markers of NK cells has been found on a subset of T cells and has been attributed to *in vivo* expansion of cytotoxic effector T cells after repeated antigenic activation. This state of replicative senescence has been described not only in the elderly but also in clinical conditions involving chronic activation of the immune system such as viral infections, rheumatic and autoimmune diseases or tumors (Tarazona et al.2000). Furthermore, NK-type T cells have been shown to produce greater amounts of IFN-γ than did NK cells (Ami et al.2002). To investigate the cytotoxic potential of these $CD4^+$ T-cell subsets, we assessed the cell-surface expression of the NK receptors CD57, NKB1 and CD161. All of them were preferentially expressed in the $CD4^+CD28^-$ T cell subset, but only $CD4^+CD28^-CD57^+$ T-cells were significantly higher in MS patients than in controls. The induction of these receptors on $CD4^+CD28^-$ T-cells might provide them, on one hand, with alternative

costimulatory pathways, and on the other hand, with characteristics of innate immunity, supplying them with a greater cytotoxic potential that may have consequences for the inflammatory responses imposed by these cells. In addition, CD57 may also contribute to cell trafficking to the CNS through the blood-brain barrier, as it has been reported to function as a neuronal homophilic adhesion molecule and to bind to L- and P-selectin (Poggi et al.1997, Ratts et al.2006).

To evaluate the migratory potential and activation requirements of these $CD4^+$ T-cell subsets we assessed the cell-surface expression of the adhesion molecules VLA-4, LFA-1, ICAM-1 and Mac-1. It is known that interaction of LFA-1 with its main ligand, ICAM-1, induces a series of transmembrane signals promoting the secretion of proinflammatory cytokines. Thus, increased expression of LFA-1 on $CD4^+CD28^-$ T cells could occur in an attempt to provide alternative costimulatory pathways. VLA-4 and LFA-1 expression was higher in the $CD4^+CD28^-$ T-cell subset in untreated MS patients than in controls, probably providing these T-cells with an alternative mechanism for T-cell activation and migration to their target tissues (Anderson and Siahaan2003), as the binding of VLA-4 to VCAM-1 has been identified as the key mediator of T cell entry into the CNS in MS (Elovaara et al.2000). On the other hand, ICAM-1 showed a defective expression in MS patients. In general, the expression of ICAM-1 on the lymphocyte surface does not seem crucial in the process of lymphocyte migration into the CNS and, although some authors have found an increased ICAM-1 expression on T lymphocytes in MS patients (Paz et al.1999), others have not (Correale and Bassani Molinas Mde2003).

Experimental autoimmune encephalomyelitis studies have shown that Mac-1 expression on T cells is critical for disease development and that targeting Mac-1 may offer the most useful β2-integrin therapeutic approach (Hu et al.2010). Although very

few T lymphocytes express Mac-1 (Hemler1990), this β2-integrin participates in transmigration of leukocytes into secondary lymphoid organs and in complement-mediated phagocytosis. In our study, as described by other authors (Hoshino et al.1993), Mac-1 expression seemed to be inversely proportional to that of CD28, as reflected in both MS patients and controls.

We also wanted to assess the apoptotic potential of these subsets. The expression of the death receptor Fas was higher in the $CD4^+CD28^+$ T cell subpopulation, data in agreement with findings described in rheumatoid arthritis, in which $CD4^+CD28^-$ T cells appear to be resistant to activation-induced cell death after occupation of the T-cell receptor, while their $CD28^+$ counterparts are subjected to apoptosis (Vallejo et al.2000). Differences in the expression of Fas between healthy subjects and MS patients are controversial. Some authors found no differences (Zipp et al.1998), while others showed a higher expression in MS patients (Ichikawa et al.1996). We found a lower Fas expression in untreated MS patients in both $CD4^+$ subsets, and this might be contributing to the pathogenesis of the disease by prolonging T-cell survival and promoting their migration to the CNS. On the other hand, it has also been reported that a persistent antigenic stimulation is responsible for the accumulation of effector $CD28^-$ T-cells accompanied by the decrease of CD95-T cells (Sansoni et al.2008) and thus, it is normal that $CD28^-$ T-cells are defective in CD95 expression.

The interactions between CD40 and CD40L provide essential signals in T-cell activation and development of effector functions during immune responses. When the expression of CD40L is increased or prolonged, the magnitude of the immune response can be expanded beyond those antigens that initially triggered the response and, consequently may develop immunity against irrelevant or self-antigens (Crow and Kirou2001). Although the constitutive expression of CD40L in $CD4^+$ T cells is minimal,

we found a significantly increased expression in the $CD4^+CD28^-$ T subpopulation as compared to their $CD28^+$ counterparts. Within the $CD4^+CD28^+$ T-cell subpopulation, MS patients always showed a higher expression than controls. Indeed, higher CD40L expression in MS patients (Kosmaczewska et al.2007) and patients with other autoimmune diseases has been reported (Desai-Mehta et al.1996). The OX40/OX40L system depends on CD28 signalling and is crucial for the final outcome of the T-cell response. Therefore, it is not surprising to find a higher expression of OX40 in the $CD4^+CD28^+$ T-cell subpopulation. Strikingly, the expression of this molecule was higher in the controls in both cell subpopulations. It is likely that the chronic antigen stimulation of T cells in MS patients, as well as leading to the progressive loss of CD28 expression, may also contribute to the loss of OX40.

The importance of regulatory T (Treg) cells in the pathogenesis of MS is growing. Most studies reported no numeric Treg alterations in peripheral blood but a functional deficit of $CD4^+CD25^{bright}$ Treg, that may contribute to disease development (Viglietta et al.2004, Haas et al.2005). In our study, $CD25^{bright}$ cells were almost absent in the $CD4^+CD28^-$ T cells compartment, as expected for a subset of T-cells with a predominant effector function. A higher expression in untreated MS patients compared to controls was observed in their $CD28^+$ counterparts, but its functional relevance remains to be clarified.

