ELSEVIER

Journal of Neuroimmunology 183 (2007) 96–103

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

An interferon-γ-producing Th1 subset is the major source of IL-17 in experimental autoimmune encephalitis

Santi Suryani, Ian Sutton *

The Garvan Institute of Medical Research, Department of Inflammation, 384 Victoria Street, Darlinghurst, Sydney, NSW 2010, Australia

Received 9 August 2006; received in revised form 19 November 2006; accepted 20 November 2006

Abstract

$Th_{IL\text{-}17}$ (IL-17+/IFN-γ−) cell lines are significantly more encephalitogenic than Th1 (IL-17−/IFN-γ+) cell lines in adoptive transfer EAE models. In actively induced EAE short *ex vivo* peptide stimulation identifies an IL-17+/IFN-γ+ population of CD4+ CNS-infiltrating MOG_{35-55}-specific T cells, which outnumber IL-17+/IFN-γ− cells by approximately 3:1 as disease develops. A decrease in numbers of IL-17+/IFN-γ+ cells following *in vitro* culture is accompanied by an increase in IL-17−/IFN-γ+ cell numbers. Together these *ex vivo* and *in vitro* observations imply that the Th1 lineage is more encephalitogenic than is suggested by adoptive transfer of Th1 (IL-17−/IFN-γ+) cell lines which have been terminally differentiated *in vitro*.

Keywords: T cell differentiation; IL-17; Interferon-γ; EAE

1. Introduction

Recent studies have demonstrated that the pro-inflammatory cytokine IL-17 has a significant role in the development of pathological changes that accompany central nervous system (CNS) inflammation in experimental autoimmune encephalitis (EAE). IL-$17^{-/-}$ mice have a delayed disease onset and reduced disease severity with early recovery (Komiyama et al., 2006), while administration of neutralizing anti-IL-17 antibodies immediately prior to the expected onset of actively induced clinical disease in wild type animals reduces both disease incidence and severity (Langrish et al., 2005; Park et al., 2005). IL-17 production by T cells is dependent on IL-23, a heterodimeric cytokine composed of a p40 subunit, shared with IL-12, and an IL-23 specific p19 subunit (Oppmann et al., 2000). Both $p19^{-/-}$ and $p40^{-/-}$ mice are resistant to actively induced EAE (Cua et al., 2003) and CNS-infiltrating CD4+ T lymphocytes derived from $p19^{-/-}$ mice do not produce IL-17 following *in vitro* re-stimulation (Langrish et al., 2005). Additional *in vitro* studies have demonstrated that IL-23 supports the differentiation of a unique T cell subset, termed $Th_{IL\text{-}17}$. $Th_{IL\text{-}17}$ differentiation is also CD28 and ICOS dependent and occurs independently of STAT4 or STAT6 signaling (Harrington et al., 2005; Park et al., 2005). The $Th_{IL\text{-}17}$ subset is characterized by the production of IL-17, IL-17F, IL-6 and TNF, but not IFN-γ, and adoptive transfer of myelin-specific $Th_{IL\text{-}17}$ cell lines produces symptomatic EAE in a dose-dependent fashion (Langrish et al., 2005). In contrast, adoptively transferred IL-17−/IFN-γ+ Th1 cell lines only have a mildly encephalitogenic action (Langrish et al., 2005). It has also been demonstrated that in certain situations IFN-γ is a potent suppressor of IL-17 production (Harrington et al., 2005; Park et al., 2005) and loss of IFN-γ-mediated inhibition of IFN-γ− T cell proliferation (Chu et al., 2000) accounts for the observations that IFN-$\gamma^{-/-}$ (Ferber et al., 1996; Wensky et al., 2005), IFN-$\gamma R^{-/-}$ (Willenborg et al., 1996) and IL-12$p35^{-/-}$ (Becher et al., 2002) mice develop a more severe EAE disease course than that seen in wild type animals. Moreover, since studies in rat models of optic nerve crush injury and spinal cord contusion

* Corresponding author. Tel.: +61 2 92958433; fax: +61 2 9295 8404.
E-mail address: i.sutton@garvan.org.au (I. Sutton).

0165-5728/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneuroim.2006.11.023

have demonstrated that myelin-specific Th1 cell lines can promote neural repair (Hauben et al., 2000; Moalem et al., 1999), understanding the mechanisms that regulate IL-17 and IFN-γ production in the course of CNS inflammation is relevant to refining immunomodulatory strategies used in the treatment of human neurological diseases.

Some debate exists as to the degree of overlap that exists between $Th_{IL\text{-}17}$ and Th1 effector cell developmental pathways. In one model it is proposed that a pre-Th1 intermediate is activated by IFN-γ or IL-27 induction of T-bet, via STAT1, leading to upregulation of IL-12Rβ2 and IL-23R. Both IL-12Rβ2 and IL-23R pair with constitutively expressed IL-12Rβ1, thereby resulting in coexpression of the IL-12R and the IL-23R. Further differentiation to a Th1 or $Th_{IL\text{-}17}$ phenotype then depends upon subsequent IL-12 or IL-23 stimulation (Bettelli and Kuchroo, 2005). An alternate hypothesis suggests that IL-17 and IFN-γ effector differentiation occurs at the naïve T cell stage. However, naïve T cells do not express the IL-23R (Aggarwal et al., 2003) and recent studies show that IL-6 and TGF-β1 are also important in the differentiation of the $Th_{IL\text{-}17}$ subset (Bettelli et al., 2006; Veldhoen et al., 2006), with IL-23R being induced by TGF-β1 (Mangan et al., 2006). While Betelli et al., were unable to induce differentiation of IL-17 producing T cells from naive 2D2 $MOG_{35–55}$ Tg T cells (Bettelli et al., 2006), Langrish et al., demonstrated that after 7 days in culture with anti-CD3/anti-CD28 antibodies and rIL-23 naïve TCR-transgenic D011.10Tg X $Rag^{-/-}$ cells had all transformed to a CD62Llo phenotype and 1% of cells produced IL-17 following stimulation with PMA/ionomycin (Langrish et al., 2005). Similarly, Harrington et al., were able to identify 2% of IL-17+/IFN-γ− cells after stimulation of naïve D011.10Tg X $Rag^{-/-}$ T cells with OVA peptide and splenic feeder cells in the presence of IL-23 and anti-IFN-γ antibody (Harrington et al., 2005).

