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*J Neuroimmunol*. Author manuscript; available in PMC 2008 December 1.

Published in final edited form as:
*J Neuroimmunol*. 2007 December ; 192(1-2): 57–67.

# High Cell Surface Expression of CD4 Allows Distinction of $CD4^+CD25^+$ Antigen-specific Effector T Cells from $CD4^+CD25^+$ Regulatory T Cells in Murine Experimental Autoimmune Encephalomyelitis

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## Abstract

Analysis of T regulatory cells (Treg) and T effector cells (Teff) in experimental autoimmune encephalomyelitis is complicated by the fact that both cell types express CD4 and CD25. We demonstrate that encephalitogenic T cells, following antigen recognition, up regulate cell surface expression of CD4. The $CD4^{high}$ sub-population contains all of the antigen response as shown by proliferation and cytokine secretion, and only these cells are capable of transferring EAE to naive animals. On the other hand, a FACS separable $CD25^+$ sub-population of cells displayed consistent levels of CD4 prior to and after antigen stimulation. These cells displayed characteristics of Treg, such as expressing high levels of the Foxp3 gene and the ability to suppress mitogenic T cell responses.

### Keywords

Experimental autoimmune encephalomyelitis; CD4; CD25; T cells; cellular proliferation; FACS; Treg

## 1. Introduction

Major effort to understand the etiology and mechanisms of multiple sclerosis (MS) has utilized experimental autoimmune encephalomyelitis (EAE) as an animal model. Although EAE is not a complete model, it does share many of the immunological features seen in MS (Swanborg 1995). EAE is characterized by infiltration of mononuclear cells into the CNS and

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Disclosures
The authors have no financial conflict of interest

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demyelination of nerve cells (Traugott, McFarlin, & Raine 1986;Traugott, Raine, & McFarlin 1985). This inflammatory reaction is initiated by $CD4^+$ T cells that recognize myelin autoantigens such as MBP (Sakai et al. 1988;Zamvil et al. 1985;Zamvil et al. 1986), MOG (Amor et al. 1994;Johns et al. 1995), and myelin proteolipid protein (PLP) (Amor et al. 1993;Trotter et al. 1987;Tuohy et al. 1989). In mice, EAE can be induced by active immunization with neuroantigen emulsified in complete Freund's adjuvant (CFA) (Traugott, Raine, & McFarlin 1985) or by passive transfer of neuroantigen-primed and in vitro-activated lymph node cells into naive recipients (Bernard 1976;Mokhtarian, McFarlin, & Raine 1984a;Pettinelli & McFarlin 1981). The initiation phase of the disease is a typical Th1 response involving production of gamma-interferon and IL-12 (Powell et al. 1990;Segal, Dwyer, & Shevach 1998). Recent studies implicate IL-23 and IL-17 in the propagation of the disease (Cua et al. 2003;Komiyama et al. 2006;Langrish et al. 2005;Sutton et al. 2006).

The nature of the effector T cells that mediate disease is still unclear. One of the difficulties in studying effector cell functions is the paucity of such cells, even after priming. We previously discovered that T cells up-regulated their cell surface expression of CD4 following antigen recognition and that antigen-specific effector cells, as analyzed by antigen-induced proliferation and limiting dilution assays, were all confined to a $CD4^{high}$ cell subpopulation isolated by flow cytometry (Ridgway, Fasso, & Fathman 1998). This observation had been confirmed in autoimmune disease models such as non-obese diabetic (NOD) mice (Lejon & Fathman 1999) and experimental autoimmune myasthenia gravis (Standifer, Kraig, & Infante 2003). The use of the $CD4^{high}$ marker should allow enrichment of antigen-specific effector T cells for further functional analysis.

Recent studies have implicated a population of regulatory T cells (Treg) in the maintenance of tolerance as well as in the control of autoimmune diseases (Sakaguchi et al. 2006). These cells typically also express the cell surface molecules CD4 and CD25 (Asano et al. 1996;Sakaguchi et al. 1995). Subsequent studies showed that CD5, OX40, $CD45RB^{lo}$, CD62L, cytotoxic T-lymphocyte antigen-4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor (GITR) are other markers associated with Treg cells (McHugh et al. 2002;Shimizu et al. 2002). As these cell surface markers are also up-regulated in activated T cells that mediate effector functions, it has been difficult to separate these two populations for further studies. In recent years, a transcription factor of the forkhead/winged-helix family called forkhead box protein 3 (Foxp3) has been identified specifically in Treg cells (Fontenot, Gavin, & Rudensky 2003;Khattri et al. 2003). Mice deficient in Foxp3 do not have Treg cells and develop lymphoproliferative diseases (Lahl et al. 2007). Scurfy mice, which have deletion of the forkhead domain of the scurfin protein Foxp3 also develop a T cell mediated X-linked autoimmune disorder (Chang et al. 2005). Foxp3 thus provides a useful marker to identify Treg cells.

Since their initial description by Sakaguchi and colleagues (Sakaguchi, Sakaguchi, Asano, Itoh, & Toda 1995), $CD4^+CD25^+$ Treg cells have been reported to be involved in several models of autoimmunity, such as diabetes, EAE, colitis, gastritis and collagen-induced arthritis (Asano, Toda, Sakaguchi, & Sakaguchi 1996;Green, Choi, & Flavell 2002;Groux et al. 1997;Huehn et al. 2004;Kohm et al. 2002). In EAE, Kohm et al. isolated $CD4^+CD25^+$ cells from the lymph nodes of naïve unprimed C57BL/6 mice and demonstrated that transfer of these cells into naive syngeneic recipient mice three days prior to active induction of EAE with $MOG_{35\text{-}55}$ significantly protected the animals from development of clinical EAE (Kohm, Carpentier, Anger, & Miller 2002). By pretreating B10.S mice, which are resistant to EAE induction with $PLP_{139\text{-}151}$, with anti-CD25 antibodies (clone PC61) prior to immunization with $PLP_{139\text{-}151}$, Reddy et al. demonstrated that a percentage of the mice became susceptible to EAE development, thus implying that EAE resistance might be maintained by “natural” $CD25^+$ cells (Reddy et al. 2004). Both studies involve activities of pre-existing Treg cells in naïve unprimed

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animals and are referred to as “natural Treg” cells. It is not clear whether Treg cells in antigen-primed animals have different characteristics and mechanisms of actions. Unfortunately, as pointed out earlier, both effector T cells and Treg cells express very similar cell surface molecules. A method that would separate these two cell populations for further functional studies is obviously needed.

In this report, we demonstrate that effector T cells and Treg display different levels of the CD4 antigen. We make use of this difference to enrich for effector T cells and Treg cells, respectively, in mice with EAE. We first demonstrate that $CD4^{high}$ cells isolated from CNS-antigen primed mice indeed contain the bulk of the antigen-specific T cell proliferative responses *in vivo* and *in vitro.* In addition, $CD4^{high}$ cells, and not $CD4^{norm}$ cells, cause EAE after adoptive transfer to naïve recipients. Based on expression of CD4 and CD25, we next identify two separable cell populations of CD4+ cells in antigen activated LN cultures, $CD4^{high}CD25^{+}$ and $CD4^{normal}CD25^{+}$. The $CD4^{high}$ cells proliferate in response to specific antigen and do not express Foxp3, while the $CD4^{normal}$ cell population does not proliferate to antigen stimulation, expresses the Foxp3 gene and are able to suppress mitogen induced T cell responses. Lastly, we show that $CD4^{high}$, but not $CD4^{normal}$ cells express the inflammatory cytokines IFN-γ and IL-17. Taken together, we demonstrate that high expression of CD4 is a reliable marker of antigen-specific EAE effector T cells and can be used to separate this cell population from Treg cells from in vitro cultures.