Cytokines play an important role in the pathogenesis of MS, a disease that occurs in defective regulation of the balance between proinflammatory and antiinflammatory cytokines (Cannella and Raine2004), with a predominant production of the former in $CD4^+$ T cells of MS patients.

IL2 was mainly produced by the $CD4^+CD28^+$ T-cell subpopulation both in MS patients and controls, due to the necessary CD28-mediated costimulation for

transcription of the IL2 gene (Boise et al.1995), as described in other diseases (Duftner et al.2005). However, the $CD4^+CD28^-$ T-cell subset was still able to synthesize IL2 at low levels, probably because its transcription may be stimulated by some other less efficient alternative costimulatory pathways. The MS patients had a higher proportion of IL2-producing $CD4^+CD28^+$ T cells than the controls, in agreement with previous studies (Clerici et al.2001).

Likewise, the $CD4^+CD28^+$ T-cell subpopulation produced higher levels of IL4 than their $CD28^-$ counterparts in the group of MS patients and of IL5 in the controls. As expected, a higher production of proinflammatory cytokines and cytotoxic markers was observed in the $CD4^+$ T-cell subpopulation lacking CD28 than in its $CD28^+$ counterpart, as previously described in some other diseases and *in vitro* studies (Duftner et al.2005, Nakajima et al.2002, Fasth et al.2004) providing these $CD28^-$ cells with the ability to lyse target cells. MS patients showed higher proportions of IFNγ-producing cells in both subsets, and of TNFα-producing cells in the $CD28^-$ subset, suggesting the contribution of these cytokines in myelin destruction. We also determined the IL-17 production in a small sample of MS patients (n=15) and controls (n=15) (data not shown), but we were unable to detect IL-17 in the $CD28^-$ subset, findings reported previously by some other authors (Miyazaki et al. 2008). It has recently been reported an increased frequency of Th17 cells in the cerebrospinal fluid of patients with RRMS during relapses but, in peripheral blood, the percentage was low and did not differ from that in patients with other neurological diseases. When these authors generated Th1 and Th17 T cell clones from MS patients they found that CD28 was found at much higher levels in the Th17 cell clones than on Th1 clones (Brucklacher-Waldert et al. 2009). All these findings support our idea that the phenotype of the $CD4^+CD28^-$ subset is mainly constituted by Th1 cells.

An impaired production of IL4 has been described in patients with active MS (Mokhtarian et al.1994, Huang et al.1999) that has been reproduced in our study within the $CD4^+CD28^-$ T-cells. On the contrary, we found a higher production of IL10 in the $CD4^+$ $CD28^+$ T-cell subset in non-treated patients when compared to controls. In this sense, several authors have shown a higher IL10 mRNA expression in MS patients (Navikas et al.1995, Rieckmann et al.1995).

Cytotoxic granules production was almost exclusively restricted to the $CD4^+CD28^-$ subset, providing these cells with the possibility to lyse target cells. Although slightly higher in the MS patients, their production did not differ significantly to that in the HC, as reported in rheumatoid arthritis (Thewissen et al.2007), suggesting that production of cytotoxic granules is more related with the $CD4^+CD28^-$ T-cell subset than with the disease itself.

To conclude, the peripheral blood $CD4^+CD28^-$ T-cell population, which is expanded in patients with MS, is not anergic or functionally ineffective. On the contrary, in MS patients, among the many phenotypic and functional changes that accompany the definitive loss of CD28 expression on $CD4^+$ T cells are a higher activation state and the exhibition of a memory phenotype. Our data suggest that these cells might have been stimulated *in vivo* and display markers of repeated antigenic stimulation such as the acquisition of the NK cell marker CD57, as well as VLA-4 and LFA-1 adhesion molecules, and the accessory molecule CD40L at significantly higher levels than in HC. Moreover, MS patients exhibit higher percentages of IFNγ and TNFα-producing $CD4^+CD28^-$ T-cells, providing these T-cells with a cytolytic profile and unique functional characteristics that might be playing an important role in the pathogenesis of MS.

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AUTHORSHIP

Contribution: L.L. and M.J.P.M. conceived and designed the project. M.J.P.M., J.A.G.L., B.O.M., C.L.G., C.M.B.. and T.O. performed the experiments. L.L., MJ.P.M. and O.F. analyzed the data and wrote the paper with input from the other authors. C.A.U., GL and OF supervised patient recruitment, provided clinical samples and provided clinical data. OF and LL contributed equally to this study.

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FIGURES LEGENDES

Figure 1. Peripheral $CD28^-CD4^+$T-cells are expanded in RRMS patients (mean: 5.39%; median: 3.72%; IR: 1.45-7.67) compared with healthy controls (HC) (mean: 2.00%; median: 0.85%; IR: 0.36-2.38).

(A) Representative dot plot of the $CD4^+CD28^-$ and $CD4^+CD28^+$ T-cell subpopulations in an untreated RRMS patient. Cells are gated on $CD3^+$ cells.

(B) Percentage of $CD4^+CD28^-$ T-cells out of the total $CD4^+$ T-cells in peripheral blood mononuclear cells of 74 HC and 78 untreated RRMS patients. Boxes extend from the 25th to the 75th percentiles; the line in the middle and the vertical lines represent the median value and both the 10th and 90th percentiles, respectively. The Mann–Whitney test was used to determine statistical differences.

Figure 2. Percentage of CTLA-4 positive cells in the $CD4^+CD28^+$ and $CD4^+CD28^-$ T-cell subsets in healthy controls and untreated MS patients.