Further evidence suggests that IL-23 is not essential for the priming of $Th_{IL\text{-}17}$ cell differentiation. T cells derived from the draining lymph nodes (DLN) of IL-23$p19^{-/-}$ mice 6 days after immunization with $MOG_{35–55}$ peptide emulsified in CFA demonstrate normal proliferative responses to $MOG_{35–55}$ restimulation (Cua et al., 2003) and following immunization of $p40^{-/-}$ mice, *in vitro* culture of DLN cells with IL-23 produces a $Th_{IL\text{-}17}$ phenotype (Langrish et al., 2005). However, while active immunization of $p19^{-/-}$ and $p40^{-/-}$ mice primes myelin-specific T cell responses in the absence of IL-23, systemic expression of IL-23 by treatment with gene transfer vectors is not sufficient to cause symptomatic disease (Cua et al., 2003), whereas reconstitution of IL-23 expression in the CNS alone induces the disease state (Cua et al., 2003). Taken together these data suggest that although $Th_{IL\text{-}17}$ cells have been induced from naïve T cells in certain *in vitro* conditions, IL-23 is not essential for *in vivo* priming of naïve T cells following immunization with myelin epitopes, but is critical for IL-17 production by activated/memory T cells when they re-encounter antigen. Therefore, mechanisms that regulate activated T cell differentiation and cytokine production within the CNS are different from those that result in priming of T cell responses in the periphery.

In this study we provide support for the concept that immunization with $MOG_{35–55}$ peptide results in the activation of a CD4+ T cell population capable of producing both IFN-γ and IL-17. We describe a hitherto unreported population of short-lived IL-17+/IFN-γ+ population of $MOG_{35–55}$-specific cells that can be isolated from the CNS and our data is consistent with this cell population representing a pre-Th1 intermediate phase of differentiation. Recent reports suggesting that the Th1 lineage is only mildly encephalitogenic are based on results of adoptive transfer studies that used Th1 cell lines which had been terminally differentiated *in vitro* to an IL-17−/IFN-γ+ phenotype. The observation that Th1 differentiation is associated with IL-17 production challenges the paradigm that the $Th_{IL\text{-}17}$ subset is the major pathogenic population in EAE.

2. Materials and methods

2.1. Mice

C57BL/6 were obtained from ARC, Perth, WA and housed under specific pathogen-free conditions. Animals were maintained according to regulations of the NH and MRC and all animal procedures were approved by the Garvan Institute Animal Ethics Committee.

2.2. EAE

EAE was induced in 8–10 week old female mice. Mice received a 50 μl injection into each flank, containing 50 μg $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) (>90% purity, Mimotopes, Clayton, Vic) emulsified with CFA supplemented with 5 mg/ml of H37 Ra Mycobacterium tuberculosis (Difco Laboratories), and received an i.p. injection containing 200 ng of pertussis toxin (List Biological Laboratories) on days 0 and 2. Animals were observed daily for signs of clinical disease. Disease severity was graded as follows: grade 0, normal; grade 1, loss of tail tone; grade 2, flaccid tail; grade 3, hindlimb paresis; grade 4, hindlimb paralysis; grade 5, forelimb paresis.

2.3. Isolation of CNS inflammatory cells

Mice were euthanized by i.p. injection of ketamine and perfused with ice-cold PBS. Inflammatory cells were recovered from the CNS (brain and spinal cord) by mechanical dissociation of tissues in 30% Percoll (4 °C), which was then layered over 70% Percoll (Amersham Biosciences). Inflammatory cells were separated from the interface after centrifugation at 500 *g* for 20 min and washed twice in PBS (4 °C). Total number of cells derived from each animal was determined using a haemocytometer.

2.4. Antibody staining of CNS inflammatory cells and flow cytometry

1×10^5 CNS-derived inflammatory cells were washed and blocked with 1 μg/ml of Fcγ block (BD Biosciences), then incubated with anti-CD45-PE, anti-CD11b-FITC and anti-CD4-APC (BD Biosciences) antibodies for 20 min at 4 °C. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickson).

2.5. $MOG_{35–55}$ peptide restimulation

After separation at 4 °C, DLN, spleen and CNS-derived inflammatory cells were incubated in T cell medium (RPMI 1640 media containing 10% FCS, supplemented with Pen-Strep and L-glutamine) at 37 °C/5% CO_2 for 2 h. Cells were then pelleted by centrifugation at 500 *g* for 5 min and resuspended in T cell medium containing Golgi Block (BD Biosciences) +/− 20 μg/ml $MOG_{35–55}$ peptide at a concentration of 1×10^6 cells/ml. 2×10^5 cells/well were then cultured in a 96-well U-bottomed plate for 6 h. For 30 hour $MOG_{35–55}$ peptide restimulation experiments cells were resuspended in T cell medium without Golgi block, which was subsequently added for the last 6 h of culture.

2.6. Intracellular cytokine staining and flow cytometry

Following culture experiments cells were transferred to a V-bottomed plate, washed and blocked with 1 μg/ml of Fcγ block (BD Biosciences), then stained with anti-CD4-APC. Cells were then washed, fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences) and intracellular stained with anti-IFN-γ-FITC and anti-IL-17-PE or FITC- and PE-conjugated isotype controls (BD Biosciences). Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickson). The percentage of $MOG_{35–55}$-specific CD4+ T cells was determined by comparing cytokine production in MOG stimulated and unstimulated cultures. The number of $MOG_{35–55}$-specific CD4+ cells derived from each animal was calculated as follows: number of CD4+ T cells = (total number of cells × % CD4+ cells)/100 and number of $MOG_{35–55}$-specific CD4+ T cells = (total number of CD4+ T cells × % cytokine+ cells)/100.

