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ORIGINAL ARTICLE

Dynamic interplay of T helpercell subsets in experimental autoimmune encephalomyelitis

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Abstract

AIM: To investigate the temporal onset and dynamic interplay of $CD4^+$ T helper cell subsets in experimental autoimmune encephalomyelitis (EAE).

METHODS: EAE was induced in C57BL/6 mice by immunization with myelin oligodendrocyte glycoprotein peptide p35-55. The clinical signs were scored and the tissue samples and immune cells isolated for analysis at different phases of EAE. The expression levels of inflammatory cytokines and related transcription factors were detected by quantitative reverse transcription polymerase chain reaction (PCR) and enzyme linked immunosorbant assay (ELISA). The percentages of Th1, Th17, Th2, Treg and memory T cell subsets in EAE were analyzed by immunostaining and flow cytometry. The data were analyzed by statistical techniques.

RESULTS: Quantitative real-time PCR analysis showed that EAE mice express elevated levels of *Th1* [interferon gamma (*IFNγ*), interleukin (*IL*)-*12p40*], *Th17* [*IL-17*, related orphan receptor gamma (*RORγ*), IL-12p40] and *Treg* [*Foxp3*, Epstein-Barr virus induced gene 3 (*EBI3*), *IL-10*] genes in the central nervous system at the peak of the disease. Whereas, the expression of *Th1* (*IFNγ*, *T-bet, IL-12p35, IL-12p40*), *Th17* (*RORγ, IL-12p40*), *Th2* (*IL-4*) and *Treg* (*Foxp3, EBI3*) response genes was reduced in the spleen during pre-disease but gradually recovered at the later phases of EAE. ELISA and flow cytometry analyses showed an increase in Th17 response in the periphery, while Th1 response remained unchanged at the peak of disease. The mRNA levels of IFNγ, IL-17 and IL-12p40 in the brain were increased by 23 ($P < 0.001$), 9 ($P < 0.05$) and 14 ($P < 0.01$) fold, respectively, on day 21 of EAE. Conversely, the mRNA expression of IL-10 was increased by 2 fold ($P < 0.05$) in the spleen on day 21. $CD4^+CD25^+Foxp3^+$Treg response was reduced at pre-disease but recovered to naïve levels by disease onset. The percentage of $CD25^+Foxp3^+$ regulatory T cells decreased from 7.7% in the naïve to 3.2% ($P < 0.05$) on day 7 of EAE, which then increased to 8.4% by day 28. Moreover, the $CD4^+CD127^+CD44^{high}$ memory T cell response was increased during the onset and recovery phases of EAE. The memory and effector cells showed an inverse relationship in EAE, where the memory T cells increased from 12.3% in naïve to 20% by day 21, and the effector cells decreased from 32% in naïve to 21% ($P < 0.01$) by day 21. The wild type C57BL/6 mice with EAE showed elevated levels of effector-memory T cells (T_{EM}) with concomitant reduction in central-memory T cells (T_{CM}), but the EAE-resistant IL-7R deficient mice showed elevated T_{CM} with no effect on T_{EM} cells in EAE.

CONCLUSION: Our findings highlight the temporal onset and dynamic interplay of effector, memory and regulatory $CD4^+$ T cell subsets and its significance to clinical outcome in EAE and other autoimmune diseases.

Key words: Autoimmune disease; Experimental autoimmune encephalomyelitis; Multiple sclerosis; T helper cells; Th1/Th17

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INTRODUCTION

The immune system has evolved to discriminate self from non-self, thereby protecting the host frominfection and malignancy. Nevertheless, a breakdown of this system often results in the pathogenesis of many infectious, cancerous and autoimmune diseases. While the nonspecific innate immunity presents the first line of defense, antigen-specific adaptive immunity is required for successful clearance of pathogens and cancer. As innate immunity relies on epithelial barriers to prevent the entry of pathogens into the body, macrophages and neutrophils proactively phagocytose and kill any invading pathogens if this barrier is compromised. The cytokines and chemokines secreted by these phagocyteshelp attract other immune cells towards the site of infection. The innate immunity also influences the subsequent development of adaptive immunity initiated through the interaction of antigen-specific naïve $CD4^+$ T cells with the professional antigen-presenting cells (APCs), co-stimulatory molecules and cytokines. This results in clonal expansion and differentiationof effector, memory and regulatory-cells with distinct cytokine profiles and biological functions in health and diseases.

