# Impaired Function of CD4+CD25high Regulatory T cells Isolated with Anti-CD25 Dynabeads® from Peripheral Blood of Patients with Multiple Sclerosis

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## Introduction

Immunoregulatory T lymphocytes of the $CD4^+CD25^+$ phenotype suppress T cell function and protect rodents from organ-specific autoimmune disease [1,2]. These naturally occurring immunoregulatory T cells originate in the thymus, constitutively express CD25 and comprise 5-10% of murine and approximately 2% of human peripheral $CD4^+$ T cells [1,3]. Within the human $CD4^+$ subset only cells co-expressing high levels of CD25 appear to have potent regulatory function [3,4]. The precise role of this distinct T cell subset in human autoimmunity is as yet undefined. Here we isolated highly pure $CD4^+CD25^{high}$ regulatory T cells (Treg) from peripheral blood of patients with multiple sclerosis (MS) using anti-CD25 Dynabeads® and assessed their suppressor function. We demonstrate that patient-derived Treg are functionally impaired, as their inhibitory effect on allogeneic T cell proliferative responses is significantly reduced compared to Treg from healthy individuals. In contrast, the number and cell surface phenotype of $CD25^{high}$ cells from MS patients was not altered. Our data suggest that dysfunction of Treg might be involved in the pathogenesis of MS.

## Methods

### *Patients*

The study included 17 untreated patients with clinically active, relapsing, remitting MS and a mean age of 34 years as well as 17 age-matched healthy control individuals. All patients had early stage MS with a mean disease duration of 15 months.

### *Cell Separation*

$CD4^+$ T lymphocytes were positively isolated from peripheral blood mononuclear cells (PBMCs) using anti-CD4 Dynabeads®. Separation of $CD4^+CD25^{high}$ Treg and $CD4^+CD25^-$ $T_{eff}$ from pure uncoated $CD4^+$ T cells was performed with anti-CD25 Dynabeads®. Attached antibodies and immunomagnetic beads were removed by the use of DETACHaBEAD® solutions.

### *Flow Cytometry*

$2 \times 10^5$ cells were directly stained with FITC- or PE-conjugated antibodies (CD4 clone M-T477, CD38 clone HB7, CD69 clone L78, Becton Dickinson, USA), (CD45RO clone UCHL1, CD45RA clone L48, PharMingen, USA). To determine the purity of sorted $CD25^{high}$ cells, we employed PE-anti-CD25 (M-A251, PharMingen). Analysis was performed using a FACScan™ flow cytometer and the CELLQuest™ software (Becton Dickinson).

### *Primary Proliferation Assays*

$10^5$ $CD4^+CD25^-$ $T_{eff}$ were incubated in 96 well plates in 200 µl RPMI 1640 (5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate) and stimulated with $10^5$ irradiated, T cell-depleted allogeneic PBMCs alone or in co-culture with increasing concentrations of $CD4^+CD25^{high}$ Treg for 4 days at 37°C in 5% $CO_2$. After 4 days of culture, 1 µCi $^3$[H] thymidine per well was added for an additional 16 hours. Proliferation was measured with a scintillation counter.

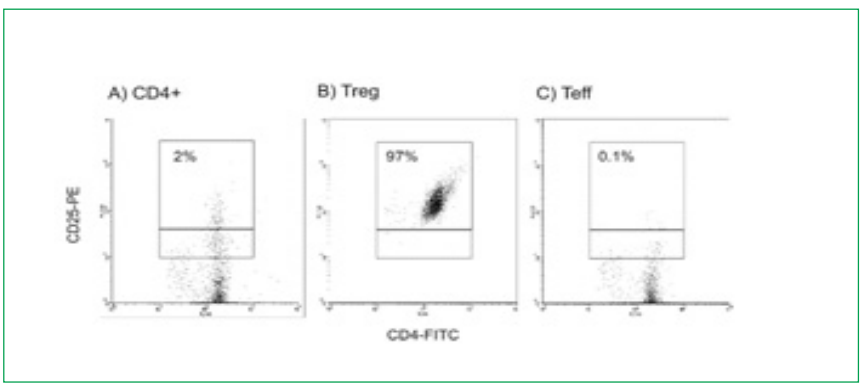


*Fig. 1: Purity of $CD4^+$ T cell subsets isolated by anti-CD4 and anti-CD25 Dynabeads®. Total $CD4^+$ T lymphocytes (A) as well as purified $CD4^+CD25^{high}$ Treg (B) and $CD4^+CD25^-$ $T_{eff}$ (C) were FACS-stained for CD4/CD25 co-expression.*

### *Statistical Analysis*

Statistical analysis was performed using Wilcoxon/Whitney/Mann/U-test for unrelated pairs. A p value of ‹0.05 was considered significant.

## Results

### *Flow Cytometry*

After positive selection with anti-CD4 Dynabeads® 1-2% of the $CD4^+$ T lymphocytes from both MS patients and normal individuals belonged to the $CD25^{high}$ T cell subset (fig. 1a). Following separation with anti-CD25 Dynabeads® 95% up to 99% of Treg expressed $CD25^{high}$ (fig. 1b), whereas virtually no CD25 coexpressing cells remained in the $T_{eff}$ subset (fig. 1c). $CD25^{high}$ cells showed a memory phenotype since ›93% of cells derived from both cohorts expressed CD45RO (fig. 2). Lymphocytes expressing the Treg phenotype did not harbour activated cells as expression levels of T cell activation markers CD38 and CD69 were extremely low and equivalent between groups (fig. 2).