2.7. Proliferation assays

2.7.1. Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling

CNS-derived inflammatory cells were washed and resuspended in 1 ml ice-cold PBS/0.1% Foetal Calf Serum (FCS) to which CFSE (Molecular Probes) was added at a final concentration of 0.05 μM. The cell suspension was then vortexed immediately and incubated in a 37 °C waterbath for 10 min, after which CFSE was quenched by addition of 10 ml ice-cold PBS/10% FCS for 5 min. After two washes with PBS/10% FCS cells were resuspended in T cell medium +/− 20 μg/ml $MOG_{35–55}$ peptide at a concentration of 1×10^6 cells/ml. 2×10^5 cells/well were then cultured in a

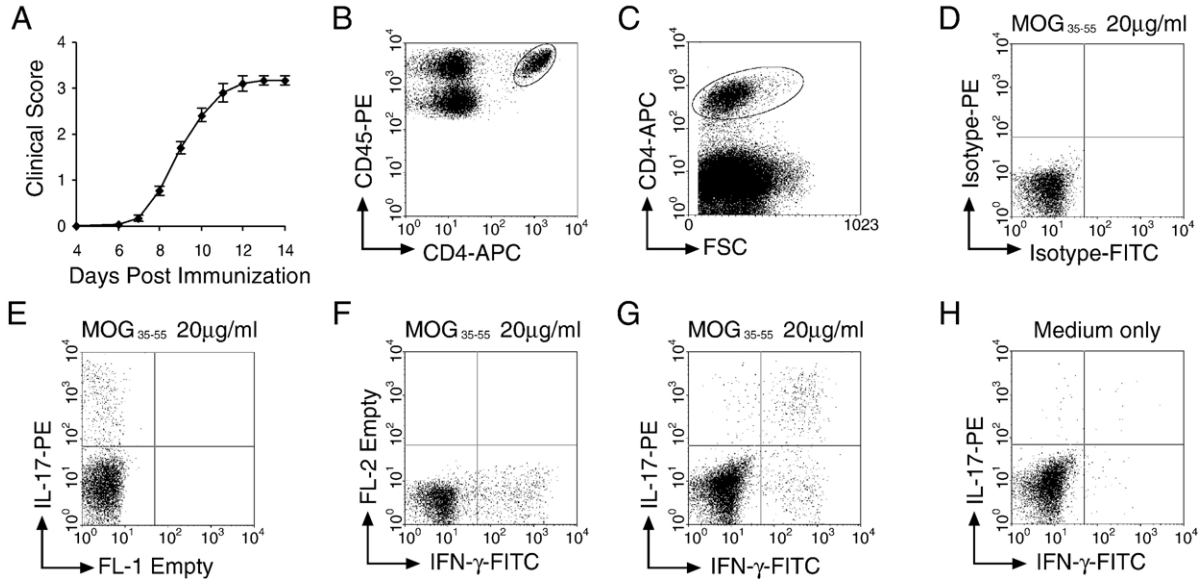

Fig. 1. Three populations of $MOG_{35–55}$-specific CD4+ T cells can be identified in the CNS of animals with EAE by intracellular cytokine staining for IL-17 and IFN-γ following a six hour *ex vivo* peptide stimulation. A. Clinical course of EAE (mean score+/−SEM). B. CD4+ T helper lymphocytes account for approximately 9% of the CD45+ inflammatory cells isolated from the CNS two days after the onset of symptomatic disease. (C–H) Analysis of IL-17 and IFN-γ expression by the CD4+ population in response to $MOG_{35–55}$ peptide stimulation. C. CD4+ cell gate. D. CD4+ cells subjected to $MOG_{35–55}$ peptide stimulation demonstrate no significant binding to isotype PE- and FITC-conjugated control antibodies. E+F. IL-17-PE and IFN-γ-FITC single staining of the CD4+ population demonstrates IL-17 (E) and IFN-γ (F) producing populations following $MOG_{35–55}$ peptide stimulation. F. Dual IL-17-PE and IFN-γ-FITC staining demonstrates IL-17+/IFN-γ−, IL-17+/IFN-γ+ and IL-17−/IFN-γ+ subsets of CD4+ $MOG_{35–55}$-specific T cells, with the majority of IL-17 expressing cells also producing IFN-γ. H. No significant cytokine production was observed in the absence of $MOG_{35–55}$ peptide stimulation.

96-well U-bottomed plate for 30 h with addition of Golgi block for the last 6 h of culture. FACS analysis was undertaken after staining with anti-CD4-PE and anti-IFN-γ-APC antibodies.

2.7.2. ^{3}[H] thymidine

2×10^{5} CNS-derived inflammatory cells/well were cultured in a 96-well U-bottomed plate +/− 20 μg/ml $MOG_{35–55}$ peptide with 1 μCi ^{3}[H] thymidine. After 30 h cells were harvested and ^{3}[H] thymidine incorporation was quantified using a beta-scintillation counter.

2.8. Statistical analysis

A two-tailed Mann–Whitney *U* test was used to compare i) the frequencies of IL-17+/IFN-γ−, IL-17+/IFN-γ+ and IL-17−/IFN-γ+ T cell populations derived from paired samples following 6 and 30 hour $MOG_{35–55}$ peptide restimulation and ii) ^{3}[H] thymidine incorporation between unstimulated and $MOG_{35–55}$ peptide stimulated cultures.

3. Results

Recent studies classify Th1 and Th_{IL-17} subsets on the basis of IFN-γ and IL-17 production respectively and adoptive transfer studies demonstrate that Th_{IL-17} and Th1 cell lines have marked differences in their encephalitogenicity. Since Th1 cell lines are quantitatively less encephalitogenic than Th_{IL-17} cell lines and can also potentially contribute to repair processes within the CNS following injury, we sought to determine the relative frequencies of $MOG_{35–55}$-specific Th1 and Th_{IL-17} cells infiltrating the CNS of animals with actively induced EAE. In initial experiments inflammatory cells were separated from the CNS of animals two days after the onset of symptomatic disease (Fig. 1A) and it was determined that approximately 9% of CD45+ cells were CD4+ T cells (Fig. 1B), with macrophages/granulocytes (CD45hiCD11b+) and microglia (CD45loCD11b+) making up the major immune cell populations (data not shown). Approximately 10% of CNS-infiltrating CD4+ T cells demonstrated $MOG_{35–55}$ specificity as measured by intracellular cytokine staining for IFN-γ and IL-17. In contrast to previous studies which have reported that IL-17 and IFN-γ production is mutually exclusive, a 6 hour *ex vivo* restimulation with $MOG_{35–55}$ peptide defined three discrete populations of $MOG_{35–55}$-specific CD4+ T cells. The majority of $MOG_{35–55}$-specific CD4+ T cells produced IFN-γ, with approximately half of these cells also producing IL-17, whereas IL-17+/IFN-γ− T cells accounted for only 1% of CNS-infiltrating CD4+ T cells (Fig. 1C–H).