The $CD4^+$Th1 effector cells eliminate intracellular pathogens and cancer but also contribute to the pathogenesis of experimental autoimmune encephalomyelitis (EAE). Earlier studies have shown that interleukin (IL)-12p40, T-bet and Stat4 knockout mice became resistant to EAE, while IL-12p35 and interferon gamma (IFNγ) knockout mice develop exacerbated disease, suggesting undefined roles for Th1 cells in autoimmune diseases[1-6]. Recent studies have also demonstrated a role for IL-17-producing Th17 cells in the pathogenesis of autoimmune diseases. While the activation of naïve T cells in the presence of IL-6 and transforming growth factor (TGF) induces the differentiation of Th17 cells in culture, *in vivo* studies have shown that IL-12p40 or IL-23p19 and related orphan receptor gamma (RORγ) knockoutmice became deficient in Th17 response and resistant to EAE[3,7-10]. Additionally, IL-17 knockout mice showed attenuated EAE demonstrating the pivotal role played by Th17 cells in the pathogenesis of autoimmune diseases[11,12]. In view of the reciprocal regulation of Th17 and Th1 differentiation in IFNγ- and IL-17-deficient mice, respectively, and the essential role played by T-bet and RORγ for the encephalitogenicity of Th1/Th17 cells, it is conceivable that the $CD4^+$Th1 and Th17 effector cells play distinct but complementary roles in the pathogenesis of autoimmune diseases[11,13].

Moreover, the anti-inflammatory Th2 and T regulatory cells (Tregs) play important roles in modulating autoimmune diseases. The activation of naïve T cells in the presence of IL-4 results in the differentiation of IL-4-producing Th2 cells thatassociates withallergic responses, infection with nematode parasitesandresistance to autoimmune diseases. Additionally, $CD4^+CD25^-$ $Foxp3^+$ natural Tregs (nTreg) and $CD4^+CD25^+Foxp3^+$ inducible Tregs (iTreg) develop following activation of naïve T cells in the presence of TGF-β, and function as potent regulators of autoimmune diseases. After the successful clearance of pathogenic antigens, most of these effector and regulatory T cells die rapidly by apoptosis to restore immune homeostasis. However, some antigen-experienced cells remain as long-lived memory cells that are capable of mounting a rapid recall response to specific antigen. These resting $CD4^+$ memory T cells depend on signals from IL-7 and IL-15, but not major histocompatibility complex class Ⅱ, for their survival and intermittent homeostatic proliferation[14]. The number and activity of these T cell subsets are tightly controlled to avoid reactivity to self antigens and excessive reactions to non-self antigens.

In fact, dysregulated effector, memory and regulatory T cells drive the pathogenesis of autoimmune diseasesincluding rheumatoid arthritis, thyroiditis, systemic lupus erythematosus, type 1 diabetes and multiple sclerosis (MS). Many animal models have been established to study the complex mechanisms in the pathogenesis of autoimmune diseases. EAE is an autoimmune disease of the central nervous system that serves as an animal model for MS. EAE can be induced in susceptible rodents and primates by immunization with myelin antigensor antigenic peptides[11]. Earlier studies have shown that neural antigen-specific $CD4^+$ Th1 and Th17 cells function as effector cells, while Th2 and Treg cells confer resistance and recovery in EAE. However, the regulation of different T cell subsets and their distinct functional significance to clinical outcomein EAE is not well defined. In this study we analyzed the profile of effector, memory and regulatory $CD4^+$ T cellsubsets during the course of EAE. We show a decrease in Treg cells during early diseasefollowed by an increase in memory and Th17 effector cells at the onset and peak of EAE. These findings suggest the temporal onset of effector, memory and regulatory $CD4^+$ T cells in EAE. Understanding the dynamic interplay between these distinct T cell subsetsmay help design novel therapiesfor MS and other autoimmune diseases.