## 2. Materials and Method

### 2.1. Mice

B10.PL and C57BL/6 (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the Stanford Medical Center Department of Comparative Medicine or the Wayne State University Department of Laboratory Animal Research. Mice were used between 6 and 12 weeks of age. All study related protocols were approved either by the Stanford University or Wayne State University animal investigation committees prior to performing the studies.

### 2.2. Antigens

MOG peptide p35-55 (MEVGWYRPSFSRVVHLYRNGK) and MBP peptide Ac1-11 (AcASQKRPSQRHG) was synthesized by Genemed Synthesis (South San Fransisco CA) and purity confirmed by HPLC.

### 2.3. Immunization

Groups of 3-5 mice were immunized subcutaneously at four sites in the flanks with 50 μl (each site) of an emulsion containing IFA plus 10 mg/ml heat killed *Mycobacterium tuberculosis,* H37RA (Difco Laboratories Inc, Detroit MI) plus 200 μg/mouse antigen (MOG35-55 or MBPAc1-11) suspended in an equal volume of Dulbecco's PBS.

### 2.4. In vitro culture

Eight to ten days after immunization, draining inguinal and axillary lymph nodes were removed and single cell suspensions prepared. LN cells were activated with the priming antigen (50 ug/ml), 8 x $10^6$ cells/well in 24-well flat-bottom plates in EAE medium at 37° C supplemented with 5% $CO_2$. EAE media consisted of RPMI 1640 supplemented with 2 mM L-Glutamine, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, and 10 mM hepes buffer (Gibco Laboratories, Grand Island, NY), 50 mM 2-ME (Sigma Chemical Co., St. Louis MO), and 10% FCS. For long term cultures, cells were allowed to “rest” for 10 days without media addition. Cells were then restimulated as follows: T cells were harvested and cultured (2 x

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$10^6$ cells) with APC's (2.5 x $10^7$ syngeneic irradiated spleen cells) and antigen (50 ug/ml), in T25 flasks in 10 ml EAE media.

### 2.5. FACS analysis

For studies *in vitro*, aliquots of lymph node cells were taken from culture, washed with FACS buffer (Dulbecco's PBS with 2% FCS), stained for 1 or 2 color flow cytometric analysis with fluorescein and phycoerythrin antibodies at a predetermined optimal concentration for 20 minutes at 4° C, washed just prior to analysis. Anti-CD4 (clone GK1.5), anti-CD25 (clone 7D4) fluoresceinated antibodies were obtained from BD-Pharmingen, Inc. (San Diego, CA ). Anti-CD4 and CD25 (same clones)-phycoerythrin antibodies were also obtained from BD-Pharmingen. Just prior to analysis, propidium iodide (P.I.-1ug/ml) was added to the cells. Positive P.I. staining was used to gate dead cells from the samples. 2 x $10^5$ cells were analyzed by two color flow cytometry on a Becton-Dickinson Facscan cytometer. The data was analyzed using WinMDI 2.8 software.

### 2.6. FACS sorting and proliferation assays

Single cell suspensions were obtained either from 4 day cultures or directly from the lymph nodes of immunized animals (in both cases, harvested 8-10 days after immunization, as above). The cells were stained with anti-CD4 antibody (and in some experiments also with anti-CD25 antibodies). The cultured cells were analyzed by FACS for determination of appropriate gates, sterile sorted into the indicated populations for further analysis. For proliferation assays, cells were plated into 96 well plates at the given cell number, pulsed with [$^3$H] thymidine for 18 hours, and then counted on a Beta plate.

### 2.7. Adoptive transfer of cell populations for determination of encephalitogenicity

Cell populations were tested for encephalitogenicity using the adoptive transfer and challenge protocol of Shaw et. al. (Shaw et al. 1992). Briefly, donor B6 mice were immunized with MOG35-55 peptide as described above. Ten days later draining LN cells were activated by *in vitro* culture with the same antigen for four days. After FACS sorting or *in vitro* culture, cells were washed and resuspended in PBS. Viable cells were assayed by tryphan blue exclusion counting. Cells were then resuspended to appropriate volumes for transfer to naïve irradiated (500R) recipient mice, which received the indicated number of viable cells by tail vein injection in 200 ul sterile PBS. Recipient mice were examined daily for clinical signs of disease and were graded according to the following scale: 0-no abnormality; 1-loss of tail tonicity; 2-paralysis in a single hind limb; 3-dual hind limb paralysis; 4-paralysis involving the forelimbs; 5-moribund; 6-death. Subcutaneous injections of normal saline were administered to all animals losing significant body weight (> 20%). EAE was induced in B10.PL mice in a similar manner except that the antigen used was MBP Ac1-11 and recipient mice were not irradiated. After thirty days those mice that did not succumb to disease were challenged with a sub-encephalitogenic dose of antigen (50 ug/mouse) in CFA, and the mice examined for signs of disease for the next sixty days. Disease was charted using the following criteria, 1) average disease grade: average disease of those mice which succumbed to disease; 2) average day of onset: average day of onset of those mice which succumbed to disease; 3) incidence of disease: number of mice with disease/total mice treated.

### 2.8. Intracellular staining of LN cells for IFN-g, and IL-17

Antigen activated LN cultures were stained for intracellular cytokine expression using the Cytofix/Cytoperm Plus Kit (BD Pharmingen) according to the manufacturer's instructions. Protein transport inhibitor was added 4 hours prior to staining. Anti IFN-γ (clone XMG1.2) and anti-IL-17 (clone TC11-18H10) were both purchased from BD Pharmingen and used at

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predetermined optimal concentrations. Specificity of staining was determined in control experiments by pre-staining with unconjugated cytokine antibodies.

### 2.9. Intracellular staining for FoxP3

Antigen stimulated LN cultures were stained for intracellular FoxP3 expression using the mouse Treg staining kit (eBioscience) according to manufacturers instructions.