Figure 3. Expression of the memory/effector phenotype ($CD45RA^-CD45RO^+$) in $CD4^+CD28^-$ and $CD4^+CD28^+$ T-cell subsets. A clear predominance of the memory/effector phenotype is shown in the $CD4^+CD28^-$ T cell subset both in HC and MS patients.

Figure 4. A representative example of the expression of surface molecules in $CD4^+CD28^-$ and $CD4^+CD28^+$ T cell subsets in an MS patient.

(A) Cells gated on $CD3^+$ and $CD4^+$ cells are expressed as dot-plots.

(B) Cells gated on $CD4^+$ cells are expressed as dot-plots.

Figure 5. Expression of the early activation marker CD69 in $CD4^+CD28^-$ and $CD4^+CD28^+$ T cell subsets. CD69 expression in the $CD4^+CD28^-$ T cell subset was significantly increased in untreated MS patients compared to healthy controls. The

$CD4^+CD28^-$ T cell subset showed always a higher activation state than its CD28+ counterpart.

Figure 6. Expression of the Natural killer cell receptors. The natural killer cell receptors CD57, CD161 and NKB1 were preferentially expressed on $CD4^+CD28^-$ T cells. Within this subset, CD57 expression was significantly higher in untreated MS patients than in controls.

Figure 7. Expression of Adhesion molecules. Surface adhesion molecules such as ICAM-1 (CD54), VLA-4 (CD49d), LFA-1 (CD11a) and macrophage-1 antigen (Mac-1, CD11b/CD18) were always preferentially expressed on $CD4^+CD28^-$ T cells. Within the $CD28^-$ T-cell subset, treatment-naive MS patients showed a higher expression of VLA-4 and LFA-1 and a lower expression of ICAM-1 than controls. MAC-1 expression did not differ between them. In the $CD4^+CD28^+$ T-cell subset, untreated patients showed a higher expression of VLA-4 when compared to controls.

Figure 8. Expression of Fas, costimulatory molecules and $CD4^+CD25^{bright}$ T lymphocytes, within each $CD4^+$ T-cell subset, in MS patients and controls.

(A) Fas expression was always higher in controls than in MS patients in both subsets.

(B) In the $CD4^+CD28^-$ T-cell subset, no differences in the expression of CD40L between MS patients and controls were found. In the $CD28^+$ counterpart, a lower expression of CD40L was found in controls when compared to MS patients.

(C) OX40 expression was always higher in controls than in MS patients, in both $CD4^+$ T-cell subsets.

(D) A higher proportion of $CD25^{bright}$ $CD4^+$ T-cells within the $CD28^+$ T cell subset were found in MS patients as compared to healthy controls.

Figure 9. Production of intracellular cytokines and Granzyme B, after stimulation with PMA/ionomycin and spontaneous production of Perforin, within each $CD4^+$ T-cell subset, in MS patients and controls.

(A) In the $CD4^+CD28^+$ T cells, IL2 production mainly occurred in MS patients.

(B) In the $CD4^+CD28^+$ T-cell subset, MS patients had more IL10-producing cells than controls.

(C) No differences in the production of IL5 between MS patients and controls were observed in either of the $CD4^+$ T-cell subsets.

(D) Untreated MS patients showed lower percentages of IL4-producing $CD4^+CD28^-$ T cells.

(E) MS patients produced higher amounts of IFNγ than controls in both $CD4^+$ T-cell subsets.

(F) Untreated MS patients showed higher percentages of TNF-α-producing $CD4^+CD28^-$ T cells than controls.

(G & H) Though Granzyme B and perforin were almost exclusively produced by the $CD4^+$ $CD28^-$ T-cell subset, no differences between MS patients and controls were observed. Conversely, $CD4^+CD28^+$ granzyme B-producing cells were more frequent in MS patients than in controls.

Table 1. **Demographic and MS clinical variables of all participants.**

Table 2. Comparison of surface molecule expression between $CD4^+CD28^-$ and $CD4^+CD28^+$ T cell subsets in each group of subjects.

Table 3. Comparison of cytokine and cytotoxic marker production between $CD4^+CD28^-$ and $CD4^+CD28^+$ T-cell subsets in each group of subjects.

	NON-TREATED RRMS[a] PATIENTS (n = 78)	CONTROLS (n = 74)	p
Sex: female/male ratio (%)	51/27 (65.4/34.6)	54/20 (73/27)	n.s.[b]
Age in years	36.5 ± 11.2	35.31 ± 10.1	n.s.
Mean age at onset in years	28.8 ± 10.6		
Mean disease duration in years	7.6 ± 6.3		
EDSS Score at present	1.4 ± 1.6		
Progression index (EDSS score at present/disease duration)	0.5 ± 1.8		

Table 1.

Quantitative data are presented as mean ± standard deviation (minimum–maximum).

[a]Relapsing-Remitting MS patiens

[b]n.s.= not significant

Table 2.