Next we investigated CD4+ T cell infiltration of the CNS and cytokine production in relation to the development of

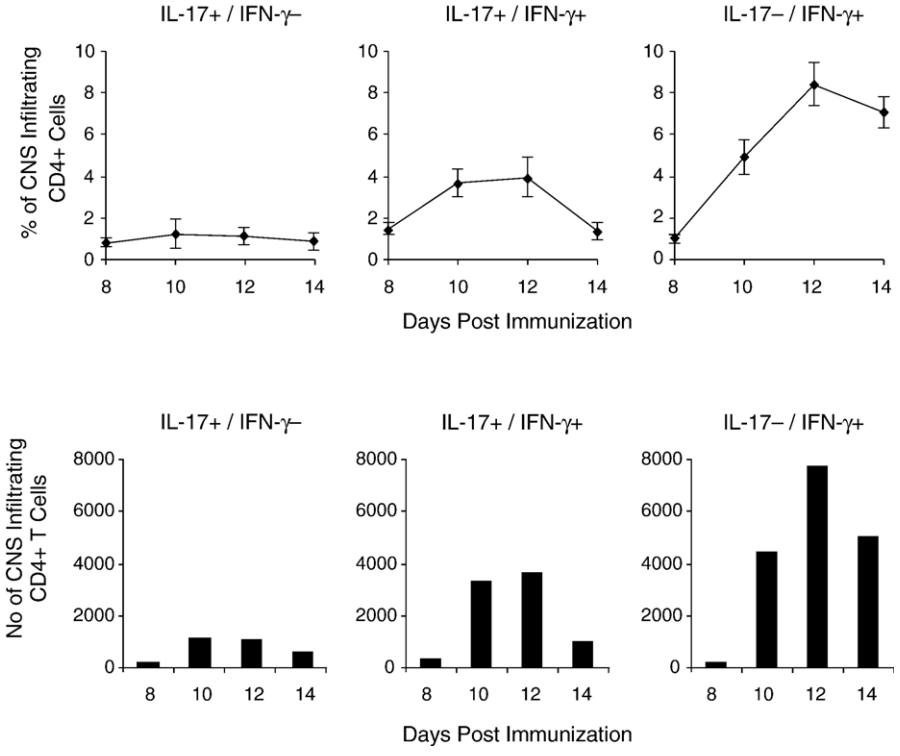

Fig. 2. The kinetics of CD4+ $MOG_{35–55}$-specific T cell subset detection within the CNS during the development of EAE. The percentage and numbers of antigen-specific CD4+ T cells infiltrating the CNS were assessed by a six hour *ex vivo* stimulation with $MOG_{35–55}$ (20 μg/ml) 8, 10, 12 and 14 days post-immunization. Top panels illustrate the percentage of CD4+ $MOG_{35–55}$-specific T cells according to cytokine production. Results expressed as a percentage of the total number of CNS-infiltrating CD4+ T cells (+/−SEM). Bottom panels illustrate the mean number of CD4+ $MOG_{35–55}$-specific T cells/animal according to cytokine production ($n=8$ at each time point).

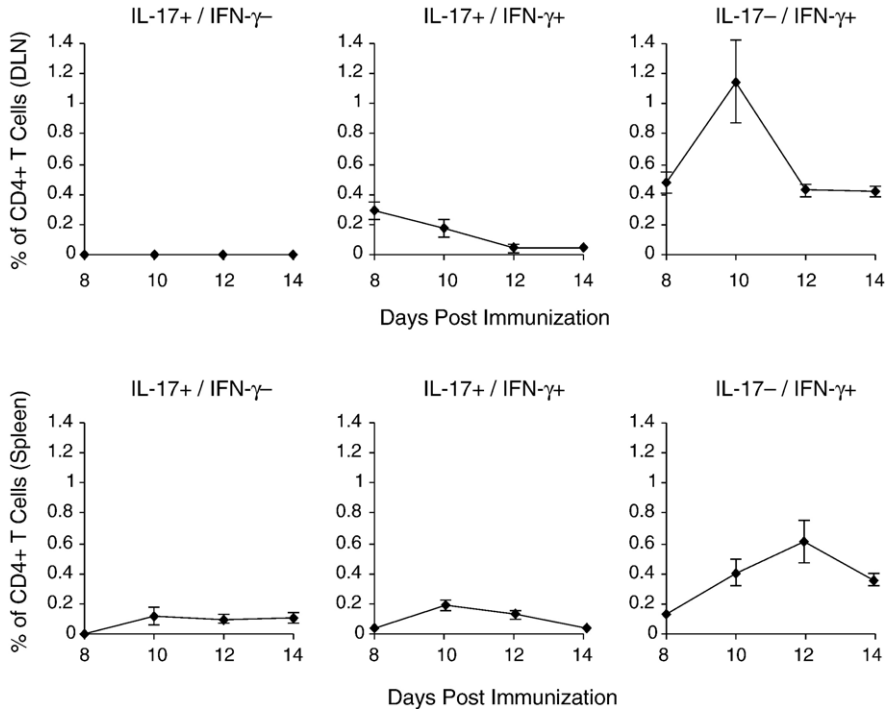

Fig. 3. The kinetics of CD4+ MOG_{35-55}-specific T cell subset detection within the DLN and spleen post-immunization. The percentage of antigen-specific CD4+ T cells in the DLN and spleen was assessed by a six hour *ex vivo* stimulation with MOG_{35-55} (20 μg/ml) 8, 10, 12 and 14 days post-immunization. Top panels illustrate the percentage of CD4+ MOG_{35-55}-specific T cells in the DLN according to cytokine production. Results expressed as a percentage of the total number of CD4+ T cells (+/− SEM). Bottom panels illustrate the percentage of CD4+ MOG_{35-55}-specific T cells in the spleen according to cytokine production. Results expressed as a percentage of the total number of CD4+ T cells (+/− SEM) ($n=8$ at each time point).