### 2.9. Real time PCR measurement of gene expression

Day 3 LN cultures of B6, MOG peptide stimulated cells were harvest and FACS sorted as described above to isolate $CD4^{high}CD25^{+}$ and $CD4^{norm}$ $CD25^{+}$ cells. Immediately following sorting, total RNA was isolated from the sorted cells using the RNeasy, RNA isolation kit (Qiagen, Valencia, CA) following the manufacturer's suggested protocol. Total RNA was quantitated by measuring A260/A280 absorbance. cDNA was produced from 1 ug total RNA using a cDNA synthesis kit (Gibco BRL) following the manufactures instructions. Real time PCR was performed in a Smartcycler (Cephied) thermocycler using taqman primer and probe sets specific for FoxP3, IFN-g and IL-17 and the housekeeping gene ubiquitin. For each cDNA, both ubiquitin and specific primers were used in separate tubes. As well, duplicate samples were run for each DNA/primer set. cDNA was subject to PCR amplification under the following conditions: 50°C for 2 min, 95°C for 5 mins, followed by 40 cycles of 15s at 95°C, 1 min at 60°C. Primer sequences are as follows:

IL-17 sense, 5′ CCCTCTGTGATCTGGGAAGC;

IL-17 antisense, 5′- TTTCCCTCCGCATTGACAC;

IL-17 taqman probe 5'- CAGTGCCGCCACCAGCGC;

IFN-γ sense, 5'- CATTGAAAGCCTAGAAAGTCTGAATAAC,

IFN-γ antisense, 5'- TGGCTCTGCAGGATTTTCATG;

IFN-γ taqman probe 5'-TCACCATCCTTTTGCCAGTTCCTCCAG;

FoxP3 sense GGCCCTTCTCCAGGACAGA;

FoxP3 antisense GCTGATCATGGCTGGGTTGT;

FoxP3 taqman probe 5'- TexRed-ACTTCATGCATCAGCTCTCCACTGTGGAT-BHQ2;

ubiquitin sense GCAAGCAGCTGGAAGATGG,

ubiquitin antisense GACCAGGTGGAGGGTGGA;

ubiquitin taqman probe 5'-Tex Red-CGGACGCTGTCAGACTAC.

Amplification of RNA (without reverse transcription) confirmed that no contaminating genomic DNA was present in the samples. All reactions were performed using the TaqMan Gold RT-PCR kit according the manufacturer's recommendation (PE Applied Biosystems). Normalization to ubiquitin expression was performed for each sample using the ΔΔCT method (Pagliarulo et al. 2004).

### 2.10 Treg suppression of CD3 stimulated T cells

Naïve $CD4^{+}CD25^{-}$ T cells (responder cells- Miltenyi CD4 bead isolated) were cultured in 96-well round bottom plates at $1\times10^{4}$ cells/well with $2 \times 10^{4}$ irradiated (3000R) syngeniec T depleted (Thy 1 Miltenyi bead isolated) spleen cells (as APC) in the presence of varying amounts of $CD4^{normal}CD25^{+}$ T cells (containing Tregs). Cell cultures were stimulated with 2μg/ml anti-CD3 Ab (Clone 2G3) for four days with the addition of 1μCi [$^{3}$H]thymidine

(Amersham) for the final 16h of culture. Cells were harvested with an automatic cell harvester and uptake of radioactivity was measured in a β-plate liquid scintillation counter (Wallac). The suppressive capacity of Tregs towards responder cells in co-culture (Tresp-Treg ratio 1:0 to 1:8) for each cell ratio was compared by expressing proliferation as a stimulation index (si=specific proliferation-cpm/background cpm), and comparing the si of each cell ratio to proliferation of stimulated Teff alone.

## 3. Results

### 3.1 MBP and MOG-specific T cells up regulate CD4 expression

Prompted by the fact that T cells responding to conventional antigens up regulate expression of CD4 (Ridgway, Fasso, & Fathman 1998), we questioned whether T cells responding to CNS autoantigens could be similarly phenotyped. To test this possibility, MOG p35-55 mice were immunized with an encephalitogenic peptide of MOG (p35-55) at four sites in the flanks. Eight days after immunization, draining lymph nodes (LN) were harvested and the cells were stained with anti-CD4 antibodies and analyzed by flow cytometry. As shown in Figure 1A, a distinct population of cells, comprising less than 25% of total lymph node cells expressed the CD4 molecule. A small subpopulation in each sample, representing 1.2% of the total cells, can also be discerned to express a higher level of CD4. The LN cells were then cultured in vitro with the priming peptide for four days. On each subsequent day, aliquots of the cells were analyzed by FACS. The percentage of $CD4^{high}$ cells in the $MOG_{p35-55}$ activated culture increased steadily throughout the culture period, from 1.2% of live cells initially to almost 12% of the cells by 96 hours in culture (Fig. 1B). This increased percentage could be the result of death of other cells over time, as well as an absolute increase in the number of $CD4^{high}$ cells due to antigen stimulation. In similar experiments we also observed the up regulation of CD4 expression in LN cells from B10.PL mice immunized and cultured with the dominant epitope of MBP Ac1-11. In this case, the $CD4^{high}$ population of cells was less discernable in the uncultured LN cells, constituting only 0.7% of total cells (Figure 1C). However, upon culture for 4 days, a prominent $CD4^{high}$ population was again observed accounting for 8.4% of total cells (Figure 1D). In similar experiments we have detected up regulation of CD4 in response to a number encephalitogenic CNS autoantigens. B6 mice immunized with $PLP_{p171-191}$ (Tompkins et al. 2002)or $MBP_{p60-80}$ (Shaw et al. 1996), SJL mice immunized with MBP (whole molecule) and Balb/c mice immunized with $PLP_{p180-199}$ (Lyons et al. 2002) all display discrete populations of $CD4^{high}$ T cells (data not shown). The fact that CD4 up regulation occurs in all of these mouse strains immunized with different encephalitogenic peptides suggests that this is a general phenomenon of CD4 T cell reactivity to CNS autoantigens.

By extension, we anticipated that long term T cell lines, which are more homogeneous in their antigen specificity, would be more uniformly $CD4^{high}$. To test this hypothesis, $MBP_{Ac1-11}$-primed and activated LN cells were carried *in vitro* for two months by repeated stimulation with antigen and syngeneic APC's at 10 day intervals. Upon FACS analysis, the $CD4^+$ cells were found to be greater than 87 % $CD4^{high}$ (Figure 1E). The remaining $CD4^{+,}$ but $CD4^{norm}$ cells are likely still viable cells derived from the APC's used for stimulation. To confirm this fact, the cultured cells were allowed to rest for an additional 7 days (during which time the irradiated APC's die and can be gated by positive propidium iodide staining) and reanalyzed. In a resting culture, the percentage of $CD4^{high}$ cells is 98% (Figure 1F). Interestingly, this indicates that the $CD4^{high}$ phenotype is persistent, and not dependant upon the recent activation of the T cells.

### 3.2 $CD4^{high}$ cells contain the bulk of the proliferative response

To test the proliferative capacity of the different CD4 cell populations, total CD4, $CD4^{high}$ and $CD4^{norm}$ cells from 72-hour cultures of $MOG_{p35-55}$-primed B6 LN cells or MBP $_{Ac1-11}$ primed



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B10.PL LN cells were sorted by flow cytometry. The sorted cells ($1 \times 10^5$) were re-cultured for 18 hours and were pulsed with tritiated thymidine during this culture period. As shown in Table 1, the CD4$^{high}$ cell population exhibited vigorous proliferation while the CD4$^{norm}$ cells had minimal proliferation. Whole lymph node cell response to antigen from the same mice was also tested. Since CD4$^{high}$ cell constituted only a fraction of the total lymph node cells, a multiplier (percent of total LN cells) was used to normalize the responses for comparison. When the proliferation data are corrected by such a multiplier, it is seen that the majority of the proliferative responses to MOG$_{35-55}$ and MBP$_{1-11}$ are contained in the CD4$^{high}$ population (Table I). Little proliferation was contributed by the CD4$^{norm}$ cell fraction and that seen is probably the result of sorting error. (0.5-1.5% as determined by post sort analysis)