### *Primary Proliferation Assays*

Following allogeneic stimulation the mean proliferation of $10^5$ $CD4^+CD25^-$ $T_{eff}$ was 32 x $10^3$ cpm and 43 x $10^3$ cpm in MS patients and controls, respectively (fig. 3a). $CD4^+CD25^{high}$ Treg from both cohorts were anergic to allogeneic stimuli proliferated at equivalent low levels (3 x $10^3$ cpm). Co-culture of increasing concentrations of Treg with $T_{eff}$ reduced the effector cell proliferation in a dose-dependent manner (data not shown). However, this suppression was significantly lower when Treg originated from MS patients (fig. 3b). Treg reduced $T_{eff}$ proliferation from 32 x $10^3$ to 22 x $10^3$ cpm in the MS group and from 43 x $10^3$ to 19 x $10^3$ cpm in the control group when cultured at a 1:1 ratio. Overall, Treg obtained from MS patients inhibited the proliferative response of $T_{eff}$ by 23%, while Treg from control individuals induced 55% ihibition (p ‹0.001, Wilcoxon-signed rank sum test) (fig. 3b).

## Conclusion

We used anti-CD4 and anti-CD25 Dynabeads® to obtain highly pure $CD4^+CD25^{high}$ regulatory T cells from peripheral blood specimens of MS patients and healthy controls and found a significantly reduced suppressive capacity of Treg in MS patients. The impaired inhibitory effect of Treg on the proliferation of autologous $CD4^+CD25^-$ responder T cells was detectable following allogeneic T cell stimulation. This finding was associated with an equal number of $CD25^{high}$ T cells within the $CD4^+$ T cell pool. Treg positively selected with anti-CD25 Dynabeads® did not appear to be contaminated by activated $CD4^+$ T lymphocytes which transiently upregulate CD25 as the enriched $CD4^+CD25^{high}$ T lymphocyte subsets derived from both groups did not express relevant amounts of T cell activation markers CD38 and CD69. In addition, both subsets were anergic to allogeneic stimuli. Overall, these findings suggest that in patients with MS a functional deficiency rather than a reduced generation or survival of $CD4^+CD25^{high}$ regulatory T lymphocytes accounts for the impaired inhibitory properties of these cells.

## References

1. Shevach EM. (2002) Nature Rev Immunol. 2:389-400

2. Kohm AP. *et al* (2002) J Immunol. 169:4712-4716

3. Baecher-Allan C. *et al* (2001) J Immunol. 167:1245-1253

4. Wing K. *et al* (2002) Immunology.106:190-199.



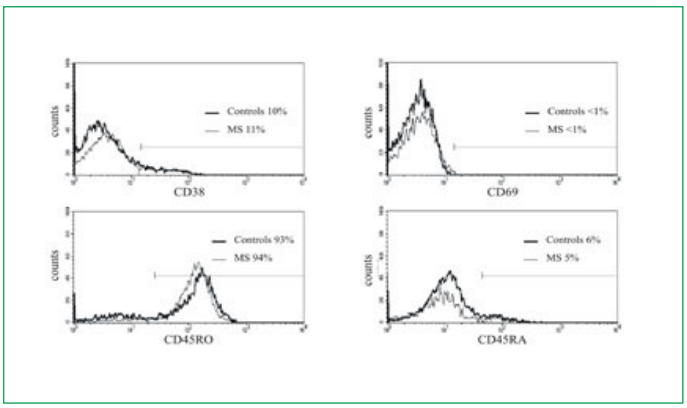


*Fig. 2: Phenotypical characterisation of highly pure $CD4^+CD25^{high}$ regulatory T cells sorted with anti-CD25 Dynabeads®. Treg isolated from MS patients and control individuals were FACS-stained for expression of CD38, CD69, CD45RA, and CD45RO.*

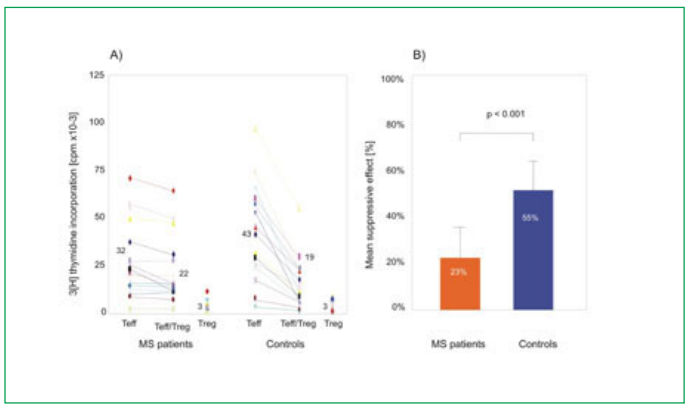


*Fig. 3: (A) Allogeneic response of T lymphocytes isolated from 17 MS patients and 17 healthy controls. $10^5$ $CD4^+CD25^-$ effector T cells alone or in co-culture with $10^5$ $CD4^+CD25^{high}$ Treg were stimulated with $10^5$ irradiated allogeneic T cell-depleted PBMCs. (B) Mean suppressive effect of $10^5$ $CD4^+CD25^{high}$ T on $10^5$ allogeneic stimulated $CD4^+CD25^-$ $T_{eff}$.*

## Ordering Information

| Product Name | Product No. | Product Volume |
|---|---|---|
| **Dynal® $CD4^+CD25^+$ Treg Kit** | 113.23 | 10 ml kit |
| **Dynal MPC®-L** sample | 120.21 | Holds 2-15 ml |