	$CD4^+CD28^-$ vs. $CD4^+CD28^+$ subsets			
Surface molecule expression		**$CD4^+CD28^-$ median (IR)[d]**	**$CD4^+CD28^+$ median (IR)**	**P values**
CD69	HC[a]	7,22 (3,71 – 10,87)	1,59 (1,11 – 2,52)	$p = 6.75 \times 10^{-13}$
	Untreated MS[b]	9,11 (4,95 – 15,38)	1,89 (1,10 – 3,30)	$p = 1.87 \times 10^{-10}$
CD57	HC	40,00 (26,87 – 69,15)	1,27 (0,97 – 2,13)	$p = 5.26 \times 10^{-24}$
	Untreated MS	62,55 (36,58 – 80,92)	1,42 (1,15 – 2,56)	$p = 5.49 \times 10^{-19}$
NKB1	HC	1,37 (0,00 – 4,64)	0,26 (0,17 – 0,42)	p = 0.018
	Untreated MS	1,10 (0,44 – 4,90)	0,23 (0,19 – 0,50)	$p = 3{,}44 \times 10^{-6}$
CD161	HC	5,35 (1,54 – 8,63)	3,85 (1,58 – 6,73)	n.s.[c]
	Untreated MS	5,88 (2,29 – 11,43)	3,86 (1,60 – 5,65)	p = 0.019
ICAM-1	HC	19,23 (8,12 – 33,08)	2,86 (1,38 – 4,32)	$p = 1.20 \times 10^{-17}$
	Untreated MS	13,59 (7,23 – 22,71)	2,35 (1,55 – 3,97)	$p = 1.41 \times 10^{-10}$
VLA-4	HC	66,67 (47,46 – 83,95)	23,88 (19,08 – 31,42)	$p = 1.79 \times 10^{-13}$
	Untreated MS	85,34 (51,50 – 96,00)	32,85 (21,61 – 48,38)	$p = 4.88 \times 10^{-10}$
LFA-1	HC	100,00 (98,67 – 100,00)	99,61 (99,35 – 99,80)	$p = 9.07 \times 10^{-5}$
	Untreated MS	100,00 (100,00 – 100,00)	99,74 (98,74 – 99,91)	$p = 1.12 \times 10^{-10}$
MAC-1	HC	15,00 (7,59 – 27,78)	2,89 (0,78 – 6,39)	$p = 8.95 \times 10^{-10}$
	Untreated MS	11,28 (4,42 – 26,93)	1,93 (0,66 – 5,76)	$p = 5.68 \times 10^{-7}$
Fas	HC	21,82 (8,74 – 33,51)	37,16 (18,21 – 42,34)	p = 0.003
	Untreated MS	11,43 (4,27 – 26,78)	24,89 (10,23 – 36,88)	p = 0.002
$CD25^{bright}$	HC	0,00 (0,00 – 0,40)	0,84 (0,50 – 1,52)	$p = 1.24 \times 10^{-12}$
	Untreated MS	0,00 (0,00 – 0,27)	1,20 (0,70 – 2,71)	$p = 8.00 \times 10^{-15}$
CD40L	HC	0,00 (0,00 – 0,51)	0,09 (0,05 – 0,30)	p = 0.005
	Untreated MS	0,06 (0,00 – 0,77)	0,20 (0,10 – 0,45)	n.s.

OX40	HC	2,36 (1,00 – 4,52)	2,54 (1,68 – 4,05)	n.s.
	Untreated MS	1,09 (0,18 – 3,50)	1,85 (0,94 – 2,82)	p = 0.050

[a]HC: healthy controls; [b]Untreated MS: untreated multiple sclerosis patients
[c]n.s.= not significant. [d]IR= interquartile ranges
Grey cells show those markers preferentially expressed in the $CD4^+CD28^-$ T cell subset

Table 3.

	$CD4^+CD28^-$ vs. $CD4^+CD28^+$ subsets			
		$CD4^+CD28^-$ median (IR)[d]	**$CD4^+CD28^+$ median (IR)**	**P values**
IL2 production	HC[a]	3,24 (1,93 – 8,61)	13,83 (6,36 – 19,39)	$p = 1.8 \times 10^{-9}$
	Untreated MS[b]	3,54 (1,70 – 6,55)	16,94 (11,90 – 23,89)	$p = 5.9 \times 10^{-13}$
IFN-γ production	HC	26,57 (21,91 – 37,02)	8,13 (4,28 – 9,57)	$p = 3.7 \times 10^{-15}$
	Untreated MS	32,90 (25,37 – 42,90)	11,34 (8,28 – 14,63)	$p = 9.3 \times 10^{-18}$
TNF-α production	HC	30,51 (25,21 – 34,37)	19,36 (14,95 – 27,09)	$p = 4.0 \times 10^{-6}$
	Untreated MS	34,00 (27,02 – 41,98)	22,74 (16,40 – 33,73)	$p = 3.3 \times 10^{-5}$
IL4 production	HC	1,24 (0,87 – 2,20)	1,51 (0,82 – 2,19)	n.s.[c]
	Untreated MS	0,97 (0,58 – 1,61)	1,58 (1,00 – 1,99)	p = 0.003
IL5 production	HC	0,58 (0,26 – 0,83)	0,77 (0,56 – 1,02)	p = 0.018
	Untreated MS	0,80 (0,22 – 1,28)	0,77 (0,56 – 1,83)	n.s.
IL10 production	HC	0,87 (0,27 – 1,21)	0,91 (0,77 – 1,32)	n.s.
	Untreated MS	1,36 (0,69 – 2,28)	1,45 (1,14 – 3,04)	n.s.
Granzyme B production	HC	14,74 (7,24 – 25,44)	0,78 (0,53 – 1,23)	$p = 1.1 \times 10^{-15}$
	Untreated MS	13,19 (7,22 – 26,84)	1,11 (0,78 – 1,59)	$p = 3.2 \times 10^{-18}$
Perforin production	HC	77,75 (66,45 – 82,14)	2,72 (2,18 – 4,09)	$p = 1.4 \times 10^{-17}$
	Untreated MS	76,48 (55,26 – 91,10)	2,65 (1,58 – 4,41)	$p = 1.5 \times 10^{-19}$

[a]HC: healthy controls; [b]Untreated MS: untreated multiple sclerosis patients

[c]n.s.= not significant. [d]IR= interquartile ranges
Grey cells show those markers preferentially expressed in the $CD4^+CD28^-$ T cell subset.

