

**CD39<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells suppress pathogenic Th17 cells and are  
impaired in multiple sclerosis<sup>1</sup>**

Jean M. Fletcher<sup>\*</sup>, Roisin Lonergan<sup>†</sup>, Lisa Costelloe<sup>†</sup>, Katie Kinsella<sup>†</sup>, Barry Moran<sup>\*</sup>, Cliona  
O'Farrelly<sup>\*</sup>, Niall Tubridy<sup>†</sup> and Kingston H.G. Mills<sup>\*2</sup>

<sup>\*</sup>School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland; <sup>†</sup>Department of  
Neurology, St. Vincent's University Hospital, Elm Park, Dublin 4, Ireland

Running title: CD39<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells suppress Th17 cells

## Abstract

Despite the fact that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Treg cells) play a central role in maintaining self-tolerance and that IL-17-producing CD4<sup>+</sup> T cells (Th17 cells) are pathogenic in many autoimmune diseases, evidence to date has indicated that Th17 cells are resistant to suppression by human Foxp3<sup>+</sup> Treg cells. It was recently demonstrated that CD39, an ectonucleotidase which hydrolyses ATP, is expressed on a subset of human natural Treg cells. We found that although both CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells suppressed proliferation and IFN- $\gamma$  production by responder T cells, only the CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup>, which were predominantly FoxP3<sup>+</sup>, suppressed IL-17 production, whereas CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells produced IL-17. An examination of T cells from multiple sclerosis (MS) patients revealed a normal frequency of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup>FoxP3<sup>+</sup>, but interestingly a deficit in the relative frequency and the suppressive function of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup>FoxP3<sup>+</sup>CD39<sup>+</sup> Treg cells. The mechanism of suppression by CD39<sup>+</sup> Treg cells appears to require cell contact and can be duplicated by adenosine, which is produced from ATP by the ectonucleotidases CD39 and CD73. Our findings suggest that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>CD39<sup>+</sup> Treg cells play an important role in constraining pathogenic Th17 cells and their reduction in MS patients might lead to an inability to control IL-17 mediated autoimmune inflammation.

## Introduction

In healthy individuals naturally occurring Treg (nTreg) cells play an essential role in maintaining tolerance to self antigens and preventing autoimmune diseases. Multiple sclerosis (MS) is an autoimmune disease caused by pathogenic T cells specific for myelin-antigens in the central nervous system. T cell responses to myelin antigens can be detected in both healthy controls as well as MS patients (1), suggesting that in MS patients the auto-reactive T cells may not be adequately controlled by regulatory mechanisms. Whilst the frequency of peripheral nTreg cells does not appear to be reduced in MS (2, 3), Treg cells from MS patients sorted on the basis of CD4<sup>+</sup>CD25<sup>hi</sup> have been found to be defective in their ability to suppress effector T cell responses(3-6). Subsequently however, it has been shown that when sorted on the basis of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup>, Treg cells from MS patients displayed normal Treg function (7).

The pathology in MS and in experimental autoimmune encephalomyelitis (EAE), a mouse model for MS, was previously thought to be mediated by Th1 cells, however IL-17 producing CD4<sup>+</sup> T cells (Th17) cells have recently emerged as a crucial effector/pathogenic cell type in this and other autoimmune diseases. CD4<sup>+</sup> T cells from MS patients secrete higher levels of IL-17 than healthy donors (8). IL-17 has been detected in the post mortem brain tissue of MS patients (9) and IL-17 mRNA was elevated in the cerebrospinal fluid and blood of patients with MS (10). Although nTreg cells can suppress proliferation and IFN- $\gamma$  production by Th1 cells in vitro, studies to date have failed to demonstrate that human nTreg cells suppress IL-17 production, suggesting that Th17 cells may be more resistant to suppression than Th1 cells (11, 12). However, the apparent ineffectiveness of human nTreg cells in regulating Th17 cells is at variance with data from mouse models, which have demonstrated that CD4<sup>+</sup>CD25<sup>+</sup> Treg cells can control autoimmune inflammation (13, 14).

nTreg cells in mice and humans express the transcription factor Foxp3, which is required for directing their function (15, 16). Foxp3 and CD25<sup>hi</sup> expression have also been negatively correlated with CD127, and exclusion of CD127<sup>hi</sup> cells eliminates recently activated cells (17, 18). Recently, CD39 was found to be expressed on a subpopulation of Treg cells (19, 20). CD39 is an ectonucleotidase which cleaves ATP in a rate limiting step to form AMP, which can then be cleaved by CD73 to form adenosine (19, 20). Extracellular ATP has multiple pro-inflammatory effects, and its removal may therefore have a net anti-inflammatory influence. Furthermore, ligation of adenosine to its A2A receptor, which is expressed on T cells, results in elevation of intracellular cAMP and suppression of effector T cell function. CD39 is expressed on all Foxp3<sup>+</sup> T cells in mice; however the situation in humans is less clear with only a subset of nTreg cells co-expressing Foxp3 and CD39 (20). Treg cells from CD39<sup>-/-</sup> mice had reduced suppressive capacity *in vitro* and these mice failed to prevent allograft rejection *in vivo* (19). These studies suggest that hydrolysis of ATP and/or generation of adenosine via the expression of CD39 and CD73 represents an important mechanism of immunoregulation. However, the role of CD39<sup>+</sup> Treg cells in humans is unclear. Furthermore, there is no evidence to date that Treg cells from MS patients can suppress IL-17 and it is unclear whether nTreg cells are defective in this autoimmune disease (3, 7). Here we have examined the suppressive function of CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells, with particular relevance to their ability to suppress Th17 cells and their potential role in MS. Our findings reveal a hitherto unrecognized function of CD39<sup>+</sup> Treg cells in controlling Th17 cells, which may explain the uncontrolled function of these pathogenic T cells in MS patients.

## **Materials and Methods**

### *Patients and blood samples*

MS patients attending out-patient clinics at St. Vincent's University Hospital, Dublin were recruited for this study. Written informed consent was obtained from each patient and the study received ethical approval from the St. Vincent's Ethics and Medical Research Committee and Trinity College Faculty of Health Sciences Research Ethics Committee. For phenotypic studies, 23 MS patients were RRMS (EDSS 0-3.5) of which 15 had been previously treated with immunomodulatory therapy and 16 patients were SPMS (EDSS 3-6.5). For functional studies, 10 treatment naïve relapsing remitting MS patients (male/female = 2/8, average age =  $40.3 \pm 7.5$ , EDSS range 0-2) were recruited. Leukocyte enriched buffy coats from anonymous healthy donors were obtained with permission from the Irish Blood Transfusion Board, St James's Hospital, Dublin and ethical approval was granted by Trinity College Faculty of Health Sciences Research Ethics Committee. PBMC were isolated by density gradient centrifugation.