MATERIALS AND METHODS

Animals

The C57BL/6 mice and IL-7Rα$^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in the animal care facility at Methodist Research Institute. Six to eight week old male mice were used in the experiments. Animal protocols were approved by the Methodist Research Institute Animal Care and Use Committee.

Reagents

The 21 amino acid peptide [MEVGWYRSPFSRVVH-LYRNGK] corresponding to mouse MOGp35-55 (96.81% pure) was obtained from Genemed Synthesis Inc. (San Francisco, CA, United States). Recombinant mouse IFN-γ, IL-17, IL-4, IL-10, IL-12 and IL-23 were purchased from R and D Systems (Minneapolis, MN, United States). CD25-APC Cy7, anti-CD16/32, isotype control conjugated to APC Cy7, BD Cytofix/Cytoperm, and anti-IL-17 capture and detection antibodies were obtained from BD Pharmingen (Franklin Lakes, NJ, United States). The anti-IFNγ capture and detection antibodies were obtained from Endogen (Rockford, IL, United States). The capture and detection antibodies specific to IL-4, IL-10 and IL-23p19, and the fluorochrome-conjugated antibodies specific to CD4, CD25, Foxp3, IL-17 and IFN-γ, isotype controls and Foxp3 Staining Buffer were obtained from e-Biosciences (San Diego, CA, United States). Anti-mouse Abs specific to IL-12/IL-23p40 and RORγ were purchased from Santa Cruz (Santa Cruz, CA, United States) and the anti-rabbit DyLight 633 secondary antibody was from Thermo Scientific (Rockford, IL, United States). Anti-mouse IL-12/IL-23p40-biotin and anti-mouse IL-12p35-biotin antibodies were obtained from Biolegend (San Diego, CA, United States) and MabTech (Mariemont, OH, United States), respectively.

Induction and evaluation of experimental allergic encephalomyelitis

To induce EAE, six to eight week old C57BL/6 wild-type or IL-7Rα$^{-/-}$male mice were immunized with 100 μg MOGp35-55 peptide Ag in 150 mL allemulsion of Complete Freund's Adjuvant (Sigma Chemicals, St Louis, MO, United States) in the lower dorsum on days 0 and 7. The mice also received (i.p.) 100 ng of pertussis toxin (Sigma Chemicals, St. Louis, MO, United States) on days 0 and 2. The clinical symptoms were scored on the day of sacrifice as follows: 0, normal; 0.5, stiff tail; 1, limp tail; 1.5, limp tail with inability to right; 2, paralysis of one limb; 2.5 paralysis of one limb and weakness of one other limb; 3, complete paralysis of both hind limbs; 4 moribund; 5, death.