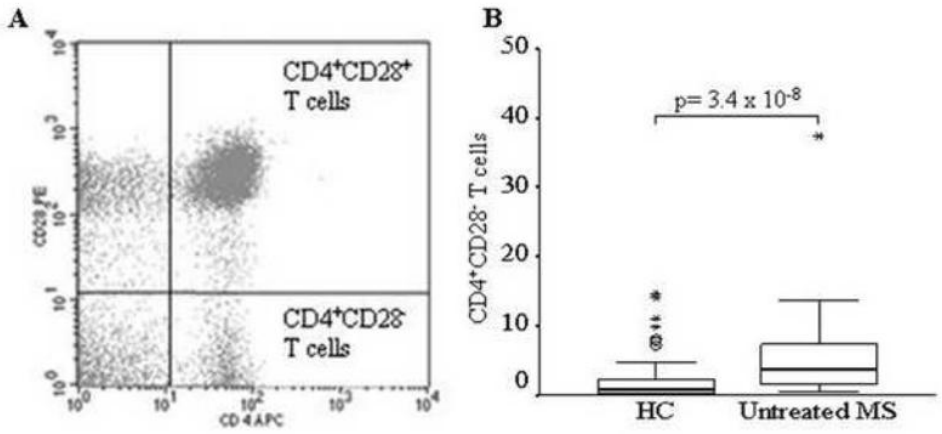


Figure 1

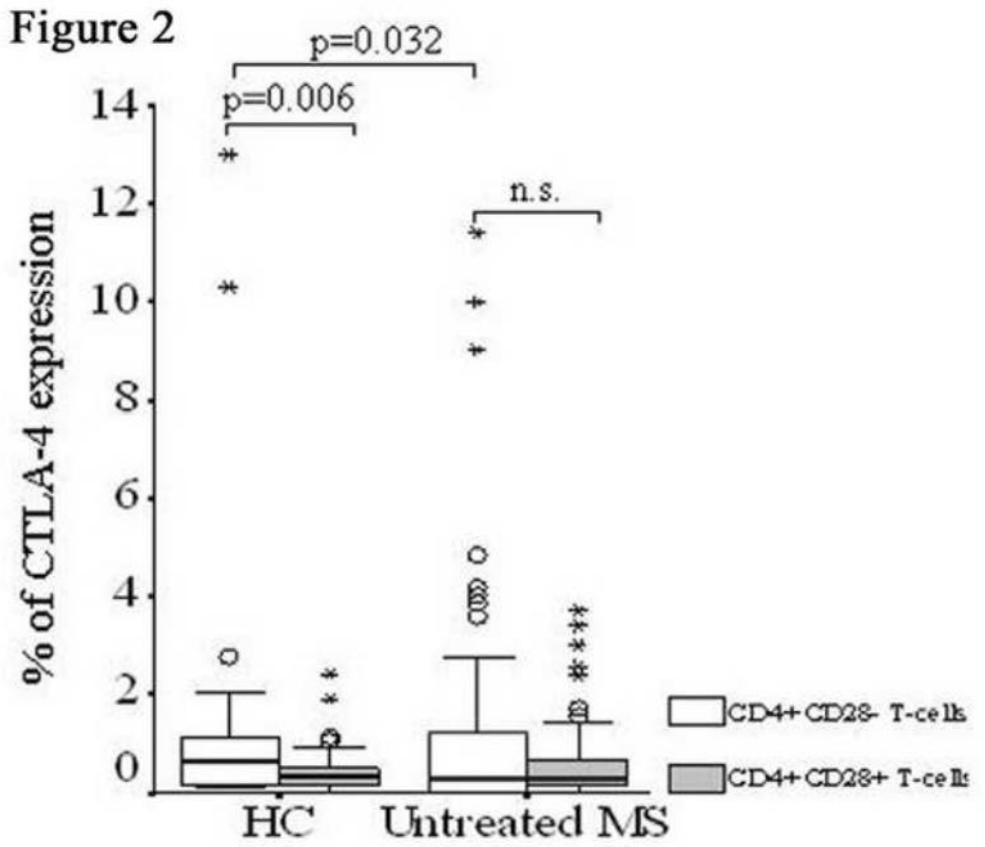