### *Cell sorting*

CD4<sup>+</sup> T cells were isolated from PBMC using MACS microbeads (Miltenyi Biotech) and an autoMACS cell sorting instrument. CD4<sup>+</sup> T cells were then stained with CD39-FITC, CD25-PE, CD4-PerCP-Cy5.5 and in some experiments also CD127-Alexa647 (all eBiosciences) and sorted using a MoFlo cell sorter (Beckman Coulter). CD4<sup>+</sup>CD25<sup>hi</sup> Treg cells as well as CD4<sup>+</sup>CD25<sup>-</sup> responder cells were sorted first, followed by CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> Treg cell subsets. For suppression assays using cells from MS patients, the CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> Treg cells were also sorted on the basis of CD127<sup>lo</sup>.

### *Suppression assays*

CD4<sup>+</sup>CD25<sup>-</sup> responder T cells ( $5 \times 10^4$ ) were stimulated with 0.5  $\mu\text{g/ml}$  plate-bound anti-CD3 (eBioscience) plus  $1 \times 10^5$  irradiated PBMC as antigen presenting cells (APC) and co-cultured with different ratios of Treg cell subsets. After 3 days supernatants were harvested and the concentrations of IL-17 and IFN- $\gamma$  determined by ELISA (R&D Systems). Cell proliferation was then measured by thymidine incorporation after addition of 1  $\mu\text{Ci/well}$  of tritiated thymidine for 18 h. For CFSE based suppression assays, CD4<sup>+</sup>CD25<sup>-</sup> responder cells were labelled with 1  $\mu\text{M}$  CFSE for 10 min at 37°C. CFSE<sup>+</sup> responder cells were co-cultured in the presence or absence of CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> T cells and stimulated with anti-CD3 plus irradiated APC for 4 days and then re-stimulated with 50 ng/ml PMA and 500 ng/ml Ionomycin for 4 hr in the presence of 5  $\mu\text{g/ml}$  Brefeldin A. Cells were then stained for intracellular expression of IL-17 and IFN- $\gamma$  (both eBioscience) using Intrastain reagents (Dako).

The adenosine agonist 2-chloro-adenisine (Tocris) was used at 1 or 10  $\mu\text{M}$ . The adenosine antagonist ZM241385 or the CD39 ectonucleotidase inhibitor POM-1 (both Tocris), were added to suppression assays at a concentration of 1  $\mu\text{M}$ . Neutralising antibodies specific for IL-10 (R&D Systems) and TGF- $\beta$  (a kind gift from Louis Boon, Bioceros BV, Utrecht, The Netherlands) were added to suppression assays at 10  $\mu\text{g/ml}$ .

#### *Analysis of Treg cell subsets by flow cytometry*

PBMC from MS patients and healthy controls were stained ex vivo with CD4-PerCP (BD Biosciences), CD39-FITC, CD25-PECy7, CD127-Alexa647 followed by fixation, permeabilisation and intracellular staining with Foxp3-PE (all eBioscience). Cells were acquired on a Beckman Coulter CyAn flow cytometer and analysed using FlowJo software. Analysis of cell surface TGF- $\beta$  and LAP was performed on sorted Treg cell subsets stimulated for 5 days with anti-CD3 plus irradiated APC. Cells were stained using TGF- $\beta$ -PE (IQ Products) and

biotinylated latency associated protein (LAP) (R&D Systems) followed by detection with streptavidin-PerCP (Becton Dickinson).

## Results

### *CD39<sup>+</sup> Treg cells suppress IL-17 production by effector T cells.*

It has previously been reported that human CD4<sup>+</sup>CD25<sup>+</sup> nTreg cells do not suppress IL-17 production by Th17 cells (11, 12). This prompted us to ask how Th17 cells are regulated in the healthy immune system. We demonstrated that CD4<sup>+</sup>CD25<sup>hi</sup> T cells suppressed proliferation and IFN- $\gamma$  when co-cultured with CD4<sup>+</sup>CD25<sup>-</sup> responder T cells, but failed to suppress IL-17 production by responder T cells (Fig. 1).

CD39 is expressed on approximately 50% of human CD25<sup>+</sup>Foxp3<sup>+</sup> nTreg cells in healthy controls (Fig. 2A). Since the expression of cell surface TGF- $\beta$  has been associated with certain subsets of Treg cells (21), we compared TGF- $\beta$  expression on the CD39<sup>+</sup> and CD39<sup>-</sup> subsets of Treg cells. The majority of CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> T cells expressed TGF- $\beta$  (65%) following stimulation with anti-CD3 and 10% of these cells also expressed LAP (Fig. 2B). Expression of TGF- $\beta$  and LAP was substantially lower on CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> cells and on CD4<sup>+</sup>CD25<sup>-</sup> T cells (Fig. 2B). We next determined the expression of FoxP3 on the CD39<sup>+</sup> and CD39<sup>-</sup> subsets of CD4<sup>+</sup>CD25<sup>hi</sup> cells. CD4<sup>+</sup>CD25<sup>-</sup>, CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells were sorted to a purity of at least 93-98% (Fig. 2C). Staining for intracellular FoxP3 expression revealed 98% and 81% positive cells in the CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> populations respectively (Fig 2C). The intensity of FoxP3 expression was somewhat higher on CD39<sup>+</sup> cells compared with CD39<sup>-</sup> cells (MFI 51.8 vs 27.2 respectively).

We examined the relative capacity of sorted CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells to suppress cytokine production and proliferation by CD4<sup>+</sup>CD25<sup>-</sup> responder T cells over a range of suppressor to responder T cell ratios. CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> T cells suppressed IFN- $\gamma$  production at a ratio as low as one Treg to 10 responder T cells, with almost complete



suppression at a ratio of 0.5:1 (Fig. 3A). The CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> Treg cells also suppressed IL-17, which was enhanced with increasing numbers of Treg cells. CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> cells were capable of suppressing IFN- $\gamma$  production by responder T cells, though not as completely as that seen with CD39<sup>+</sup> cells (Fig. 3A). In contrast, CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells did not suppress IL-17 and in fact enhanced IL-17 production over a range of Treg to responder T cell ratios (Fig. 3A). The results of multiple experiments with a range of donors confirmed that while total CD4<sup>+</sup>CD25<sup>hi</sup> T cells and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells could suppress responder cell proliferation and IFN- $\gamma$  production, they did not suppress IL-17, whereas CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> Treg cells, consistently suppressed IL-17 production by responder T cells (Fig. 3B).