Immunostaining and flow cytometry

Spleen cells (2 × 10^6/sample) from wild-type male mice induced to develop EAE wereincubated with anti-CD16/32 antibody to block Fc receptors (0.5 μg/million cells). Cells were then subjected to surface staining, followed by fixation and permeabilization with BD Cytofix/Cytopermor eBioscience Foxp3 staining buffer, and subsequent staining for intracellular cytokines or transcription factors. Cell surface proteins were detected using anti-CD4-PE, anti-CD8-PE, anti-CD25-APCCy7, anti-CD44-FITC, anti-CD62L-APC or anti-CD127-PE Cy5. Intracellular proteins were detected with anti-Foxp3-FITC, anti-IFNγ-FITC, anti-IL-17-FITC or anti-RORγ followed by anti-rabbit DyLight 633. Samples stained for the intracellular cytokines IL-17 and IFNγ were restimulated with 1 μg/mLionomycin and 50 nmol/L phorbol 12-myristate 13-acetate (PMA) for 1 h at 37 ℃ followed by BD GolgiStop (containing monensin) for an additional 5 h. Spectral overlap was eliminated by appropriate compensation based on single stained samples andgates were determined based on isotype controls.The cells were analyzed by flow cytometry using a BD FACSCalibur™ or a BD LSRII™ flow cytometer (Becton Dickinson, San Jose, CA, United States) and analyzed with FlowJo software (Ashland, OR, United States).

Quantitative real-time polymerase chain reaction

The quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using the ABI Prism 7900 fast sequence detection system (Applied Biosystems) according to the manufacturer's instructions. Spleen and brain samples were isolated, flash frozen and stored at -80 ℃ until use. Total RNA was extracted from each sample (RNeasy Mini kit, Qiagen, Valencia, CA, United States) and transcribed into cDNA using random hexamer primers and TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA, United States). The concentration of cDNAwas determined using 18S, and equivalent amounts of cDNA were combined for each time point (2 animals/group) and subjected to qPCR analysis in quadruplicate in fast optical 96-well plates(TaqMan probes, Applied Biosystems). The data were analyzed using the ABI Prism 7900 relative quantification (ΔΔCt) study software (Applied Biosystems). In this study, we used primer sets for 12 selected genes with 18S (Applied Biosystems) as the internal control. The expression levels of inflammatory genes normalized to control are presented as arbitrary fold changes compared with naïve or day 14 as indicated.

Cytokine enzyme-linked immunosorbant assay

Isolated spleen cells were cultured in RPMI medium (1 × 10^6/mL/well) with 0 or 5 μg/mL MOGp35-55 peptide. The culture supernatants were collected after 48 h and frozen at -80 ℃ until use. Equal volumes of culture supernatants from 2 independent samples per group were combined and the presence of secreted cytokines analyzed by enzyme-linked immunosorbent assay (ELISA). Flat-bottomed 96-well plates were coated with capture antibody at 4 ℃ overnight. Plates were washed and subsequently blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Recombinant standards and