### 3.3 CD4$^{high}$ cells are encephalitogenic

Since the bulk of the antigen responsiveness was contained in CD4$^{high}$ T cells, we next tested if the cells were also encephalitogenic in a series of adoptive transfer experiments. LN cells from MOG$_{p35-55}$-primed B6 donor mice were cultured in vitro with antigen for 4 days. Viable cells were then separated into CD4$^{high}$ and CD4$^{normal}$ populations by FACS. The cells were transferred into naive syngeneic recipients at the indicated cells numbers (Table 2). Unsorted cells were also transferred to serve as controls. As is shown, transfer of $6.3 \times 10^6$ CD4$^{high}$ cells elicited EAE in recipient mice by day 15 post cell transfer, demonstrating their encephalitogenic potential. As expected, unseparated LN cells also transferred disease, with similar kinetics. We noticed a slight difference in disease grade, which may be due to the fact that an equivalent number of CD4$^{high}$ cell were not transferred. Due to limitations in FACS sorting we were only able to collect sufficient cells to transfer 82% the unsorted equivalent. Alternatively, the difference in disease grade could also be due to non-CD4$^+$ cells present in the unsorted cell population. More importantly, CD4$^{norm}$ cells were unable to transfer disease even though $5 \times 10^7$ cells were transferred to recipients.

In a second experiment, CD4$^{high}$ and CD4$^{norm}$ cells from MBP $_{Ac1-11}$ primed B10.PL mice were similarly transferred to naïve recipient mice. In this case however, only adoptive transfer of the unsorted cell population could induce disease in naïve recipients (Table 3A). The fact that none of the sorted cell populations produced EAE in host animals was not completely unexpected, since the threshold number of cells necessary to elicit disease under these conditions is unknown. We previously reported that low encephalitogenicity in B6 and SJL mice could be enhanced to detectable levels by challenging the recipients with antigen (Shaw, Kim, Ho, Lisak, & Tse 1992;Shaw et al. 2007). The antigenic challenge apparently served to boost the activities of the transferred cells since challenge prior to cell transfer or depletion of the donor cells prior to challenge did not elicit EAE (Shaw, Li, Ho, Hoa, Lisak, & Tse 2007). To further test the encephalitogenicity of the sorted cell populations, the groups of recipients that did not succumb to EAE were immunized with a sub-encephalitogenic dose of MBPAc1-11 at four sites in the flanks. As shown in Table 3B, antigenic challenge resulted in disease development only in mice that received CD4$^{high}$ cells. In addition, onset of clinical disease correlated with the graded number of cells transferred. Mice that received $4 \times 10^6$ CD4$^{high}$ cells succumbed to EAE in less than five days. Mice that received only $1 \times 10^5$ sorted CD4$^{high}$ cells developed EAE in fifteen days post challenge. Mice that received high numbers of CD4$^{norm}$ cells, on the other hand, did not develop EAE even after antigenic challenge. As a control, naïve B10.PL mice were immunized with the same MBP peptide emulsion used for challenge. None of these mice developed EAE. These experiments strongly support the concept that in EAE, antigen-specific encephalitogenic T cells primarily reside in the CD4$^{high}$ subpopulation, similar to that reported for type I diabetes and EAMG (Lejon & Fathman 1999;Standifer, Kraig, & Infante 2003).

### 3.4 CD4$^{high}$CD25$^{+}$ effector cells can be distinguished from CD4$^{norm}$CD25$^{+}$ regulatory T cells

Since their initial description by Sakaguchi and colleagues (Sakaguchi, Sakaguchi, Asano, Itoh, & Toda 1995), CD4$^{+}$CD25$^{+}$ Treg cells have been reported to be involved in several models of autoimmunity, such as diabetes, EAE, colitis, gastritis and collagen-induced arthritis (Asano, Toda, Sakaguchi, & Sakaguchi 1996;Green, Choi, & Flavell 2002;Groux, O'Garra, Bigler, Rouleau, Antonenko, de Vries, & Roncarolo 1997;Huehn, Siegmund, Lehmann, Siewert, Haubold, Feuerer, Debes, Lauber, Frey, Przybylski, Niesner, de la, Schmidt, Brauer, Buer, Scheffold, & Hamann 2004;Kohm, Carpentier, Anger, & Miller 2002). These cells typically also express the cell surface molecules CD4 and CD25 (Kuniyasu et al. 2000). As the CD25 marker is also up-regulated in activated T cells that mediate effector functions, it has been difficult to separate these two populations for further studies. Based on our observation that encephalitogenic T cells up regulate their CD4 expression upon immunization of the animals with neuro-antigens, we speculated that this feature may differentially distinguish antigen-specific effector cells from regulatory T cells. To test this hypothesis, B6 mice were immunized with MOG35-55 peptide. Ten days after immunization, draining lymph node cells were isolated and cultured with antigen for 4 days. At the end of the culture period, cells were stained with anti-CD4 and anti-CD25 antibodies and analyzed on FACS. As can be seen in the upper right quadrant of Figure 2, two distinct populations of CD4$^{+}$/CD25$^{+}$ cells can be identified, one with intermediate level and one with high level of CD4 expression. These two populations are referred to as, CD4$^{normal}$CD25$^{+}$ (gate R2) and CD4$^{high}$CD25$^{+}$ (gate R3) respectively.

To differentiate the function of these cell populations, we next examined the expression of genes associated with both Treg and T effector cells in EAE. We expected that the R2 population of cells would contain Treg cells which would express high levels of FoxP3. Alternatively, we expected that the R3 population of cells contained effector T cells which would produce the inflammatory cytokines IFN-γ and IL-17, (Ando et al. 1989;Langrish, Chen, Blumenschein, Mattson, Basham, Sedgwick, McClanahan, Kastelein, & Cua 2005). To test this hypothesis, B6 mice were immunized with $MOG_{35-55}$ and draining LN cells cultured with the peptide. Three days later the cells were harvested and stained for FACS analysis of intracellular FoxP3. As can be seen in Figure 3, the vast majority of the CD4$^{normal}$CD25$^{+}$ cells (R2 gate) stained positive for FoxP3, while the CD4$^{high}$ cells (R3 gate) were uniformly negative.

In similar experiments we stained for inflammatory cytokines, and found that the CD4$^{high}$ population of cells contained the bulk of the cytokine producing cells (Figure4). Nearly half of the CD4$^{high}$ cells from these animals were found to produce IFN-γ (9.9% total CD4$^{high}$, 4.1% CD4$^{high}$/IFN-γ$^{+}$--Figure 4, upper panel). In separate experiments we found that expression of IL-17 was also contained solely in the CD4$^{high}$ sub-population, but fewer cells produced IL-17. Only 15% of the CD4$^{high}$ cells were positive for IL-17 expression (Figure 4, lower panel). To further quantitate the levels of expression of these genes in the different cells populations, B6 mice were immunized with MOG peptide, draining LN cells were cultured for four days and CD4$^{normal}$CD25$^{+}$ (gate R2) and CD4$^{high}$CD25$^{+}$ (gate R3) cells FACS sorted. The yield for R2 was $0.7 \times 10^{6}$ cells per mouse and the yield for R3 was nearly $2 \times 10^{6}$ cells per mouse. IL-17 and IFN-g expression was analyzed by real time PCR. For comparison, gene expression was compared to that of unsorted LN cells using the ΔΔCt method (Pagliarulo, George, Beil, Groshen, Laird, Cai, Willey, Cote, & Datar 2004). As shown in Figure 5, the CD4$^{normal}$CD25$^{+}$ population had greater than 300 fold increased expression of Foxp3 compared to unsorted LN cells. On the other hand, IFN-γ and IL-17 expression was highly enriched in sorted CD4$^{high}$ cells, with 52 and 187 fold increases in gene expression respectively.