In order to confirm the suppression of responder cell IL-17 production by the CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> T cells, we used a CFSE based assay and intracellular cytokine staining. CD4<sup>+</sup>CD25<sup>-</sup> responder T cells were labelled with CFSE and stimulated with anti-CD3 plus irradiated APC in the presence or absence of CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> T cells. After 4 days the co-cultures were re-stimulated using PMA and ionomycin and stained for IL-17 and IFN- $\gamma$ . CFSE<sup>+</sup> responder T cells alone proliferated extensively as demonstrated by the reduction in CFSE intensity and increase in size reflected by increased forward scatter (FSC) (Fig. 3C). Addition of CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> T cells inhibited responder cell proliferation (Fig. 3C). CFSE<sup>+</sup> responder cells were gated to exclude the CFSE<sup>-</sup> APC and Treg cells, and expression of IL-17 and IFN- $\gamma$  was examined. CD4<sup>+</sup>CD25<sup>-</sup> responder cells alone secreted substantial amounts of IFN- $\gamma$  (32.6%) and IL-17 (11.7%), with 5.6% co-producing IL-17 and IFN- $\gamma$ . Co-culture with CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> T cells suppressed the frequency of IFN- $\gamma$ -secreting CD4<sup>+</sup> T cells by approximately 5-fold (7.2% IFN- $\gamma$ <sup>+</sup>) and IL-17 by 2-fold (6.6% IL-17<sup>+</sup>).

### *CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells produce IL-17*

We next examined the contribution of cytokines produced by the Treg cells added to the co-cultures in suppression assays. We observed enhancement of IL-17 following addition of CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells to responder T cells (Fig 3), which suggested that CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells might be a source of IL-17. Consistent with the data in Fig 3, both CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells suppressed proliferation and IFN- $\gamma$  production by responder T cells (Fig. 4A). Furthermore, CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> Treg cells efficiently suppressed IL-17 and also suppressed IL-10 (Fig. 4A). In contrast, total CD4<sup>+</sup>CD25<sup>hi</sup> or CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells not only did not suppress IL-17, production of this cytokine was enhanced by addition of CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells (Fig. 4A). Furthermore, CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells secreted IL-10 in response to stimulation with anti-CD3, whereas CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> T cells secreted very low levels of IL-10 (Fig. 4A). Indeed the concentration of IL-10 was higher in the CD4<sup>+</sup>CD25<sup>-</sup> responder T cell population than the CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> T cells; IL-10 production in the former population may be from Tr1-like cells or IL-10-producing Th1 cells.

Treg cells are known to be anergic, and do not proliferate in response to TCR stimulation and do not produce cytokines such as IFN- $\gamma$  or IL-2. Consistent with this we found that after stimulation of total CD4<sup>+</sup>CD25<sup>hi</sup>, CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> or CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cell subsets with anti-CD3 plus irradiated APC in the absence of responder T cells, they did not proliferate or produce IFN- $\gamma$  (Fig. 4A). Furthermore, CD39<sup>+</sup> Treg cells did not secrete IL-17. However, total CD4<sup>+</sup>CD25<sup>hi</sup> T cells as well as the CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> subtype secreted IL-17 when stimulated with anti-CD3 in the absence of responder T cells (Fig. 4A). These data indicated that the inability of the total CD4<sup>+</sup>CD25<sup>hi</sup> T cells to suppress IL-17 reflects the opposing effects of the different subsets contained within CD25<sup>hi</sup> Treg cell population. While the CD39<sup>+</sup> subset of Treg

cells efficiently suppressed IL-17, the CD39<sup>-</sup> subset secreted significant amounts of IL-17 which masked the suppressive effect of the CD39<sup>+</sup> Treg cells.

We next confirmed the production of IL-17 by FoxP3<sup>+</sup> Treg cells using intracellular staining and flow cytometry. CD4<sup>+</sup> T cells were stimulated with PMA and Ionomycin for 4 hours and then stained for intracellular FoxP3 and IL-17. While the majority of IL-17 secreting CD4<sup>+</sup> T cells were found within the Foxp3<sup>-</sup> population, a small percentage of the IL-17-secreting CD4<sup>+</sup> T cells were FoxP3<sup>+</sup> (Fig. 4B). Gating on Foxp3 revealed that 5.5% of these cells secreted IL-17 (Fig. 4B). Together with the data on IL-17 production assessed by ELISA (Fig. 4A), our findings suggest a proportion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells secrete IL-17 and that these are within the CD39<sup>-</sup> subset.

#### *Reduction in the frequency and suppressive function of CD39<sup>+</sup> Treg cells in MS patients*

Th17 cells have a crucial role in the pathogenesis of autoimmune disease, and Treg cells are thought to be important in constraining these pathogenic T cells. Having shown that CD39<sup>+</sup> Treg cells could effectively control IL-17 production by responder T cells, we next investigated the potential role of CD39<sup>+</sup> Treg cells in individuals with MS. There has been conflicting data regarding a possible deficit in total Treg cells in MS patients compared with normal individuals, with some studies finding no difference and others a reduced frequency of Treg cells in MS patients (2, 3, 5, 22). This may reflect the use of different Treg cell markers and the fact that markers such as CD25 and Foxp3 can also be expressed on activated cells. In the present study, we examined the frequency of total CD4<sup>+</sup>CD127<sup>lo</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in MS patients, as well as the CD39<sup>+</sup> and CD39<sup>-</sup> subsets thereof. We first showed that the majority of CD4<sup>+</sup>CD127<sup>lo</sup>CD25<sup>+</sup> Treg cells were Foxp3<sup>+</sup>, and that these Treg cells could be divided in two

populations on the basis of expression of CD39 (Fig. 5A). We found no difference in the frequency of total CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells between RRMS or SPMS patients and normal individuals (Fig. 5B). In contrast, when we examined the frequency of Treg cells on the basis of expression of CD39, we found a significant reduction in CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>CD39<sup>+</sup> Treg cells, in RRMS patients compared with normal controls (Fig. 5C). However there was no difference in the frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>CD39<sup>+</sup> Treg cells in SPMS patients. We also did not find any significant difference between treatment naïve RRMS and those that had previously been treated (data not shown). There was a reciprocal increase in frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>CD39<sup>-</sup> cells in RRMS patients (Fig. 5C).