Figure 2

Figure 3

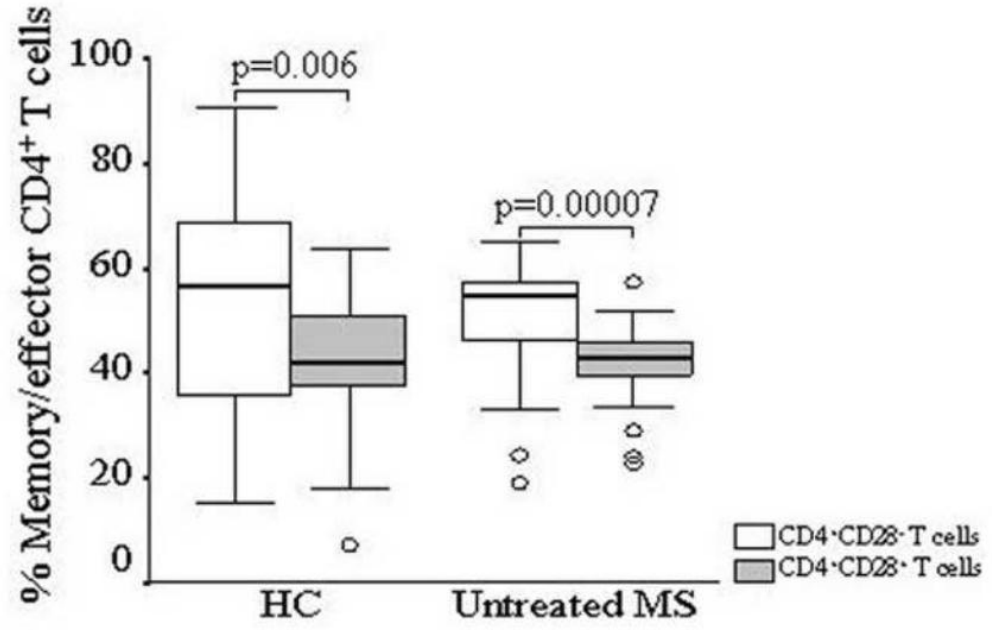
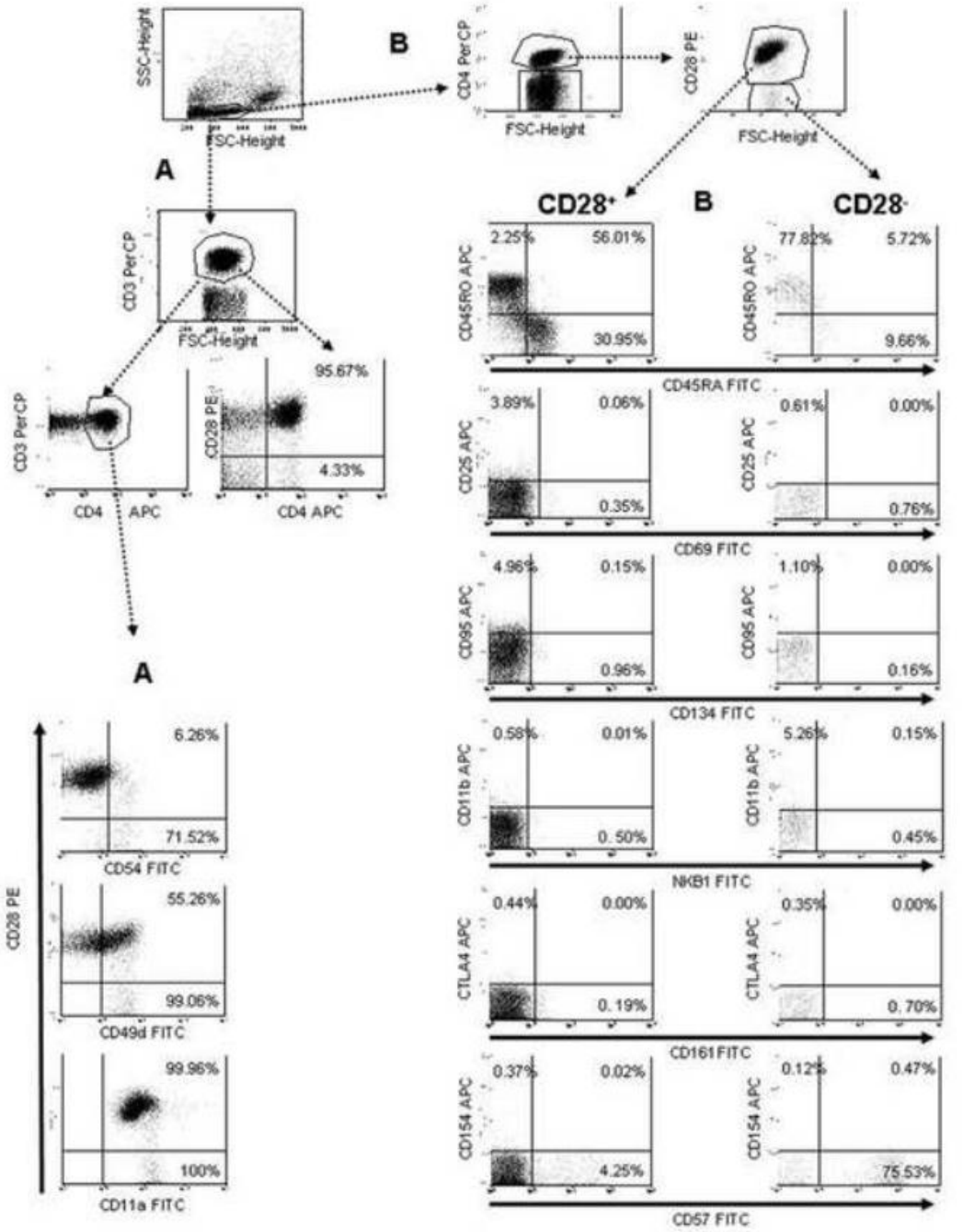