symptomatic disease. Disease onset typically occurred 8 days post-immunization and animals then developed a rapidly ascending paralysis over the next 4 days, following which time the acquired disability stabilized (Fig. 1A). At the onset of clinical signs the majority of MOG_{35-55}-specific CD4+ T cells were IL-17+/IFN-γ+ (Fig. 2) and disease progression was associated with an increase in the numbers of all three populations within the CNS. Maximum numbers of IL-17+/IFN-γ− and IL-17+/IFN-γ+ cells occurred between day 10 and day 12, after which numbers of both populations declined, correlating with disease stabilization. Between day 8 (disease onset) and day 12 (stabilization of disability) IL-17+/IFN-γ+ cells were detected at a 2.8 fold higher prevalence than IL-17+/IFN-γ− cells, an observation

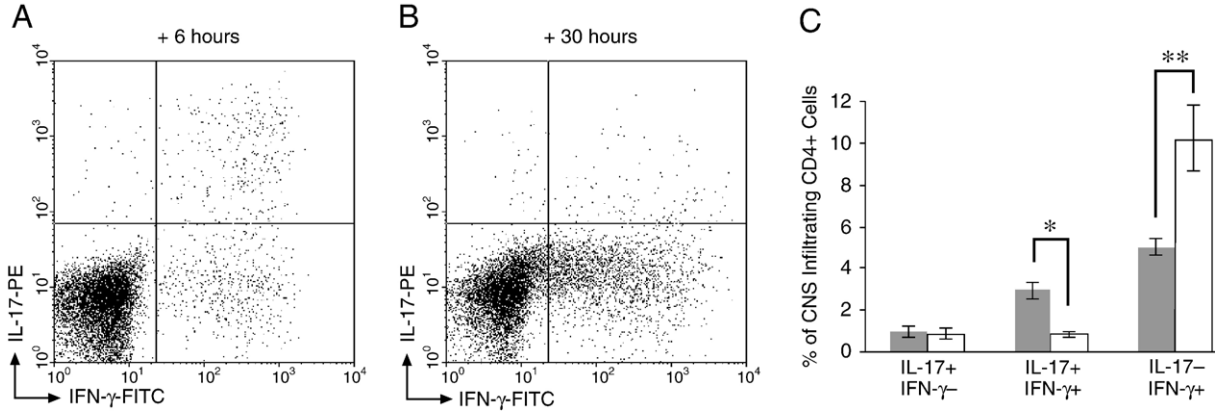

Fig. 4. IL-17+/IFN-γ+MOG_{35-55}-specific CD4+ T cells are a short-lived population. (A–C) CD4+ MOG_{35-55}-specific IL-17+/IFN-γ−, IL-17+/IFN-γ+ and IL-17−/IFN-γ+ subset frequencies were compared following 6 h and 30 h of *in vitro* peptide stimulation. A. FACS plot following a 6 hour peptide stimulation. B. FACS plot following a 30 hour peptide stimulation. C. Comparison of IL-17+/IFN-γ−, IL-17+/IFN-γ+ and IL-17−/IFN-γ+ subset frequencies after 6 h (shaded bars) and 30 h (open bars) of peptide stimulation. ($n=10$. Paired samples analyzed for frequency of T cell subsets at 6 and 30 hour time points using a two-tailed Mann–Whitney *U* test). No change in the frequency of IL-17+/IFN-γ− cells was observed between 6 and 30 h of culture ($p=$NS). A further 24 h of culture in the presence of MOG_{35-55} resulted in a marked reduction in the frequency of IL-17+/IFN-γ+ MOG_{35-55}-specific CD4+ T cells (*$p<0.0005$) and an increase in the frequency of IL-17−/IFN-γ+ MOG_{35-55}-specific CD4+ T cells (**$p<0.05$).

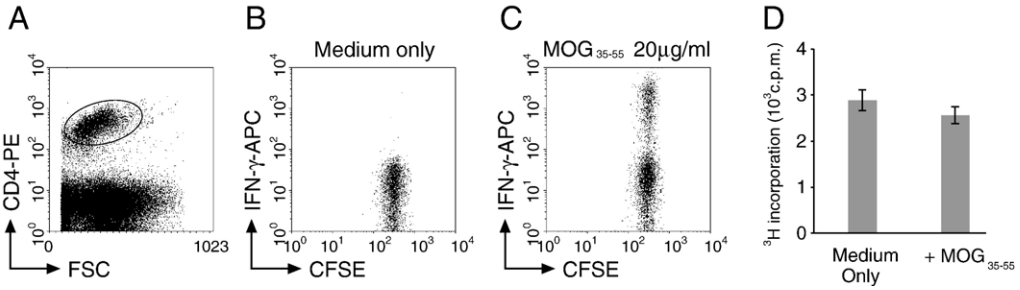

Fig. 5. Cell division is not observed in CNS-derived IFN-γ+ $MOG_{35–55}$-specific CD4+ T cells 30 h after *in vitro* peptide stimulation. A–C. CNS-derived inflammatory cells were labeled with CFSE and cultured for 30 h +/− $MOG_{35–55}$ (20 μg/ml). A. CD4+ T cells were examined for CFSE fluorescence levels and IFN-γ production. B. CFSE fluorescence in the absence of $MOG_{35–55}$ stimulation. C. With $MOG_{35–55}$ stimulation a population of IFN-γ+ cells was detected. Uniform CFSE fluorescence levels were observed in the IFN-γ+ cell population and mean CFSE fluorescence levels did not differ significantly from those seen in unstimulated cells or the IFN-γ− population. D. A ^{3}[H] thymidine incorporation assay also demonstrated that 30 h of $MOG_{35–55}$ peptide stimulation was not associated with cell proliferation ($n=8$, $p=$NS).

consistent with the former population being the major source of IL-17. Similar numbers of IL-17+/IFN-γ+ and IL-17−/IFN-γ+ cells were observed at day 10, but numbers of IL-17−/IFN-γ+ cells continued to rise with maximum frequency and absolute numbers occurring 12 days after immunization (Fig. 2).