There have been some suggestions that Treg cells in MS patients are functionally impaired (3, 6), although others have failed to demonstrate a defect (7). Furthermore, there is no reported evidence that Treg cells can suppress IL-17 production in MS patients. Here we examined the possibility that a defect in regulation of Th17 cells in MS patients reflected not only a reduction in the numbers of CD39<sup>+</sup> Treg cells, but also a defect in their function. CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup>CD39<sup>+</sup> cells were sorted via flow cytometry from both MS patients and normal controls and compared for their ability to suppress IL-17 production by responder T cells. We found that CD39<sup>+</sup> Treg cells from MS patients suppressed IFN- $\gamma$  to a similar extent as those from normal controls individuals (Fig. 6A). However, when compared with normal individuals, CD39<sup>+</sup> Treg cells from MS patients exhibited a significantly impaired capacity to suppress IL-17 production (Fig. 6A). In order to determine whether this was due to increased resistance to suppression by CD25<sup>-</sup> responder T cells from MS patients, or due to a defect in the Treg cells, we performed cross-over experiments using responder T cells from control cells co-cultured with Treg cells from MS patients and vice versa. We found that CD39<sup>+</sup> Treg cells from normal

controls effectively suppressed IL-17 production by responder T cells from control or MS patients (Fig. 6B). However, we demonstrated that CD39<sup>+</sup> Treg cells from MS patients were less effective at suppressing IL-17 production by responder T cells from MS patients (Fig. 6B). These findings indicate that the defect in regulation of IL-17 production in MS reflect a defect in numbers and function of CD39<sup>+</sup> Treg cells in these patients.

#### *The mechanism of suppression of IL-17 production by CD39<sup>+</sup> Treg cells*

Despite the vast literature on suppression of effector T cells by nTreg cells, the mechanism is still unclear. Much of the focus of studies to date has been on suppression of proliferation by responder T cells. As far as we are aware there are no reports on suppression of IL-17 production by human nTreg cells. Having shown differences in suppression of IL-17 versus IFN- $\gamma$  and proliferation, we examined the mechanism of suppression of IL-17 and IFN- $\gamma$  production by CD39<sup>+</sup> Treg cells and the possible role of CD39. CD39 is an ectonucleotidase that hydrolyses extracellular ATP to form ADP or AMP, which can be further cleaved into adenosine. Adenosine has immunosuppressive effects on T cells, mediated via binding to the A2A receptor. Furthermore, extracellular ATP released during inflammation or tissue damage is thought to be pro-inflammatory. Thus, either the removal of ATP and/or the generation of adenosine may explain the immunosuppressive role of CD39. We first examined the effect of adenosine, a downstream product of ATP cleavage by CD39, on the ability of effector cells to produce IL-17 and IFN- $\gamma$ . Increasing doses of 2-chloro-adenosine, a stable adenosine analogue, efficiently suppressed proliferation, as well as IFN- $\gamma$  and IL-17 production by responder T cells (Fig. 7A). The findings suggest that CD39<sup>+</sup> Treg cells could potentially suppress via hydrolysis of ATP and production of adenosine.

We next investigated the effects of blocking ATP hydrolysis by CD39. CD4<sup>+</sup> responder T cells were stimulated with anti-CD3 and APC in the presence of POM-1, an inhibitor of CD39 ectonucleotidase activity, or ZM241385, which inhibits the binding of adenosine to its A2A receptor, and cytokine production was measured after 3 days. Addition of POM-1 or ZM241385, did not reverse the suppressive effect of CD39<sup>+</sup> Treg cells (Fig. 7B). However, blockade of CD39 activity significantly enhanced IL-17 production and had little effect on IFN- $\gamma$  by responder T cells (Fig. 7B). This suggested that extracellular ATP promotes IL-17, and that CD39 ectonucleotidase activity could reduce IL-17 via removal of ATP.

Since we had shown that after stimulation CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> T cells secreted IL-10 and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> cells expressed high levels of TGF- $\beta$ , we next investigated a possible role for these cytokines in mediating suppression. However, addition of a neutralising antibody specific for TGF- $\beta$  failed to abrogate suppression by CD39<sup>+</sup> Treg cells (Fig 7C). Furthermore, we could find no evidence of a role for IL-10 in mediating suppression by the CD39<sup>+</sup> or CD39<sup>-</sup> subsets, since a blocking IL-10 antibody did not reverse suppression (Fig 7D).

We next examined the requirement for cell contact versus soluble factors. Using a transwell culture system, we found that suppression of IFN- $\gamma$  by CD39<sup>-</sup> Treg cells and IFN- $\gamma$  and IL-17 by the CD39<sup>+</sup> Treg cell subset required contact between the regulatory and responder T cells, as suppression was abrogated when they were separated by a semi-permeable membrane (Fig. 7E). This is consistent with our findings that anti-IL-10 or anti-TGF- $\beta$  failed to reverse suppression by CD39<sup>+</sup> or CD39<sup>-</sup> Treg cells. These findings suggest that suppression by CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> cells is not mediated by IL-10 and TGF- $\beta$  in vitro, but does require cell contact and while it could not be reversed by inhibitors of an inhibitor of CD39 ectonucleotidase activity, could be duplicated by adenosine.

## Discussion

There is now convincing evidence from animal models that the failure to regulate inflammatory T cells that secrete IL-17 is central to the development of autoimmune diseases (23). However, all the evidence to date suggests that human Foxp3<sup>+</sup> Treg cells, while capable of suppressing proliferation and IFN- $\gamma$  production, do not suppress IL-17 production by effector T cells (11, 12, 24). In this study we demonstrate for the first time that a subset of human CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells that express CD39 can suppress IL-17 production. We also found that Foxp3<sup>+</sup>CD39<sup>-</sup> cells, although capable of inhibiting proliferation and IFN- $\gamma$  production by responder T cells, did not inhibit IL-17 production and when stimulated with anti-CD3 in the absence of responder T cells produced significant concentrations of IL-17 and IL-10. Thus, our findings reveal that previous failures to demonstrate suppression of IL-17 production by total Foxp3<sup>+</sup> Treg cells reflects the fact that the Foxp3<sup>+</sup>CD39<sup>-</sup> Treg cell subset produce IL-17, thus masking the suppressive effect of the Foxp3<sup>+</sup>CD39<sup>+</sup> Treg cells.