Figure 4



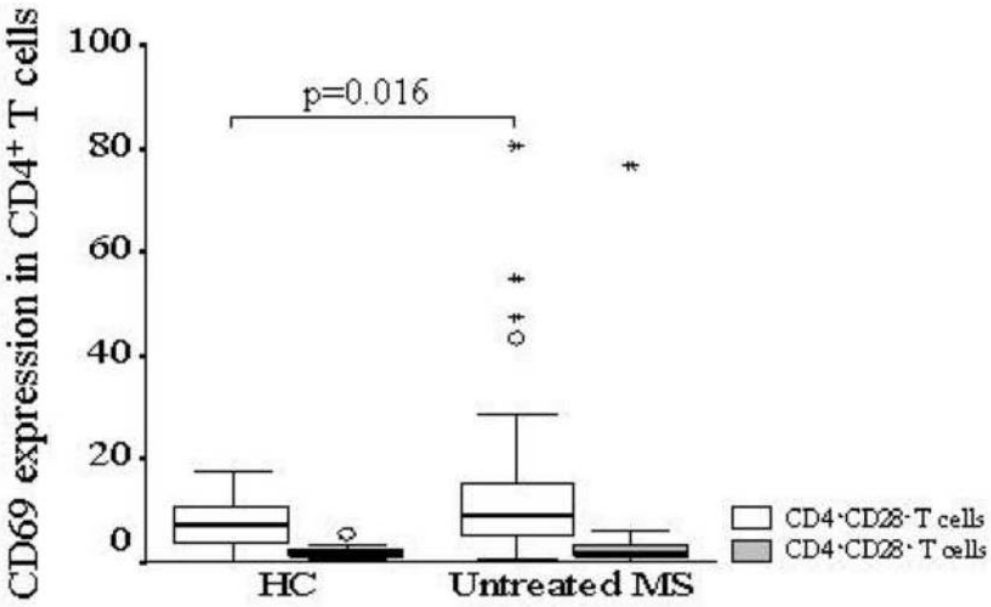
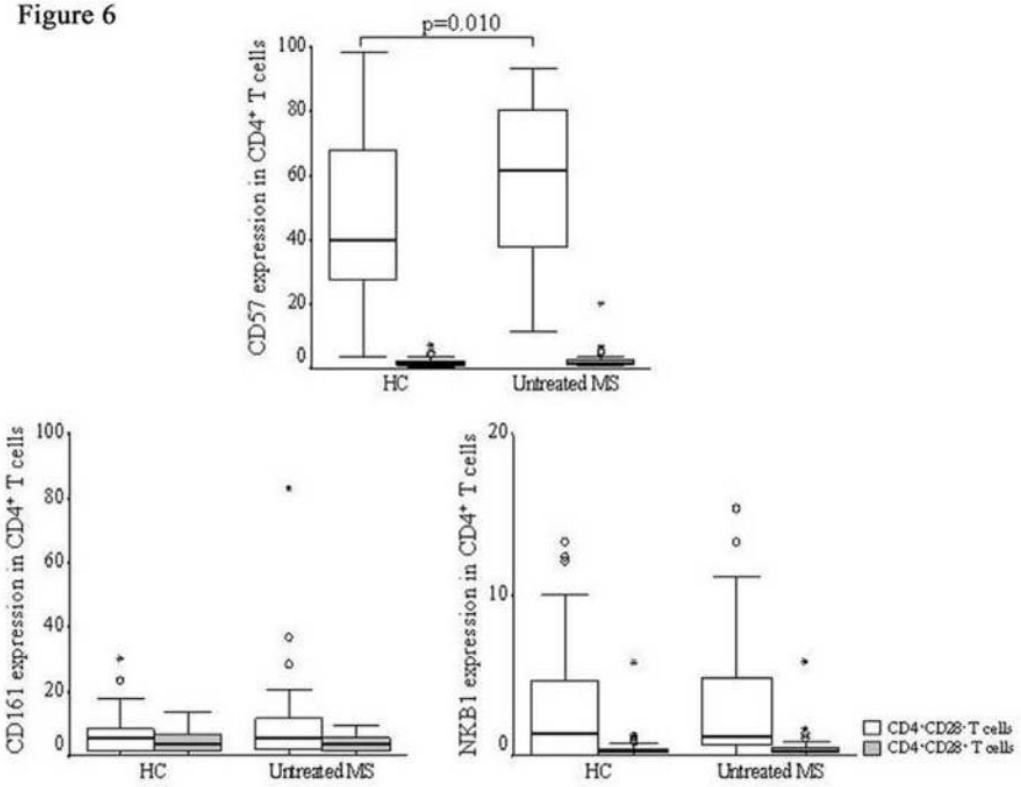