Since the bulk of the FoxP3 expression was confined to the CD4$^{normal}$CD25$^{+}$ cell population, these cells were tested for their ability to inhibit the proliferative responses of anti-CD3 stimulated naïve CD4$^{+}$CD25$^{-}$ spleen cells (Pace, Pioli, & Doria 2005). Figure 6 shows that



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$CD4^{normal}CD25^{+}$ cells are extremely effective in suppressing the proliferative responses even at Treg:target (Effector) cell ratio of 1:8. $CD4^{normal}CD25^{+}$ Treg cells by themselves did not respond to anti-CD3 stimulation (data not shown). These results show clearly that the $CD4^{normal}CD25^{+}$ cell population contains the bulk of Treg cells. Thus, this approach has achieved separation of encephalitogenic effector T cells ($CD4^{high}CD25^{+}$) and Treg cells ($CD4^{normal}CD25^{+}$) in mice primed with a neuroantigen ten days earlier.

## 4. Discussion

The results presented here demonstrate that CNS-antigen-specific T cells up regulate CD4 expression both in vitro and in vivo. The entire proliferative response to an autoantigen was found within this population of cells as shown by thymidine incorporation assays as well as by limiting dilution analysis of precursor frequency (data not shown). We have also demonstrated that the encephalitogenic response to CNS autoantigens was contained in the $CD4^{high}$ population of cells by adoptive transfer of disease using this sub-population of cells. Moreover, $CD4^{high}$ cells exclusively produce two key inflammatory cytokines implicated in EAE pathogenesis, namely IFN-γ and IL-17.

The frequency of T cells specific for an auto-antigen is extremely low, on the order of 1:50,000-300,000 T cells in unprimed mice, and approximately 1:1000-10,000 in primed mice (Ford & Burger 1983;Gebel et al. 1983;Kojima et al. 1988). Although several methods have been developed to study autoreactive T cell responses *in vivo* (e.g. adoptive transfer of T cell clones and TCR transgenic mice) (Mendel et al. 2004;Mokhtarian, McFarlin, & Raine 1984b;Zamvil, Nelson, Trotter, Mitchell, Knobler, Fritz, & Steinman 1985), the characterization of autoantigen-specific T cell responses requires manipulation of the normal physiology of the immune response. T cell clones and T cells from TCR transgenic mice utilize a single TCR αβ heterodimer and these T cells generate a homogenous, single affinity response to antigen. The methods used to isolate T cell clones, and also the T cells from which TCR transgenic mice are derived, have used culture conditions predisposed to selecting only the best growing T cells from culture; the T cells with the proliferative response "most favorable" for growth. Thus, although these methods have provided important insights into autoreactive T cell responses *in vivo*, they share some drawbacks in studies of conventional immune response.

We find that $CD4^{high}$ effector cells expressed both IFN-γ and IL-17, the major inflammatory mediators implicated in EAE development. Our experiments indicate that $CD4^{high}$ effector cells express IFN-γ more than twice as frequently as they did IL-17. This is consistent with previous reports in SJL and B6 mice in which IFN-γ producing cells outnumbered IL-17 producing cells (Harrington et al. 2005;Langrish, Chen, Blumenschein, Mattson, Basham, Sedgwick, McClanahan, Kastelein, & Cua 2005), and indicates that the $CD4^{high}$ phenotype encompasses all EAE effector cell types. Interestingly, we found no T cells expressing both cytokines simultaneously. This is in contrast to a recent report by Suryani and Sutton, who found that the majority of IL-17 producing cells recovered from the CNS of B6 mice with active EAE also, expressed IFN-γ (Asano, Toda, Sakaguchi, & Sakaguchi 1996). This difference may due to the cell source since our studies concentrated on cells from the draining LN's not the CNS. The difference seen could also be due to the length of the in vitro activation. Suryani found that cytokine double positive cells became single positive IL-17+ producing cells within 30 hours after in vitro activation. It is not known if this phenotype change is an artifact of ex vivo antigenic stimulation. We have found that encephalitogenic $CD4^{high}$ T cells can be detected in the CNS of mice with active EAE (data not shown) and are currently determining the cytokine expression profile of these cells. The use of the $CD4^{high}$ phenotype should allow us to examine these cells without the need for ex vivo stimulation.

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Recent studies have implicated a population of regulatory T cells (Treg) in the maintenance of tolerance as well as controlling EAE. Kohm et al. (Kohm, Carpentier, Anger, & Miller 2002) isolated $CD4^{+}CD25^{+}$ cells from the lymph nodes of naïve unprimed C57BL/6 mice and demonstrated that transfer of these cells into naive syngeneic recipient mice three days prior to active induction of EAE with $MOG_{35\text{-}55}$ significantly protected the animals from development of clinical EAE. The difficulty in isolating Treg cells from in vitro cultures of antigen stimulated LN cells lies in the fact that both cell types express CD4 and CD25. We have demonstrated that CD4+CD25+ Treg cells can be differentiated from CD4+ effector cells on the basis of differential CD4 expression. $CD4^{high}$ effector cells were found to be uniformly negative for the transcription factor FoxP3, a marker for Treg cells. Treg cells were contained in a distinct population of cells expressing intermediate levels of CD4 ($CD4^{normal}$) and high levels of CD25 after three to four days of in vitro culture with antigen. These cells expressed high levels of FoxP3 and did not express the inflammatory cytokines IFN-γ or IL-17. Nearly all of these cells expressed FoxP3 indicating that the majority of the cells in this population were Treg. The use of antigen-specific tetramers (Korn et al. 2007) should allow for more specific study of antigen-specific Treg without resorting to the use of genetically manipulated mice such as FoxP3-GFP knock-in animals (Korn, Reddy, Gao, Bettelli, Awasthi, Petersen, Backstrom, Sobel, Wucherpfennig, Strom, Oukka, & Kuchroo 2007).

### Acknowledgments

The authors would like to acknowledge the expert technical assistance of Ms. Cariel Taylor and the invaluable administrative assistance of Ms Robin Kizer and Ms Lynette Rae. The authors would also like to thank Dr. Jeannette Thai for review and thoughtful discussion of the manuscript.

Grant Support

This work was sponsored by the National Institutes of Health grants DK39959 and DK44837 and National Multiple Sclerosis Society Grant RG 3112-A-2. Michael K. Shaw was a fellow of the National Multiple Sclerosis Society for a portion of this work.