Our findings suggest that Foxp3<sup>+</sup>CD39<sup>+</sup> Treg cells play an important role in constraining pathogenic Th17 cells and in preventing autoimmune diseases. Indeed we found that the frequency of Foxp3<sup>+</sup>CD39<sup>+</sup> Treg cells was reduced in RRMS patients. Furthermore the residual Foxp3<sup>+</sup>CD39<sup>+</sup> Treg population in MS patients, while capable of suppressing IFN- $\gamma$  were less effective at suppressing IL-17 production than the same cells from normal individuals, demonstrating that these patients have a defect in the control of Th17 responses. Studies in other autoimmune diseases, such as type 1 diabetes and rheumatoid arthritis, have shown that an increased resistance to suppression by effector cells in the inflammatory milieu contributes to autoimmune pathology (25). However, our data suggests that the unregulated Th17 cells in MS

may be due to impaired function of the Treg cells rather than a resistance to suppression by responder T cells. This is the first study to demonstrate defective regulation of IL-17 in MS.

Thus far the role of Treg cells in MS has been controversial. Although a reduction in the frequency of Foxp3<sup>+</sup> Treg cells has been reported in MS patients (5), the majority of studies, including our own, found that there are no significant differences in the frequency of total Foxp3<sup>+</sup> Treg cells between MS patients and normal control individuals (2, 3). However, consistent with a previous report (20) we found a reduced frequency of CD4<sup>+</sup>FoxP3<sup>+</sup>CD39<sup>+</sup> Treg cells in RRMS patients. In contrast the frequency of CD4<sup>+</sup>FoxP3<sup>+</sup>CD39<sup>+</sup>T cells was not reduced in the progressive phase of disease. A number of studies have identified an impaired suppressive capacity by Treg cells from MS patients (2-5, 26), however, a more recent study showed that Treg cells from MS patients exhibited normal suppressive function (7). It was suggested that this discrepancy could be accounted for by the fact that the latter study used CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Treg, whereas the CD4<sup>+</sup>CD25<sup>+</sup> Treg cells used in earlier studies may have contained activated effector T cells. This is in agreement with our study, where we have shown that Treg cells from MS patients sorted on the basis of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup>CD39<sup>+</sup> displayed no significant defect in ability to suppress IFN- $\gamma$  production. However the study by Michel and colleagues (7) did not examine the ability of Treg cells from MS patients to suppress IL-17 production. We found that CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup>CD39<sup>+</sup> cells from MS patients were less effective in suppressing IL-17 production than those from normal individuals.

There has been some suggestion that Th17 cells may be more difficult to suppress than Th1 cells. For example in an autoimmune gastritis model Th1 and Th2 cells were more effectively suppressed than Th17 cells by polyclonal Treg cells (27). Indeed our data from experiments involving intracellular cytokine staining coupled with CFSE-labelling of responder



T cells, indicated that IFN- $\gamma$  is more efficiently suppressed than IL-17. Our previous results have demonstrated that while IL-10 effectively suppressed Th1 cells, it was much less effective in suppressing Th17 cells, whereas TGF- $\beta$  effectively suppressed both Th1 and Th17 cells (28). Thus different regulatory mechanisms may be involved in the control of Th17 versus Th1 cells. Certainly the IL-17 producing cells within the CD39<sup>-</sup> Treg cell population would appear to be resistant to suppression by the CD39<sup>+</sup> Treg cell subset; given that total Treg cells secrete significant amounts of IL-17.

The mechanism of suppression of effector T cell responses by Treg cells is still unclear. While IL-10 and TGF- $\beta$  clearly play an important role in Treg cell mediated suppression in vivo, many studies have failed to demonstrate a role for these cytokines in suppression by nTreg cells in vitro. Suppression appears to require cell contact, and CTLA-4, cytotoxicity and competition for access to the APC have all been implicated (29). Our data demonstrating that Foxp3<sup>+</sup>CD39<sup>+</sup> but not Foxp3<sup>+</sup>CD39<sup>-</sup> Treg cells can suppress IL-17 production suggests that CD39 may have a critical role in suppression. There are a number of possible mechanisms involved. Firstly, the hydrolysis of ATP by CD39 could reduce IL-17 production by CD4<sup>+</sup> T cells. Although we found that neither an inhibitor of CD39 ectonucleotidase activity nor an A2A receptor antagonist could reverse the suppression mediated by CD39<sup>+</sup> Treg cells, we did find that blocking CD39 ectonucleotidase activity significantly enhanced IL-17 production by responder T cells. Indeed it has recently been shown that ATP can promote Th17 cells in vivo (30) and that in vitro treatment of dendritic cells with ATP enhanced the production of IL-23 while reducing IL-12, thereby likely promoting Th17 over Th1 cells (31). Secondly, we found that an analogue of adenosine, the final breakdown product of ATP, which binds to the A2A receptor on T cells, effectively inhibited IL-17 as well as IFN- $\gamma$  production and T cell proliferation. Consistent with our

findings, CD39-defective mice have reduced Treg cell activity and impaired ability to control allograft rejection (19).

It has recently been reported that human Treg cells can express Foxp3 and ROR $\gamma$ T and secrete IL-17 (32-37), and this may be explained by our finding that CD4<sup>+</sup>Foxp3<sup>+</sup> T cells include a subset of CD4<sup>+</sup>Foxp3<sup>+</sup>CD39<sup>-</sup> cells that secrete IL-17. The ability of Treg cells to secrete IL-17 may represent inherent plasticity in this population to convert to effector T cells under conditions of inflammation, such as in the presence of IL-2 or IL-15 (34), IL-1 $\beta$  and IL-2 (36), IL-1 $\beta$  and IL-6 (37) or dendritic cells activated under specific conditions (33, 35). Although Foxp3<sup>+</sup>CD39<sup>-</sup> Treg cells also secreted IL-10, this cytokine did not play a role in their suppression, since the addition of neutralising antibodies to IL-10 did not reverse suppression. Although IL-10 is thought to have an immunoregulatory role in MS, our findings suggest that this may not be mediated through CD39<sup>+</sup>Foxp3<sup>+</sup> Treg cells.

Our study has demonstrated for the first time that a subset of human Treg cells can suppress Th17 cells, refuting the current view that Th17 cells are resistant to suppression by Treg cells. Given the importance of Th17 cells in mediating autoimmune disease, our finding has major implications for understanding the control of pathogenic Th17 cells. Furthermore, we demonstrate a role for CD39<sup>+</sup> Treg cells in MS where we found a reduced number and impaired ability of patient CD39<sup>+</sup> Treg cells to suppress IL-17. This suggests that Foxp3<sup>+</sup> Treg cells in MS patients would be less able to constrain auto-reactive Th17 cells that mediate the autoimmune pathology. Finally, our findings have important implications for the use of Treg cells as a therapy for autoimmune and other inflammatory conditions. Injection of Treg cells selected on the basis of expression of CD25 or Foxp3 will include a population of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>CD39<sup>-</sup> T cells that secrete the pro-inflammatory cytokine IL-17, which is pathogenic in many of these diseases.