Since CNS-infiltrating T cells are derived from peripherally activated precursor cells we also examined IL-17 and IFN-γ production in the CD4+ T cell populations within the draining lymph nodes (DLN) and spleen. In the DLN no $MOG_{35–55}$-specific IL-17+/IFN-γ− could be detected above background levels, IL-17+/IFN-γ+ cells declined in number after day 8, and IL-17−/IFN-γ+ cell numbers peaked at day 10 (Fig. 3). Within the spleen *ex vivo* stimulation with $MOG_{35–55}$ peptide demonstrated a similar temporal profile of cytokine producing $MOG_{35–55}$-specific CD4+ T cells to that observed in the CNS, with maximum numbers of IL-17+/IFN-γ+ cells present at day 10–12 and IL-17−/IFN-γ+ cell numbers reaching maximum frequency at day 12 (Fig. 3). The temporal profile of these observed responses and >10-fold increase in the frequency of CD4+$MOG_{35–55}$-specific cells in the CNS compared to the spleen was consistent with priming of $MOG_{35–55}$-specific T cells in the DLN with subsequent retention of antigen-specific cells within the CNS.

In all three organ systems it was noted that numbers of IL-17−/IFN-γ+ cells continued to increase for 24 h after the number of IL-17+/IFN-γ+ cells had stabilized. This increase in numbers of IL-17−/IFN-γ+ CD4+ T cells within the CNS might reflect further recruitment and retention of peripherally derived T cells, proliferation of recruited T cells within the CNS, differentiation of IL-17+/IFN-γ+ cells into a IL-17−/IFN-γ+ phenotype or a combination of these mechanisms. To determine whether IL-17+/IFN-γ+ cells could represent a pre-Th1 intermediate subset that differentiates into an IL-17−/IFN-γ+ population, CNS-derived CD4+ cells were restimulated with $MOG_{35–55}$ peptide and *ex vivo* cytokine profiles 6 h after stimulation were compared with those obtained from paired samples after 30 h of culture. An additional 24 h in culture resulted in a marked decrease in the frequency of IL-17+/IFN-γ+$MOG_{35–55}$-specific T cells ($p<0.0005$) and an increase in the frequency of IL-17−/IFN-γ+ T cells ($p<0.05$) (Fig. 4).

In order to determine whether the increase in frequency of IL-17−/IFN-γ+ cells could be due to a proliferation effect, CNS-derived cells were labeled with CFSE and stimulated with $MOG_{35–55}$ peptide for 30 h, with addition of Golgi block for the last 6 h of culture. CD4+ cells were then probed for interferon-γ production and CFSE fluorescence levels compared between IFN-γ+ and IFN-γ− populations. Both IFN-γ+ $MOG_{35–55}$-specific T cells and IFN-γ− T cell populations demonstrated the same mean CFSE fluorescence intensity after 30 h of culture and no IFN-γ+ $MOG_{35–55}$-specific T cells had a reduced CFSE fluorescence intensity that would have been observed with cell division (Fig. 5A–C). Similarly, no significant differences were observed when ^{3}[H] thymidine uptake was compared between $MOG_{35–55}$-stimulated and unstimulated cultures (Fig. 5D). Therefore, the rise in frequency of IL-17−/IFN-γ+ cells that accompanies the decline in frequency of IL-17+/IFN-γ+ cells occurs in the absence of cell proliferation and is consistent with the hypothesis that a pre-Th1 intermediate IL-17+/IFN-γ+ population differentiates into an IL-17−/IFN-γ+Th1 population.

4. Discussion

The important role of the pro-inflammatory cytokine IL-17 in the development of EAE is illustrated by the delayed onset and less severe disease course observed in IL-17$^{-/-}$ mice (Komiyama et al., 2006). Recent studies have defined a unique T cell subset, termed $Th_{IL\text{-}17}$, that is identified by a cytokine profile distinct from that observed in Th1 or Th2 subsets (Langrish et al., 2005). The differentiation of the $Th_{IL\text{-}17}$ subset requires different cytokines and transcription factors to those needed for Th1/Th2 differentiation and the presence or absence of IL-6 with TGF-β1 stimulation also determines a separate $Th_{IL\text{-}17}/T_{reg}$ dichotomy (Bettelli et al., 2006). Increased IL-17 levels are associated with the human inflammatory CNS disorders, multiple sclerosis (Lock et al.,

2002) and neuromyelitis optica (Ishizu et al., 2005), and adoptive transfer studies in mice using myelin-specific T cell lines have demonstrated that EAE is induced by $Th_{IL\text{-}17}$ cells in a dose-dependent fashion, whereas Th1 cell lines are only mildly pathogenic. While these adoptive T cell transfer studies demonstrate the clear pathogenic role of the $Th_{IL\text{-}17}$ subset, the T cell lines used were derived from DLN of immunized mice that had been cultured for 10–14 days with either IL-12 or IL-23 prior to adoptive transfer (Langrish et al., 2005). Thus important questions regarding the *in vivo* regulation of activated T cell differentiation within the CNS still require further investigation.