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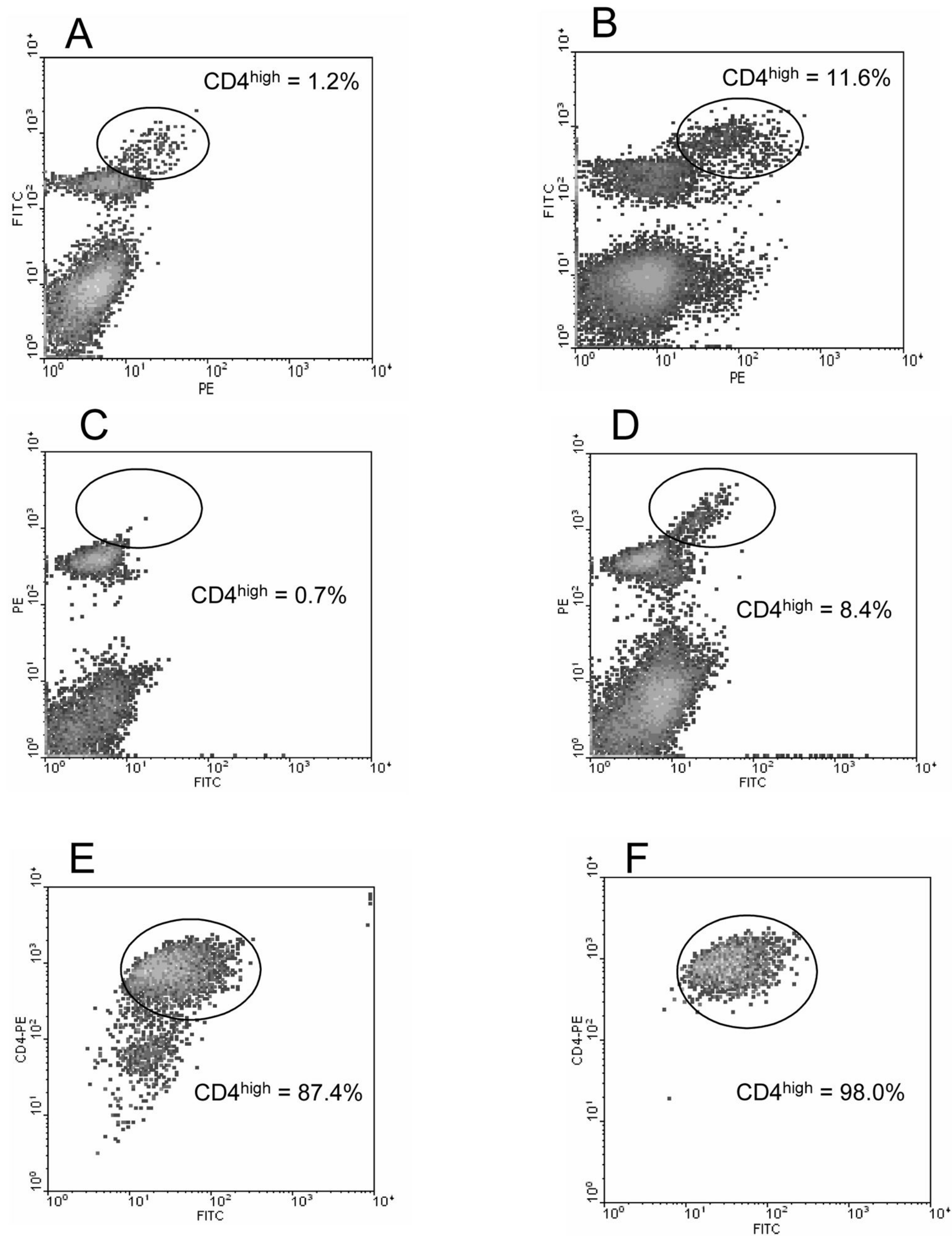


**Figure 1. FACS staining of LN cells demonstrates the presence of CD4$^{high}$ cells**

LN cells from MOG p35-55 primed B6 mice (panel A-B), or LN cells from MBP Ac1-11 primed B10.PL mice (panel C-F) were stained with CD4 antibodies. In panels A and B the CD4 antibody is FITC conjugated. In panels C-F, the CD4 antibody is PE conjugated. For the sake of clarity, the cells are plotted against an empty channel (x axis-no stain) in a density plot. The percentage CD4$^{high}$ cells are determined by the indicated gates. Panel A shows LN cells isolated from a $MOG_{35-55}$ primed B6 mouse without in vitro culture; Panel B: shows the same LN cells after in vitro culture with MOG peptide for four days; Panel C shows LN cells from a MBP Ac1-11 primed B10.PL mouse without culture; Panel D shows the same LN cells after four days in vitro culture with MBP peptide; Panel E shows the same LN cells after 6 rounds

of in vitro culture with MOG peptide and syngeneic APC's, 4 days after stimulation; Panel F shows the same cells as in Panel E except 11 days after stimulation.

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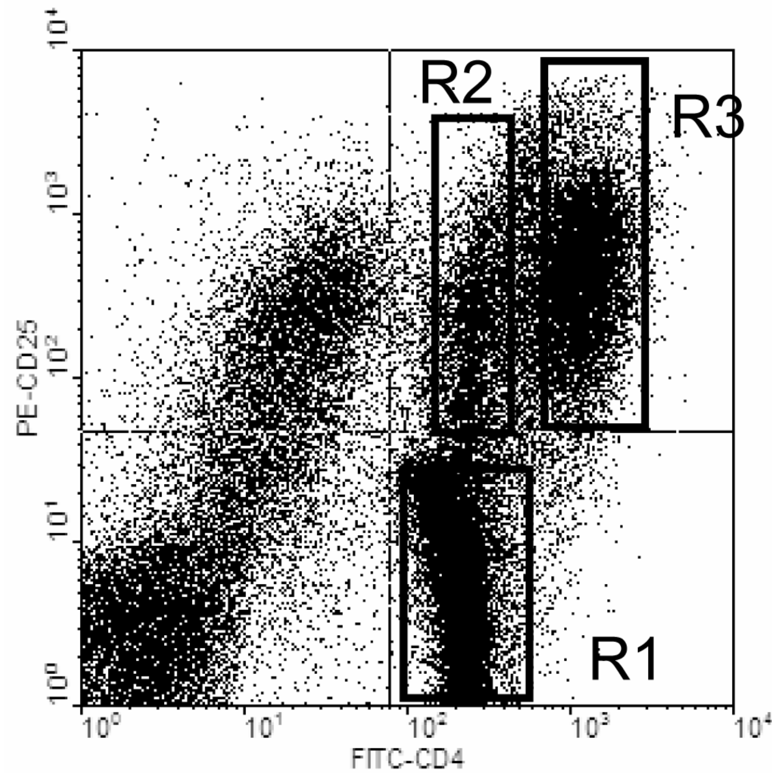


**Figure 2. FACS analysis of MBP-primed lymph node cells from B6 mice after a four-day culture and stained with anti-CD4 and anti-CD25 antibodies**

LN cells from MOG primed B6 mice were stimulated in vitro with the priming peptide for four days. FACS analysis of CD4 (FITC) and CD25 (PE) expression reveals two distinct CD4+/ CD25+ cell populations. $CD4^{normal}$CDC25+ cells (putative Treg) are delineated by gate R2 while $CD4^{high}$CD25+ cells (putative Teff) are delineated by gate R3. FACS staining is typical of all cultured LN cells tested.