Conversely selection of cells on the basis of expression of CD39 (together with CD25 and lack of expression of CD127) would allow the use of functional Treg cells that have the capacity to suppress both Th1 and Th17 cells, as well as excluding Foxp3<sup>+</sup>CD39<sup>-</sup> T cell that are IL-17 secreting and therefore potentially pathogenic in autoimmune diseases.

## **Acknowledgements**

We are grateful to Dr Patrick Walsh for helpful discussion.

## Footnotes

<sup>1</sup> This work was supported by The Irish Health Research Board (JF) and Science Foundation Ireland (KM).

<sup>2</sup> To whom correspondence should be addressed. Prof. Kingston Mills, School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland. Tel +353-1-8963573. Fax: +353-1-6772086  
Email: [kingston.mills@tcd.ie](mailto:kingston.mills@tcd.ie)

## References

1. Martin, R., D. Jaraquemada, M. Flerlage, J. Richert, J. Whitaker, E. O. Long, D. E. McFarlin, and H. F. McFarland. 1990. Fine specificity and HLA restriction of myelin basic protein-specific cytotoxic T cell lines from multiple sclerosis patients and healthy individuals. *J. Immunol.* 145:540-548.
2. Feger, U., C. Luther, S. Poeschel, A. Melms, E. Tolosa, and H. Wiendl. 2007. Increased frequency of CD4+ CD25+ regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients. *Clin Exp Immunol* 147:412-418.
3. Viglietta, V., C. Baecher-Allan, H. L. Weiner, and D. A. Hafler. 2004. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp.Med.* 199:971-979.
4. Frisullo, G., V. Nociti, R. Iorio, A. K. Patanella, M. Caggiula, A. Marti, C. Sancricca, F. Angelucci, M. Mirabella, P. A. Tonali, and A. P. Batocchi. 2008. Regulatory T cells fail to suppress CD4(+)T-bet(+) T cells in relapsing multiple sclerosis patients. *Immunology.*
5. Venken, K., N. Hellings, M. Thewissen, V. Somers, K. Hensen, J. L. Rummens, R. Medaer, R. Hupperts, and P. Stinissen. 2008. Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. *Immunology* 123:79-89.
6. Haas, J., A. Hug, A. Viehover, B. Fritzsching, C. S. Falk, A. Filser, T. Vetter, L. Milkova, M. Korporal, B. Fritz, B. Storch-Hagenlocher, P. H. Krammer, E. Suri-Payer, and B. Wildemann. 2005. Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. *Eur J Immunol* 35:3343-3352.
7. Michel, L., L. Berthelot, S. Pettre, S. Wiertlewski, F. Lefrere, C. Braudeau, S. Brouard, J. P. Soulillou, and D. A. Laplaud. 2008. Patients with relapsing-remitting multiple sclerosis have normal Treg function when cells expressing IL-7 receptor alpha-chain are excluded from the analysis. *J Clin Invest.*
8. Vaknin-Dembinsky, A., K. Balashov, and H. L. Weiner. 2006. IL-23 is increased in dendritic cells in multiple sclerosis and down-regulation of IL-23 by antisense oligos increases dendritic cell IL-10 production. *J Immunol* 176:7768-7774.
9. Lock, C., G. Hermans, R. Pedotti, A. Brendolan, E. Schadt, H. Garren, A. Langer-Gould, S. Strober, B. Cannella, J. Allard, P. Klonowski, A. Austin, N. Lad, N. Kaminski, S. J. Galli, J. R. Oksenberg, C. S. Raine, R. Heller, and L. Steinman. 2002. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat.Med.* 8:500-508.
10. Matusevicius, D., P. Kivisakk, B. He, N. Kostulas, V. Ozenci, S. Fredrikson, and H. Link. 1999. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult Scler* 5:101-104.
11. Evans, H. G., T. Suddason, I. Jackson, L. S. Taams, and G. M. Lord. 2007. Optimal induction of T helper 17 cells in humans requires T cell receptor ligation in the context of Toll-like receptor-activated monocytes. *Proc Natl Acad Sci U S A* 104:17034-17039.
12. Annunziato, F., L. Cosmi, V. Santarlasci, L. Maggi, F. Liotta, B. Mazzinghi, E. Parente, L. Fili, S. Ferri, F. Frosali, F. Giudici, P. Romagnani, P. Parronchi, F. Tonelli, E. Maggi,

- and S. Romagnani. 2007. Phenotypic and functional features of human Th17 cells. *J Exp Med* 204:1849-1861.
13. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155:1151-1164.
  14. Huter, E. N., G. H. Stummvoll, R. J. DiPaolo, D. D. Glass, and E. M. Shevach. 2008. Cutting edge: antigen-specific TGF beta-induced regulatory T cells suppress Th17-mediated autoimmune disease. *J Immunol* 181:8209-8213.
  15. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4:330-336.
  16. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061.
  17. Seddiki, N., B. Santner-Nanan, J. Martinson, J. Zaunders, S. Sasson, A. Landay, M. Solomon, W. Selby, S. I. Alexander, R. Nanan, A. Kelleher, and B. Fazekas de St Groth. 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* 203:1693-1700.
  18. Liu, W., A. L. Putnam, Z. Xu-Yu, G. L. Szot, M. R. Lee, S. Zhu, P. A. Gottlieb, P. Kapranov, T. R. Gingeras, B. Fazekas de St Groth, C. Clayberger, D. M. Soper, S. F. Ziegler, and J. A. Bluestone. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 203:1701-1711.
  19. Deaglio, S., K. M. Dwyer, W. Gao, D. Friedman, A. Usheva, A. Erat, J. F. Chen, K. Enjyoji, J. Linden, M. Oukka, V. K. Kuchroo, T. B. Strom, and S. C. Robson. 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 204:1257-1265.
  20. Borsellino, G., M. Kleinewietfeld, D. Di Mitri, A. Sternjak, A. Diamantini, R. Giometto, S. Hopner, D. Centonze, G. Bernardi, M. L. Dell'Acqua, P. M. Rossini, L. Battistini, O. Rotzschke, and K. Falk. 2007. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* 110:1225-1232.
  21. Ito, T., S. Hanabuchi, Y. H. Wang, W. R. Park, K. Arima, L. Bover, F. X. Qin, M. Gilliet, and Y. J. Liu. 2008. Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. *Immunity* 28:870-880.
  22. Venken, K., N. Hellings, K. Hensen, J. L. Rummens, R. Medaer, B. D'Hooghe M, B. Dubois, J. Raus, and P. Stinissen. 2006. Secondary progressive in contrast to relapsing-remitting multiple sclerosis patients show a normal CD4+CD25+ regulatory T-cell function and FOXP3 expression. *J Neurosci Res* 83:1432-1446.
  23. Mills, K. H. 2008. Induction, function and regulation of IL-17-producing T cells. *Eur J Immunol* 38:2636-2649.
  24. Flores-Borja, F., E. C. Jury, C. Mauri, and M. R. Ehrenstein. 2008. Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis. *Proc Natl Acad Sci U S A* 105:19396-19401.
  25. Lawson, J. M., J. Tremble, C. Dayan, H. Beyan, R. D. Leslie, M. Peakman, and T. I. Tree. 2008. Increased resistance to CD4+CD25hi regulatory T cell-mediated suppression in patients with type 1 diabetes. *Clin Exp Immunol* 154:353-359.
  26. Venken, K., M. Thewissen, N. Hellings, V. Somers, K. Hensen, J. L. Rummens, and P. Stinissen. 2007. A CFSE based assay for measuring CD4+CD25+ regulatory T cell