In this study we have demonstrated that following EAE induction in C57BL/6 wild type animals the major population of CNS-infiltrating CD4+ $MOG_{35–55}$-specific CD4+ T cells have an IL-17−/IFN-γ+ (Th1) phenotype, whereas the IL-17+/IFN-γ− $Th_{IL\text{-}17}$ subset only accounts for approximately 1% of the CNS-infiltrating CD4+ T cells. Although previous studies using the C57BL/6-$MOG_{35–55}$ and SJL-$PLP_{139–151}$ EAE models have suggested that IL-17 and IFN-γ production is mutually exclusive in CD4+ T cells derived from spleen and DLN, in both studies cells were restimulated with peptide for 18 and 24 h with addition of Golgi block and PMA/ionomycin for the last 4–5 h of the culture (Harrington et al., 2005; Langrish et al., 2005). In contrast, in this study an *ex vivo* 6 hour peptide stimulation of CNS-derived CD4+ T cells, in the presence of Golgi block, revealed that a significant proportion of $MOG_{35–55}$-specific CD4+ T cells produced both IL-17 and IFN-γ. The identification of this large population of IL-17+/IFN-γ+ CD4+ population raises important issues in relation to the existing Th1/$Th_{IL\text{-}17}$ classification. In using a short period of T cell stimulation our work shares some similarities with the seminal paper of Kelso and Gough which reported the identification of both Th1 and Th2 cytokines in alloreactive T cell clones, thereby challenging the traditional Th1/Th2 dichotomy that had also been established by work on long-term T cell clones (Kelso and Gough, 1988). While the study of Kelso and Gough stimulated T cells on average for 22 days after cloning, CNS-derived T cells obtained in this study were stimulated immediately following separation. Therefore, in order to determine whether the IL-17+/IFN-γ+ phenotype is stable over time cytokine profiles were examined after 30 h of $MOG_{35–55}$ peptide stimulation. The results obtained demonstrated a dramatic decline in the frequency of IL-17+/IFN-γ+ T cells after a further 24 h of *in vitro* culture.

Presently it cannot be discounted that survival of this CNS-derived $MOG_{35–55}$-specific IL-17+/IFN-γ+ T cell population depends upon CNS factors that are absent in culture. However, two observations indirectly support the notion that this IL-17+/IFN-γ+ subset is a short-lived pre-Th1 intermediate population which transiently expresses IL-17. Firstly, the results from the 6 hour *ex vivo* peptide stimulation at different points in the disease course demonstrate that at the time of disease onset the majority of $MOG_{35–55}$-specific CD4+ T cells are IL-17+/IFN-γ+, then as disease progresses the IL-17−/IFN-γ+ becomes the major subset and continues to increase in number after IL-17+/IFN-γ+ cell numbers stabilize. Secondly, the disappearance of IL-17+/ IFN-γ+ cells that occurs following *in vitro* culture for 30 h is accompanied by an increase in numbers of IL-17−/IFN-γ+ cells in the absence of cell division/proliferation. While the existence of such a "pre-Th1" subset that can differentiate into either a Th1 or $Th_{IL\text{-}17}$ phenotype according to the local immune environment was recently proposed by Bettelli and Kuchroo (2005) it is unclear from our data whether the CNS-derived IL-17+/IFN-γ+ subset is capable of differentiating into a $Th_{IL\text{-}17}$ phenotype. However, this IL-17+/IFN-γ+ population outnumbered IL-17+/IFN-γ− cells by approximately 3:1 from the onset of disease to the development of maximum disability suggesting that the IL-17+/IFN-γ+ subset is the major source of IL-17 as the EAE disease course develops.

In conclusion, the results obtained in this study indicate that priming of a T cell response results in a subset of cells that are capable of producing both IL-17 and IFN-γ and further differentiation of activated T cells can potentially occur within the CNS. Additional studies are now required to determine whether activated T cells entering the CNS are irreversibly committed to a particular phenotype and what factors influence intracerebral T cell differentiation, cytokine production and survival. This is of importance since unlike naïve T cells, mature pathogenic $Th_{IL\text{-}17}$ effector cells are not susceptible to the inhibitory effects of IFN-γ or IL-4 and expansion of this differentiated population following *in vitro* incubation of cultures with IL-23 is due to selective outgrowth (Harrington et al., 2005). This latter observation is consistent with Kelso's hypothesis that ultimately T cell polarization within a particular environment is achieved at the population level by altering frequencies of expression among cells that have acquired different cytokine expression patterns (Kelso, 1999). While recognition of the $Th_{IL\text{-}17}/T_{reg}$ axis has added a new dimension to the understanding of CNS inflammation, this study establishes that the Th1 lineage is the major population within the CNS CD4+ infiltrate in EAE. Furthermore, several observations suggest that cells of the Th1 lineage can contribute to CNS injury, notably that IFN-γ exacerbates multiple sclerosis (Panitch et al., 1987a,b) and differentiated IL-17−/IFN-γ+ Th1 cell lines can also be mildly encephalitogenic in adoptive transfer EAE models. The pathogenicity of any cell subset or cytokine ultimately depends on the presence of other synergistic or inhibitory factors and it cannot be discounted that IFN-γ has pathological effects when acting synergistically with TNF-α, IL-6 and IL-1 after the production of these cytokines has been induced by the transient expression of IL-17 (Chabaud et al., 1998; Dal Canto et al., 1999; Jovanovic et al., 1998; Okuda et al., 1998; Samoilova et al., 1998; Sutton et al., 2006) during intracerebral Th1 differentiation. Therefore, definitive conclusions regarding Th1 effector function should not necessarily be directly inferred from adoptive

transfer studies that use cell lines/clones which have been differentiated *in vitro* and Th1 differentiation/effector function during the course of CNS inflammation should remain an important area of investigation.

Acknowledgements

This study was funded by grants from St Vincent's Clinic Foundation, Pfizer Neurosciences Australia and MS Research Australia. The authors are grateful to Dr Cecile King for critically reading the manuscript.

References

Aggarwal, S., Ghilardi, N., Xie, M.H., de Sauvage, F.J., Gurney, A.L., 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J. Biol. Chem. 278, 1910–1914.

Becher, B., Durell, B.G., Noelle, R.J., 2002. Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. J. Clin. Invest. 110, 493–497.

Bettelli, E., Kuchroo, V.K., 2005. IL-12- and IL-23-induced T helper cell subsets: birds of the same feather flock together. J. Exp. Med. 201, 169–171.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L., Kuchroo, V.K., 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441, 235–238.

Chabaud, M., Fossiez, F., Taupin, J.L., Miossec, P., 1998. Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. J. Immunol. 161, 409–414.