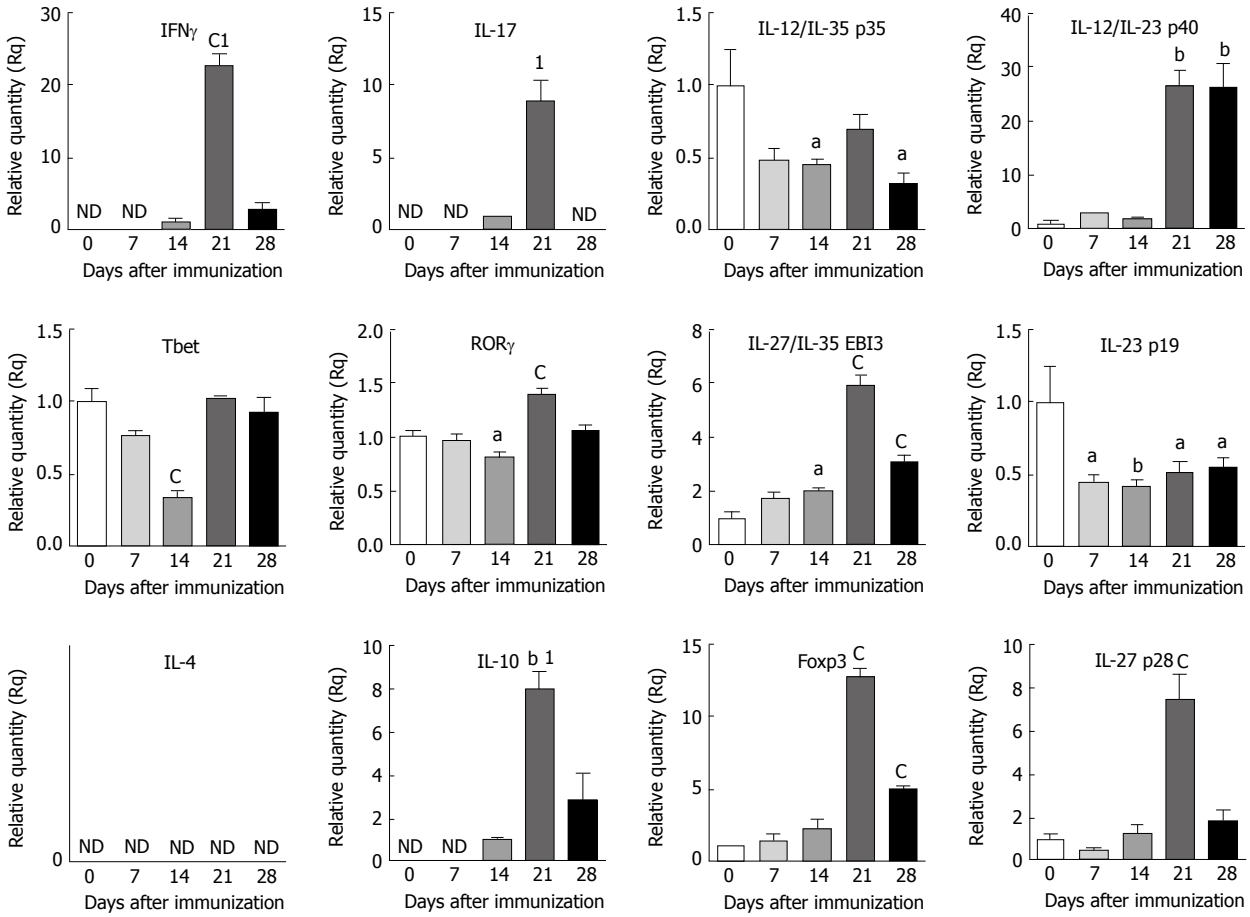

Figure 1 Expression of cytokines and transcription factors in the central nervous system of mice with experimental autoimmune encephalomyelitis. Brain samples were isolated from C57BL/6 mice on different days as indicated following induction of experimental autoimmune encephalomyelitis (EAE). Total RNA was extracted from individual tissue samples and reverse transcribed into cDNA. Equal quantities of cDNA from two mice per group were combined and analyzed by quantitative reverse transcription polymerase chain reaction using 18S as the internal control. The fold changes in the expression of selected genes in the central nervous system of mice with EAE were calculated based on naïve as control. [1]If naïve samples were undetectable, fold changes in the expression of cytokines in EAE mice were calculated based on day 14 samples as control. The data shown are the mean ± SE of quadruplicates and the statistical significance is shown as $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, *vs* naïve or day 14. ND: Not detectable; IFNγ: Interferon gamma; IL: Interleukin; RORγ: Related orphan receptor gamma.

samples were added and incubated at 4 ℃ overnight followed by detection antibody for 1 h at room temperature, Avidin-alkaline phosphatase for 30 m and colorimetric assessment with 1 mg/mL p-nitrophenylphosphate prepared in 1 mol/L diethanolamine. Results were interpolated from a standard curve and expressed as ng/mL. All values reported were greater than the minimum detection threshold (125 pg/mL) for the standard curve. Samples that fell below the minimum detection threshold were reported as not determined. IL-23 was detected using purified p19 and biotinylated p40 antibodies and IL-12 was detected using purified p40 and biotinylated p35 antibodies.

Statistical analysis

To determine statistical significance, unpaired *t* tests or one-way analysis of variance tests were performed using GraphPad Prism v5.0 (GraphPad Software, La Jolla, CA, United States). Post-hoc analyses were performed using Dunnett's correction. Bar graph values are mean ± SE.