- mediated suppression of auto-antigen specific and polyclonal T cell responses. *J Immunol Methods* 322:1-11.
27. Stummvoll, G. H., R. J. DiPaolo, E. N. Huter, T. S. Davidson, D. Glass, J. M. Ward, and E. M. Shevach. 2008. Th1, Th2, and Th17 effector T cell-induced autoimmune gastritis differs in pathological pattern and in susceptibility to suppression by regulatory T cells. *J Immunol* 181:1908-1916.
  28. Rowan, A. G., J. M. Fletcher, E. J. Ryan, B. Moran, J. E. Hegarty, C. O'Farrelly, and K. H. Mills. 2008. Hepatitis C virus-specific Th17 cells are suppressed by virus-induced TGF-beta. *J Immunol* 181:4485-4494.
  29. Miyara, M., and S. Sakaguchi. 2007. Natural regulatory T cells: mechanisms of suppression. *Trends Mol Med* 13:108-116.
  30. Atarashi, K., J. Nishimura, T. Shima, Y. Umesaki, M. Yamamoto, M. Onoue, H. Yagita, N. Ishii, R. Evans, K. Honda, and K. Takeda. 2008. ATP drives lamina propria T(H)17 cell differentiation. *Nature* 455:808-812.
  31. Schnurr, M., T. Toy, A. Shin, M. Wagner, J. Cebon, and E. Maraskovsky. 2005. Extracellular nucleotide signaling by P2 receptors inhibits IL-12 and enhances IL-23 expression in human dendritic cells: a novel role for the cAMP pathway. *Blood* 105:1582-1589.
  32. Voo, K. S., Y. H. Wang, F. R. Santori, C. Boggiano, Y. H. Wang, K. Arima, L. Bover, S. Hanabuchi, J. Khalili, E. Marinova, B. Zheng, D. R. Littman, and Y. J. Liu. 2009. Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc Natl Acad Sci U S A* 106:4793-4798.
  33. Osorio, F., S. LeibundGut-Landmann, M. Lochner, K. Lahl, T. Sparwasser, G. Eberl, and C. Reis e Sousa. 2008. DC activated via dectin-1 convert Treg into IL-17 producers. *Eur J Immunol* 38:3274-3281.
  34. Koenen, H. J., R. L. Smeets, P. M. Vink, E. van Rijssen, A. M. Boots, and I. Joosten. 2008. Human CD25<sup>high</sup>Foxp3<sup>pos</sup> regulatory T cells differentiate into IL-17-producing cells. *Blood* 112:2340-2352.
  35. Radhakrishnan, S., R. Cabrera, E. L. Schenk, P. Nava-Parada, M. P. Bell, V. P. Van Keulen, R. J. Marler, S. J. Felts, and L. R. Pease. 2008. Reprogrammed FoxP3+ T regulatory cells become IL-17+ antigen-specific autoimmune effectors in vitro and in vivo. *J Immunol* 181:3137-3147.
  36. Deknuydt, F., G. Bioley, D. Valmori, and M. Ayyoub. 2009. IL-1beta and IL-2 convert human Treg into T(H)17 cells. *Clin Immunol* 131:298-307.
  37. Beriou, G., C. M. Costantino, C. W. Ashley, L. Yang, V. K. Kuchroo, C. Baecher-Allan, and D. A. Hafler. 2009. IL-17 producing human peripheral regulatory T cells retain suppressive function. *Blood*.



## Figure Legends

**FIGURE 1.** CD4<sup>+</sup>CD25<sup>hi</sup> T cells suppress proliferation and cytokine secretion but not IL-17 production by responder T cells. CD4<sup>+</sup>CD25<sup>-</sup> responder T cells and CD4<sup>+</sup>CD25<sup>hi</sup> T cells from a healthy donor were sorted by flow cytometry. The suppressive ability of the Treg cells was determined by co-culture with responder T cells stimulated with anti-CD3 plus irradiated APC. Cytokines and proliferation were analysed on day 3. \*\*\* p<0.001 Student's t-test.

**FIGURE 2.** Distinct populations of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells based on expression of CD39. *A*, PBMC from healthy controls were stained for CD4, CD25, Foxp3 and CD39; CD4<sup>+</sup> T cells were gated on the basis of CD25<sup>+</sup>Foxp3<sup>+</sup> and the dot plots show the expression of CD39 versus CD25 or FoxP3. *B*, CD4<sup>+</sup>CD25<sup>-</sup> responder T cells, CD4<sup>+</sup>CD25<sup>hi</sup> T cells and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> subsets of Treg cells were sorted by flow cytometry (as indicated), stimulated for 5 days with anti-CD3 plus APC and then stained with anti-TGF-β and anti-LAP, or IgG-PE and streptavidin-PerCP respectively as negative controls. *C*, MACS purified CD4<sup>+</sup> T cells were stained with CD39-FITC, CD25-PE and CD4-PerCP-Cy5.5 and sorted using a MoFlo into CD4<sup>+</sup>CD25<sup>-</sup>, CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> subsets. The cells were stained with FoxP3-Alexa647 and analysed by flow cytometry for purity and FoxP3 expression (% FoxP3<sup>+</sup>: 98%, 81%; MFI: 51.8, 27.2 for CD39<sup>+</sup> (dashed line) and CD39<sup>-</sup> (dotted line) respectively relative to CD4<sup>+</sup>CD25<sup>-</sup> cells (filled histogram)).