Chu, C.Q., Wittmer, S., Dalton, D.K., 2000. Failure to suppress the expansion of the activated CD4 T cell population in interferon gamma-deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. J. Exp. Med. 192, 123–128.

Cua, D.J., Sherlock, J., Chen, Y., Murphy, C.A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., Zurawski, S., Wiekowski, M., Lira, S.A., Gorman, D., Kastelein, R.A., Sedgwick, J.D., 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 421, 744–748.

Dal Canto, R.A., Shaw, M.K., Nolan, G.P., Steinman, L., Fathman, C.G., 1999. Local delivery of TNF by retrovirus-transduced T lymphocytes exacerbates experimental autoimmune encephalomyelitis. Clin. Immunol. 90, 10–14.

Ferber, I.A., Brocke, S., Taylor-Edwards, C., Ridgway, W., Dinisco, C., Steinman, L., Dalton, D., Fathman, C.G., 1996. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). J. Immunol. 156, 5–7.

Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M., Weaver, C.T., 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat. Immunol. 6, 1123–1132.

Hauben, E., Butovsky, O., Nevo, U., Yoles, E., Moalem, G., Agranov, E., Mor, F., Leibowitz-Amit, R., Pevsner, E., Akselrod, S., Neeman, M., Cohen, I.R., Schwartz, M., 2000. Passive or active immunization with myelin basic protein promotes recovery from spinal cord contusion. J. Neurosci. 20, 6421–6430.

Ishizu, T., Osoegawa, M., Mei, F.J., Kikuchi, H., Tanaka, M., Takakura, Y., Minohara, M., Murai, H., Mihara, F., Taniwaki, T., Kira, J., 2005. Intrathecal activation of the IL-17/IL-8 axis in opticospinal multiple sclerosis. Brain 128, 988–1002.

Jovanovic, D.V., Di Battista, J.A., Martel-Pelletier, J., Jolicoeur, F.C., He, Y., Zhang, M., Mineau, F., Pelletier, J.P., 1998. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. J. Immunol. 160, 3513–3521.

Kelso, A., 1999. Educating T cells: early events in the differentiation and commitment of cytokine-producing CD4+ and CD8+ T cells. Springer Semin. Immunopathol. 21, 231–248.

Kelso, A., Gough, N.M., 1988. Coexpression of granulocyte-macrophage colony stimulating factor, γ interferon, and interleukins 3 and 4 is random in murine alloreactive T-lymphocyte clones. Proc. Natl. Acad. Sci. U. S. A. 85, 9189–9193.

Komiyama, Y., Nakae, S., Matsuki, T., Nambu, A., Ishigame, H., Kakuta, S., Sudo, K., Iwakura, Y., 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J. Immunol. 177, 566–573.

Langrish, C.L., Chen, Y., Blumenschein, W.M., Mattson, J., Basham, B., Sedgwick, J.D., McClanahan, T., Kastelein, R.A., Cua, D.J., 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J. Exp. Med. 201, 233–240.

Lock, C., Hermans, G., Pedotti, R., Brendolan, A., Schadt, E., Garren, H., Langer-Gould, A., Strober, S., Cannella, B., Allard, J., Klonowski, P., Austin, A., Lad, N., Kaminski, N., Galli, S.J., Oksenberg, J.R., Raine, C.S., Heller, R., Steinman, L., 2002. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. Nat. Med. 8, 500–508.

Mangan, P.R., Harrington, L.E., O'Quinn, D.B., Helms, W.S., Bullard, D.C., Elson, C.O., Hatton, R.D., Wahl, S.M., Schoeb, T.R., Weaver, C.T., 2006. Transforming growth factor-beta induces development of the T(H) 17 lineage. Nature 441, 231–234.

Moalem, G., Leibowitz-Amit, R., Yoles, E., Mor, F., Cohen, I.R., Schwartz, M., 1999. Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. Nat. Med. 5, 49–55.

Okuda, Y., Sakoda, S., Bernard, C.C., Fujimura, H., Saeki, Y., Kishimoto, T., Yanagihara, T., 1998. IL-6-deficient mice are resistant to the induction of experimental autoimmune encephalomyelitis provoked by myelin oligodendrocyte glycoprotein. Int. Immunol. 10, 703–708.

Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M., Gorman, D., Wagner, J., Zurawski, S., Liu, Y., Abrams, J.S., Moore, K.W., Rennick, D., de Waal-Malefyt, R., Hannum, C., Bazan, J.F., Kastelein, R.A., 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 13, 715–725.

Panitch, H.S., Hirsch, R.L., Haley, A.S., Johnson, K.P., 1987a. Exacerbations of multiple sclerosis in patients treated with gamma interferon. Lancet 1, 893–895.

Panitch, H.S., Hirsch, R.L., Schindler, J., Johnson, K.P., 1987b. Treatment of multiple sclerosis with gamma interferon: exacerbations associated with activation of the immune system. Neurology 37, 1097–1102.

Park, H., Li, Z., Yang, X.O., Chang, S.H., Nurieva, R., Wang, Y.H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., Dong, C., 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat. Immunol. 6, 1133–1141.

Samoilova, E.B., Horton, J.L., Hilliard, B., Liu, T.S., Chen, Y., 1998. IL-6-deficient mice are resistant to experimental autoimmune encephalomyelitis: roles of IL-6 in the activation and differentiation of autoreactive T cells. J. Immunol. 161, 6480–6486.

Sutton, C., Brereton, C., Keogh, B., Mills, K.H., Lavelle, E.C., 2006. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. J. Exp. Med. 203, 1685–1691.

Veldhoen, M., Hocking, R.J., Atkins, C.J., Locksley, R.M., Stockinger, B., 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 24, 179–189.

Wensky, A.K., Furtado, G.C., Marcondes, M.C., Chen, S., Manfra, D., Lira, S.A., Zagzag, D., Lafaille, J.J., 2005. IFN-gamma determines distinct clinical outcomes in autoimmune encephalomyelitis. J. Immunol. 174, 1416–1423.

Willenborg, D.O., Fordham, S., Bernard, C.C., Cowden, W.B., Ramshaw, I.A., 1996. IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. J. Immunol. 157, 3223–3227.