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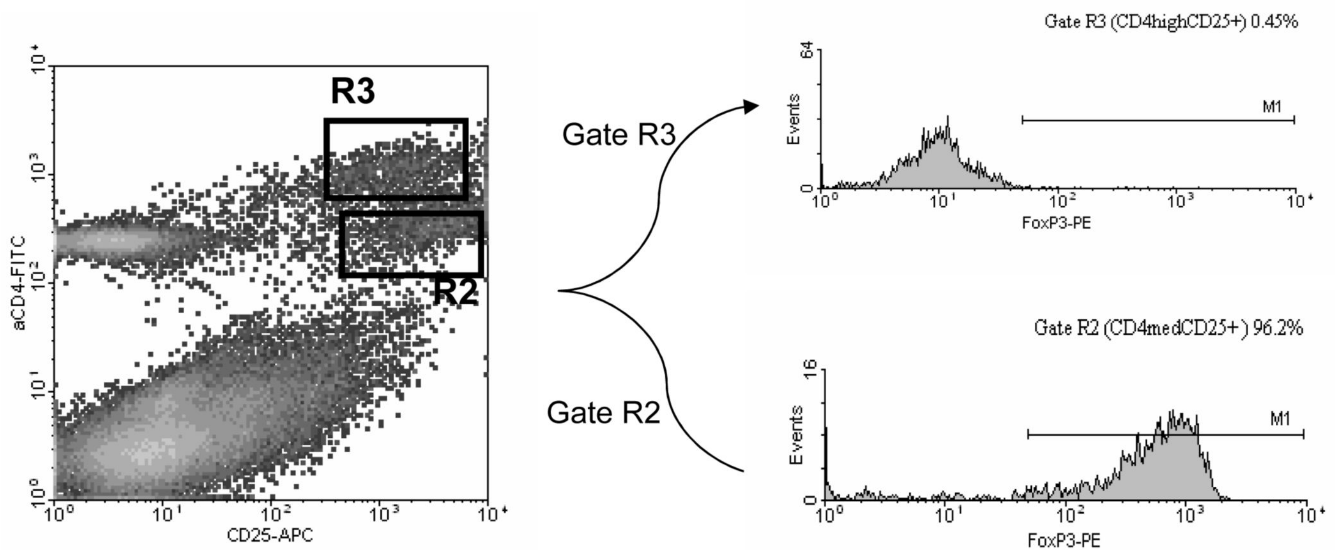


**Figure 3. FoxP3 is only is contained primarily in CD4$^{normal}$ CD25$^{+}$ cells**

LN cells from MOG p35-55 primed mice were cultured for four days with the priming peptide followed by analysis of intracellular FoxP3 expression. LN cells were stained with antibodies to CD4 (FITC) and CD25 (APC) then fixed and stained for intracellular expression of FoxP3 (PE) using a Treg staining kit (eBioscience). Cell populations defined by gates R2 and R3 in the density plot were further analyzed for FoxP3 expression in the histograms at right. Staining is representative of at least three experiments.

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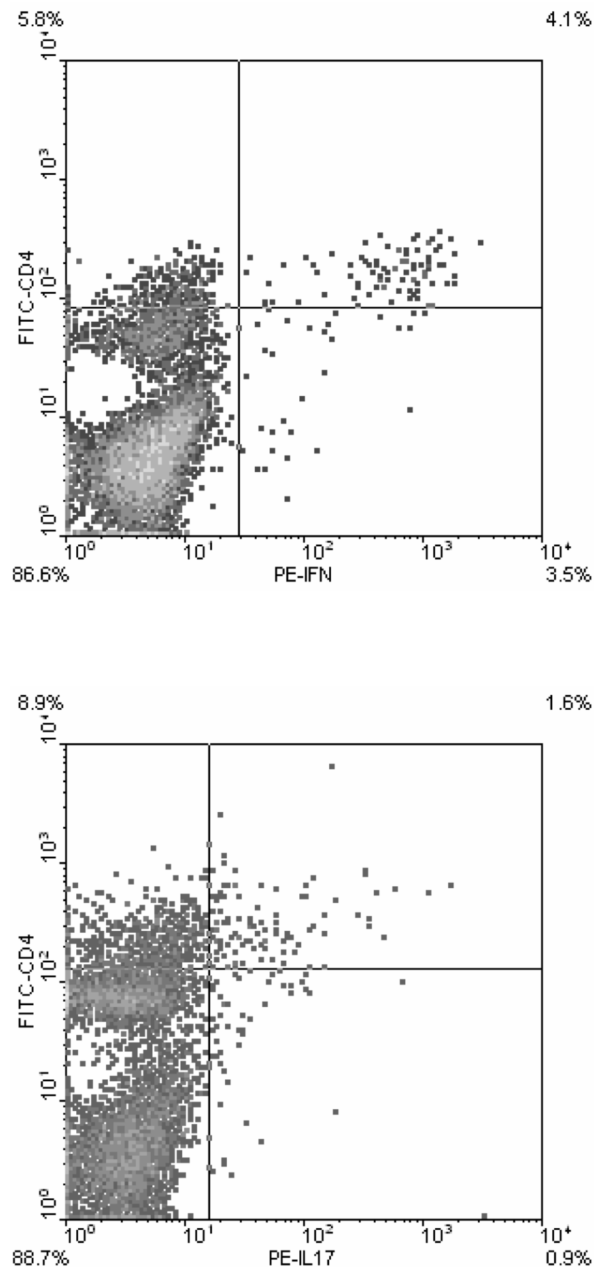


**Figure 4. Inflammatory cytokine expression is contained in the CD4$^{high}$ cell population**

LN cells from MOG p35-55 primed mice were cultured for four days with the priming peptide. Cells were then stained for analysis of CD4 (FITC) expression and intracellular IFN-γ and IL-17 expression (both PE) as described in methods. Quadrant gates are set to differentiate CD4$^{high}$ cells from CD4+ cells (set arbitrarily), and cytokine producing cells from non-producing cells (as determined by analysis of unstained control samples). Staining is representative of at least two experiments.

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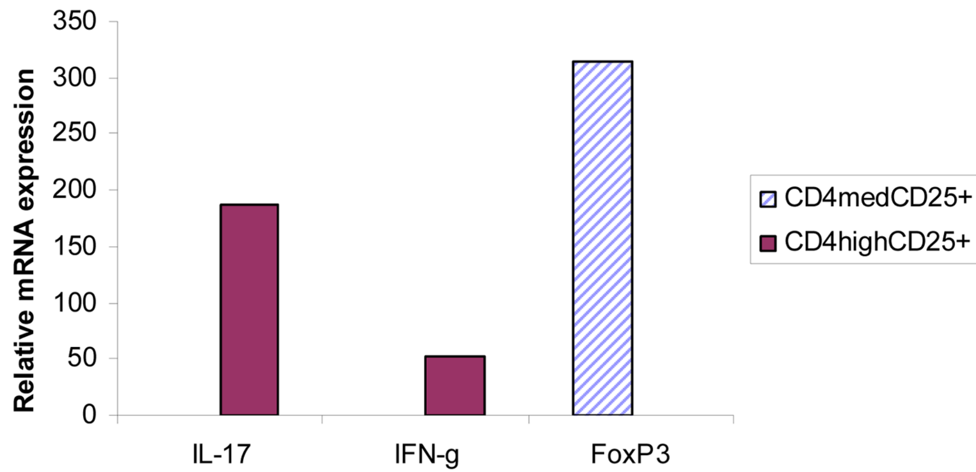


**Figure 5. Inflammatory cytokine and FoxP3 gene in sorted cell subsets**

MOG peptide primed and in vitro stimulated LN cells were stained with CD4 and CD25 antibodies as described in methods. $CD4^{high}$ Teff cells and $CD4^{normal}CD25^{+}$ Treg cells were sorted using gating similar to that shown in Figure 3. Gene expression from sorted cells ($8 \times 10^4$ cells) was analyzed by real time PCR.