RESULTS

Effector, memory and regulatory gene expressionprofile in the central nervous system of mice with EAE

To determine the dynamics of effector, memory and regulatory T cell subsetsin autoimmune diseases, we first examined the expression of associated cytokines and transcription factors in the central nervous system (CNS) of mice with EAE. Brain samples were collected from C57BL/6 mice on day 0 (naïve), 7 (pre-disease), 14 (onset), 21 (peak) and 28 (remission) following induction of EAE, and the gene expression was analyzed by qRT-PCR. As shown in Figure 1, we found no detectable expression of IFNγ or IL-17 in naïve mice or on day 7 following induction of EAE. Interestingly, by day 14 detectable levels of both IFNγ and IL-17 were expressedin the CNS whichincreased 22.8 and 8.9fold, respectively, by day 21 (peak of disease). However, by day 28 the levels of both IFNγ and IL-17 decreased significantly (Figure 1). Moreover, the mRNA levels of Th1 and Th17 transcription factors T-bet and RORγ decreased

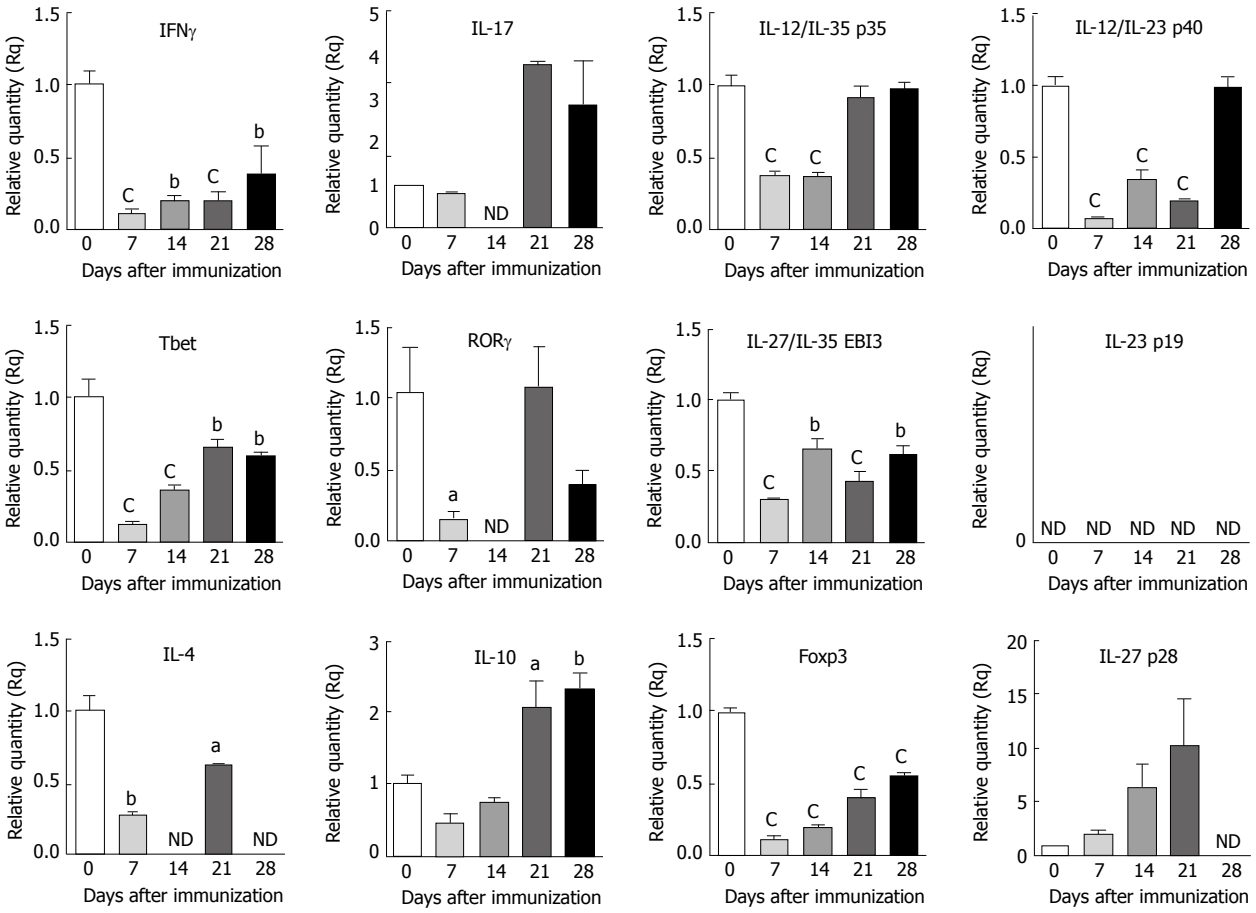

Figure 2 Expression of cytokines and transcription factors in the lymphoid organ of mice with experimental autoimmune encephalomyelitis. Spleen samples were isolated from C57BL/6 mice on different days as indicated following induction of experimental autoimmune encephalomyelitis (EAE). Total RNA was extracted from individual tissue samples and cDNA was reverse transcribed. Equal quantities of cDNA from two mice per group were combined and analyzed by quantitative reverse transcription polymerase chain reaction using 18S as the internal control. The fold changes in the expression of selected genes in the spleen of mice with EAE were calculated based on naïve as control. The data shown are the mean ± SE of quadruplicates and the statistical significance is shown as $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. ND: Not detectable.