Figure 5

Figure 6



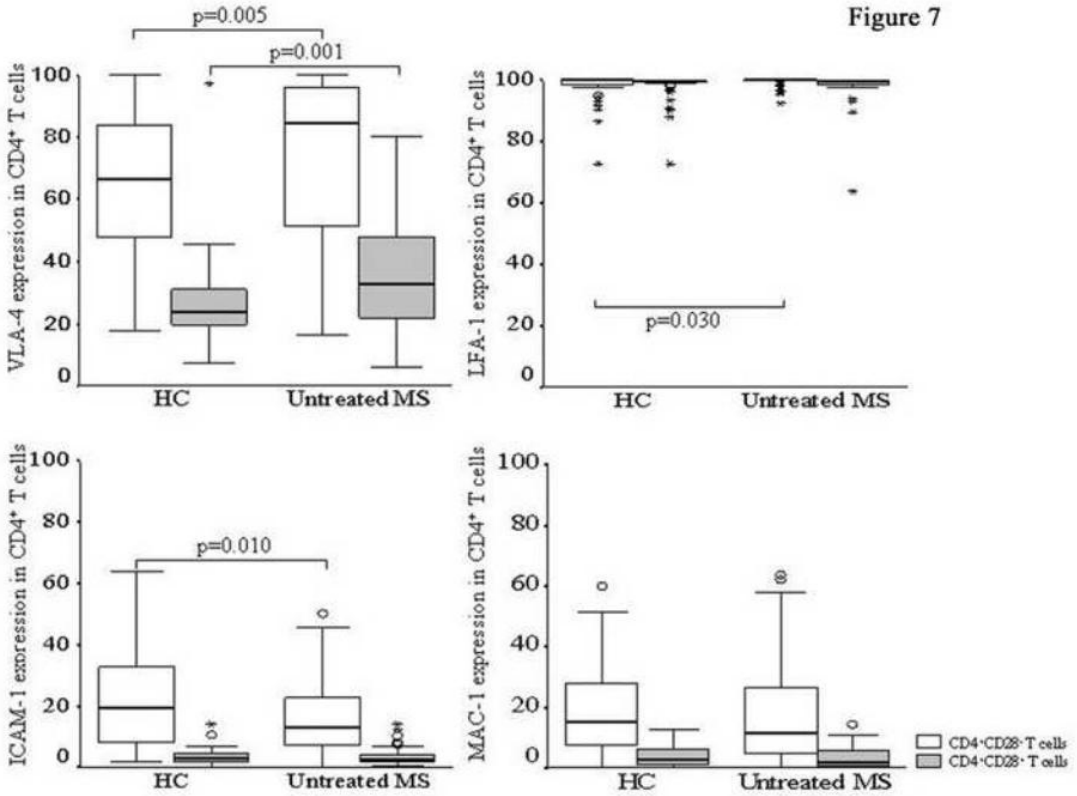


Figure 7

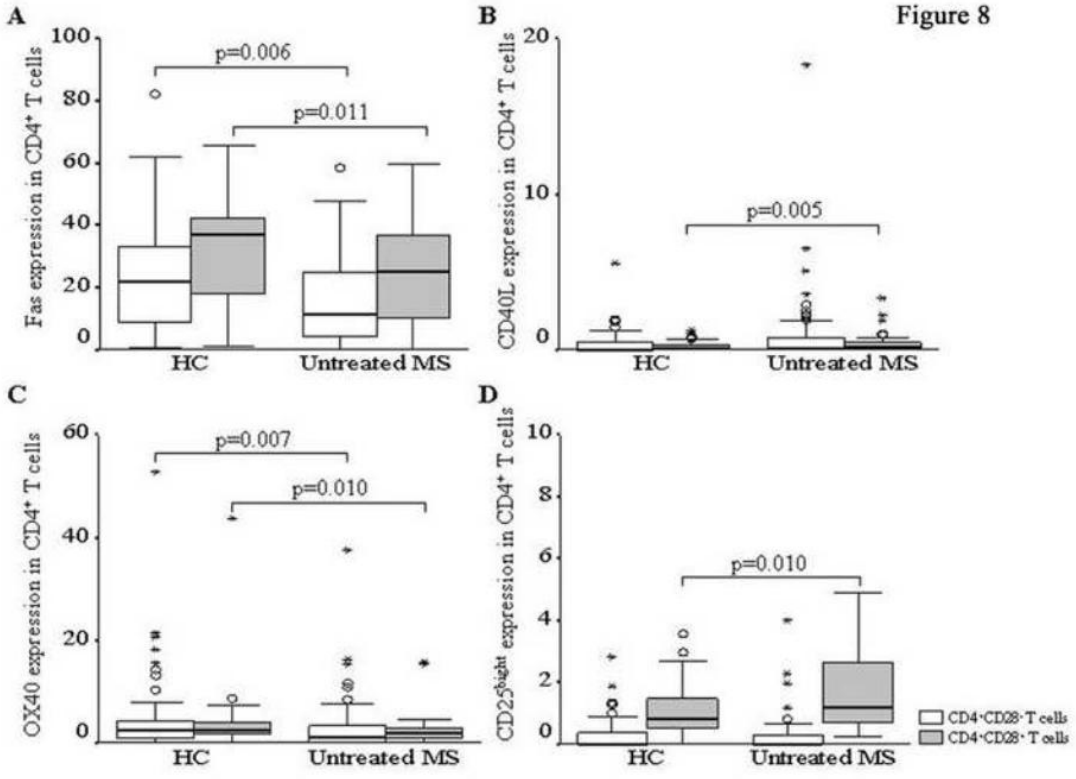


Figure 8

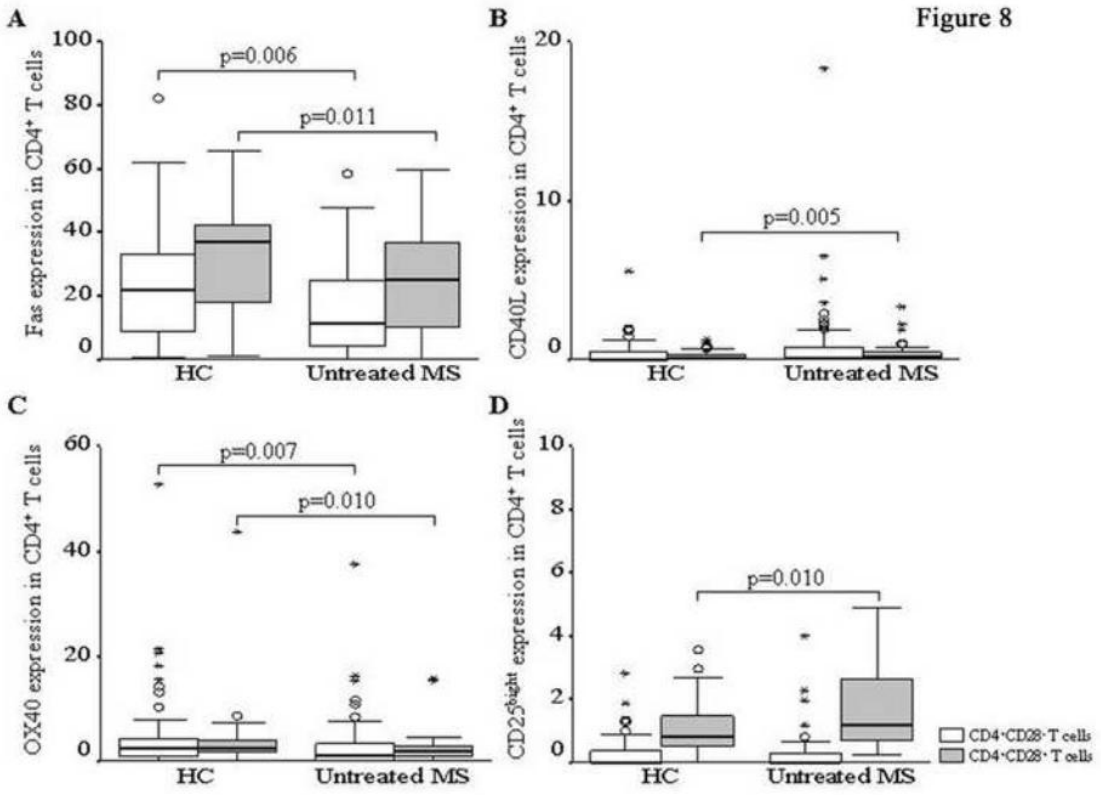


Figure 9