**FIGURE 3.** CD39<sup>+</sup> Treg cells, but not CD39<sup>-</sup> Treg cells suppress IL-17 production by responder T cells. CD4<sup>+</sup>CD25<sup>-</sup> responder T cells, CD4<sup>+</sup>CD25<sup>hi</sup> T cells and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup>

and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> subsets of Treg cells were sorted by flow cytometry. The suppressive ability of total Treg cells or Treg subsets was determined by co-culture with responder T cells stimulated with anti-CD3 plus irradiated APC. Cytokines and proliferation were analysed on day 3. *A*, CD39<sup>+</sup> or CD39<sup>-</sup> Treg cells were co-cultured with responder T cells at various ratios, and their ability to suppress IFN- $\gamma$  or IL-17 was determined. *B*, Summary data from separate donors in several experiments showing the suppressive capacity of the different Treg subsets expressed as a percentage of the control value (CD25<sup>-</sup> responder T cells) for IFN- $\gamma$  or IL-17 production. IFN- $\gamma$ : no significant difference between groups, IL-17: \* < p 0.05 by one way Anova (Holm-Sidak method). *C*, CD4<sup>+</sup>CD25<sup>-</sup> responder cells were labelled with CFSE and stimulated with anti-CD3 plus irradiated APC either alone or co-cultured with CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> T cells. After 4 days cells were re-stimulated with PMA/Ionomycin and stained for expression of IL-17 and IFN- $\gamma$ . CFSE labelled responder cells were gated to exclude the CFSE<sup>-</sup> APC and Treg cells and their expression of IL-17 vs IFN- $\gamma$  is shown.

**FIGURE 4.** Total CD4<sup>+</sup>FoxP3<sup>+</sup> or CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> Treg cells secrete IL-17 which masks their suppressive capacity. CD4<sup>+</sup>CD25<sup>-</sup> responder T cells, CD4<sup>+</sup>CD25<sup>hi</sup> T cells and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>+</sup> and CD4<sup>+</sup>CD25<sup>hi</sup>CD39<sup>-</sup> subsets of Treg cells from healthy donors were sorted by flow cytometry. *A*, Total CD4<sup>+</sup>CD25<sup>hi</sup> T cells, or CD39<sup>+</sup> or CD39<sup>-</sup> subsets were cultured alone, or with CD4<sup>+</sup>CD25<sup>-</sup> responder T cells (ratio 0.5:1). Proliferation, IFN- $\gamma$ , IL-17 and IL-10 were measured on day 3. \* p< 0.05, \*\* p<0.01 co-cultures compared with responder T cells alone, Student's t test. Data is representative of 3 separate experiments with different donors. *B*, CD4<sup>+</sup> T cells were left un-stimulated or stimulated for 4 hr with PMA/Ionomycin and

then stained for expression of FoxP3 and IL-17. Expression of IL-17 by the total CD4<sup>+</sup> population or the FoxP3<sup>+</sup> CD4<sup>+</sup> T cells are shown.

**FIGURE 5.** The frequency of FoxP3<sup>+</sup>CD39<sup>+</sup> Treg cells is reduced and FoxP3<sup>+</sup>CD39<sup>-</sup> cells enhanced in MS patients. PBMC from MS patients or healthy donors were stained for cell surface expression of CD4, CD25, CD39, CD127 and intracellular Foxp3 and analysed by flow cytometry. *A*, Cells were gated first on the basis of CD4<sup>+</sup>CD127<sup>lo</sup>CD25<sup>+</sup>, then the Foxp3<sup>+</sup> cells were analysed for expression of CD39 as shown in the histogram. *B*, Comparison of the percentage of CD4<sup>+</sup>CD127<sup>lo</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells from RRMS or SPMS patients compared with controls. *C*, The percentage of CD4<sup>+</sup>CD127<sup>lo</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells that are CD39<sup>+</sup> or CD39<sup>-</sup> in RRMS or SPMS patients compared with controls.

**FIGURE 6.** Defective function of CD39<sup>+</sup> Treg cells from MS patients. *A*, CD4<sup>+</sup>CD127<sup>lo</sup>CD25<sup>+</sup>CD39<sup>+</sup> Treg cells from healthy donors (filled bars) or MS patients (clear bars) were sorted by flow cytometry, co-cultured with autologous CD4<sup>+</sup>CD25<sup>-</sup> responder cells and suppression of IFN- $\gamma$  and IL-17 was measured. *B*, CD4<sup>+</sup>CD127<sup>lo</sup>CD25<sup>+</sup>CD39<sup>+</sup> Treg cells from MS patients (MS) were co-cultured with either autologous responder T cells, or those from controls (C), and vice versa. The suppression of responder cell IFN- $\gamma$  or IL-17 is expressed as the % of cytokine produced by responder cells alone. IFN- $\gamma$ : no significant difference between groups, IL-17: \* $p$ <0.05 by one way Anova (Holm-Sidak method).

**FIGURE 7.** The mechanism of suppression by CD39<sup>+</sup> Treg cells. *A*, CD4<sup>+</sup> T cells from healthy donors were stimulated with anti-CD3 plus APC in the presence of 1 and 10  $\mu$ M 2-

chloroadenosine, a stable adenosine agonist. The effect on T cell proliferation, IFN- $\gamma$ , or IL-17 production was determined. *B*, The effect of blocking CD39 activity or adenosine binding to its receptor on T cells was determined by adding CD39 ectonucleotidase inhibitor POM-1 (1  $\mu$ M) or the A2A receptor antagonist ZM241385 (1  $\mu$ M) respectively to a suppression assay with CD39<sup>+</sup> Treg cells. IFN- $\gamma$  and IL-17 were measured on day 3. \*\*\* $p < 0.001$  POM-1 treated versus responder T cell alone (Student's t-test). The effect of neutralising TGF- $\beta$  (*C*) or IL-10 (*D*) on suppression mediated by Treg cell subsets was determined by addition of 10  $\mu$ g/ml neutralising anti-TGF- $\beta$  or IL-10 antibodies to co-cultures. *E*, The requirement for contact in suppression by CD39<sup>+</sup> Treg cells was determined using a transwell assay. CD39<sup>+</sup> Treg cells and responder cells were co-cultured together or separated by a semi permeable transwell membrane, and stimulated with anti-CD3 plus APC. IFN- $\gamma$  and IL-17 were measured in the cell culture supernatants on day 3.