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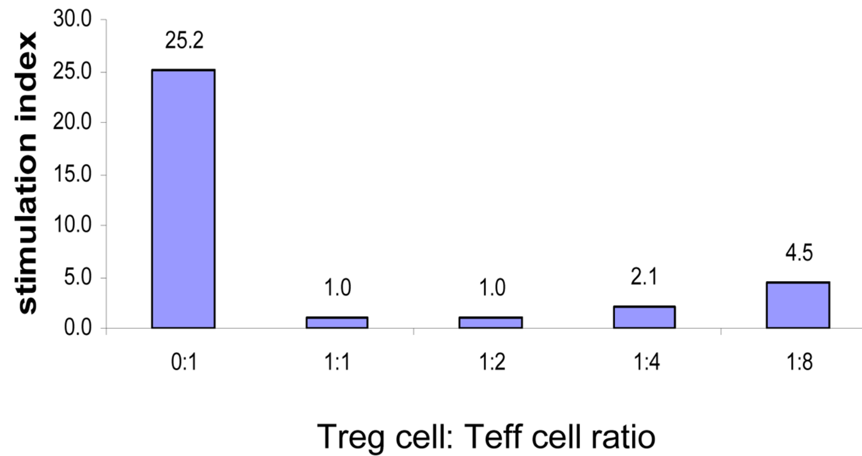


**Figure 6. CD4$^{normal}$CD25$^{+}$ cells can suppress mitogenic T cell responses**

CD4$^{normal}$CD25$^{+}$ (Treg) cells were isolated from 4-day cultures of MOG35-55-immunized B6 MOG p35-55 lymph nodes cells by sterile sorting using gates similar to that depicted in Figure 3. Naïve CD4 positive T cells ($1 \times 10^4$) were cultured for 3 days in round-bottom 96-well plates with the sorted cells and $2 \times 10^4$ APCs (T-cell-depleted splenocytes) per well in triplicate in the presence of 2.0 ug/ml of anti-CD3 antibody (clone 145-2C11) in ratios of Treg/E as indicated above. Sixteen hours before harvesting, cells were pulsed with $^{3}$H-Thymidine. Cells were harvested and incorporation of radioactive thymidine was counted in a beta- counter. Results are representative of at least two experiments.

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## Table 1

**The $CD4^{high}$ sub-population contains all of the proliferative response**

Three days after in vitro culture, encephalitogenic peptide primed and in vitro activated LN cells were sterile sorted into $CD4^{high}$ and $CD4^{norm}$ populations. Unsorted LN cells were used for comparison. The cells, $1 \times 10^5$ cells of each type, were plated and pulsed immediately with $^3H$-thimidine for 18 hours before scintillation counting. Results are shown as raw data and corrected for the percentage of each CD4 sub-population represented in the original culture (as determined by sort gating). The percentage of $CD4^{high}$ and $CD4^{norm}$ cells for each experiment is indicated as the multiplier.

| Mouse strain antigen combination | Cells type cultured ($1 \times 10^5$) | CPM | Multiplier (% of total CD4 cells in culture) | | Corrected CPM |
|---|---|---|---|---|---|
| C57BL/6 mice immunized with $MOG_{35\text{-}55}$ | Total CD4 Cells | 31,579 | 1.0 | (100%) | 31,579 |
| | $CD4^{norm}$ Cells | 2,411 | 0.858 | (85.8%) | 2,067 |
| | $CD4^{high}$ Cells | 220,932 | 0.142 | (14.2%) | 31,482 |
| B10.PL mice immunized with $MBPAc_{1\text{-}11}$ | Total CD4 Cells | 14,200 | 1.0 | (100%) | 14,200 |
| | $CD4^{norm}$ Cells | 888 | 0.919 | (91.9%) | 816 |
| | $CD4^{high}$ Cells | 165,993 | 0.081 | (8.1%) | 13,445 |

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## Table 2

**Adoptive transfer of EAE in B6 mice using isolated CD4$^{high}$ cells**

The indicated CD4 cell populations were sterile sorted from day 4 cultures of MOG activated B6 LN cells. Cells were transferred i.v. to naïve syngeneic mice. EAE is graded as described in methods.

| | **Disease Incidence** | **Average Day of Onset** | **Average Disease Grade** |
|---|---|---|---|
| Unsorted Cells ($5 \times 10^7$) | 5/5 | 12.2 | 3.0 |
| CD4$^{norm}$ Cells ($5 \times 10^7$) | 0/3 | -- | -- |
| CD4$^{high}$ Cells ($6.3 \times 10^6$) | 3/3 | 15.0 | 1.0 |

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## Table 3A/B

**Adoptive transfer of EAE in B10.PL mice by sorted CD4 cell subsets**

***A****: Sorted CD4 cell subsets do not induced EAE by adoptive transfer only*. B10.PL mice were immunized with MBP Ac1-11 and LN cells activated in vitro with the same peptide. After a four day culture the cells were FACS sorted into CD4 subpopulations and transferred to naïve recipients at the numbers indicated. Mice were observed for clinical signs of EAE for 30 days. ***B****: Clinical EAE in B10.PL mice after antigenic challenge*. After 30 days, those mice that had not displayed clinical EAE were immunized with a sub-encephalitogenic dose of MBP Ac1-11 (50 ug/mouse). Naive animals, immunized with MBP Ac1-11, served as controls, demonstrating that the dosage used is sub-encephalitogenic.

**Table 3A**

| | **Disease Incidence (pre-challenge)** | **Average Day of Onset** | **Average Disease Grade** |
|---|---|---|---|
| Unsorted Cells ($5 \times 10^7$) | 3/3 | 11.0 | 3.0 |
| CD4$^{norm}$ Cells ($1 \times 10^7$) | 0/3 | -- | -- |
| CD4$^{high}$ Cells ($1.5 \times 10^5$) | 0/3 | -- | -- |
| CD4$^{high}$ Cells ($1.5 \times 10^6$) | 0/3 | -- | -- |
| CD4$^{high}$ Cells ($4 \times 10^6$) | 0/3 | -- | -- |

**Table 3B**

| | Disease Incidence (post-challenge) | Average Day of Onset | Average Disease Grade |
|---|---|---|---|
| Unsorted Cells ($5 \times 10^7$) | na | na | na |
| CD4$^{norm}$ Cells ($1 \times 10^7$) | 0/3 | -- | -- |
| CD4$^{high}$ Cells ($1.5 \times 10^5$) | 3/3 | 15.3 | 1.0 |
| CD4$^{high}$ Cells ($1.5 \times 10^6$) | 3/3 | 8.7 | 3.0 |
| CD4$^{high}$ Cells ($4 \times 10^6$) | 3/3 | 4.7 | 3.0 |
| Naïve mice immunized with 50 ug/ml MBP Ac1-11 | 0/6 | NA | NA |