3.3 and 1.2 fold, respectively on day 14 (disease onset) as compared to naïve controls. While T-bet expression reversed to naïve levels by day 21 and 28, the expression of RORγ was significantly increased by day 21 and then reversed to naïve levels by day 28. Additionally, the IL-12 family cytokine subunits IL-12p40, IL-27p28 and EBI3 showed 14, 5.7 and 3.0 fold increase, respectively, on day 21 following induction of EAE as compared to naïve mice. Similarly, the levels of IL-10 and Foxp3 transcripts were also elevated 7.9 and 5.5fold, respectively, on day 21 compared to naïve mice. However, by day 28 the mice were recovering from the clinical disease and many of the gene transcripts examined in the CNS had returned to naïve levels. These results demonstrate that the gene expression profiles in the CNS correlate with the clinical outcome of EAE.

Effector, memory and regulatory gene expressionprofile in the lymphoid organs of mice with EAE

To further determine the dynamics of effector, memory and regulatory T cells in autoimmune diseases, we examined the mRNA levels of cytokines and transcription factors in lymphoid organs by qRT-PCR. We found thatthe mRNA expression profileof many cytokines in the spleen was different from that in the brain (Figure 2). While the expression levels of mRNA for effector (*IFN, T-bet, IL-12p35, IL-12p40*) and regulatory (*EBI3, IL-4, IL-10 and Foxp3*) genes were decreased by day 7 following immunization, they recovered steadily throughout the course of EAE. Furthermore, the expression of IL-17 and IL-10 showed 3.8 and 2.1fold increase, respectively, during the peak of disease compared to naïve mice, which remained high through day 28. Of the genes examined, the expression of IL-17 in the CNS and periphery most closely correlates with the clinical signs of EAE (Figure 3A).

Cytokine profile of neural antigen-specific immune cells

To further determine the functional significance of effector, memory and regulatory T cells in EAE, we then measured the secretion of IFNγ and IL-17 from spleen cells by ELISA during the course of EAE. We found that spleen cells stimulated *ex vivo* with 5 μg/mL of MOGp35-55 secreted higher levels of IFNγ protein on day 7 that further increased by day 14 compared to naïve

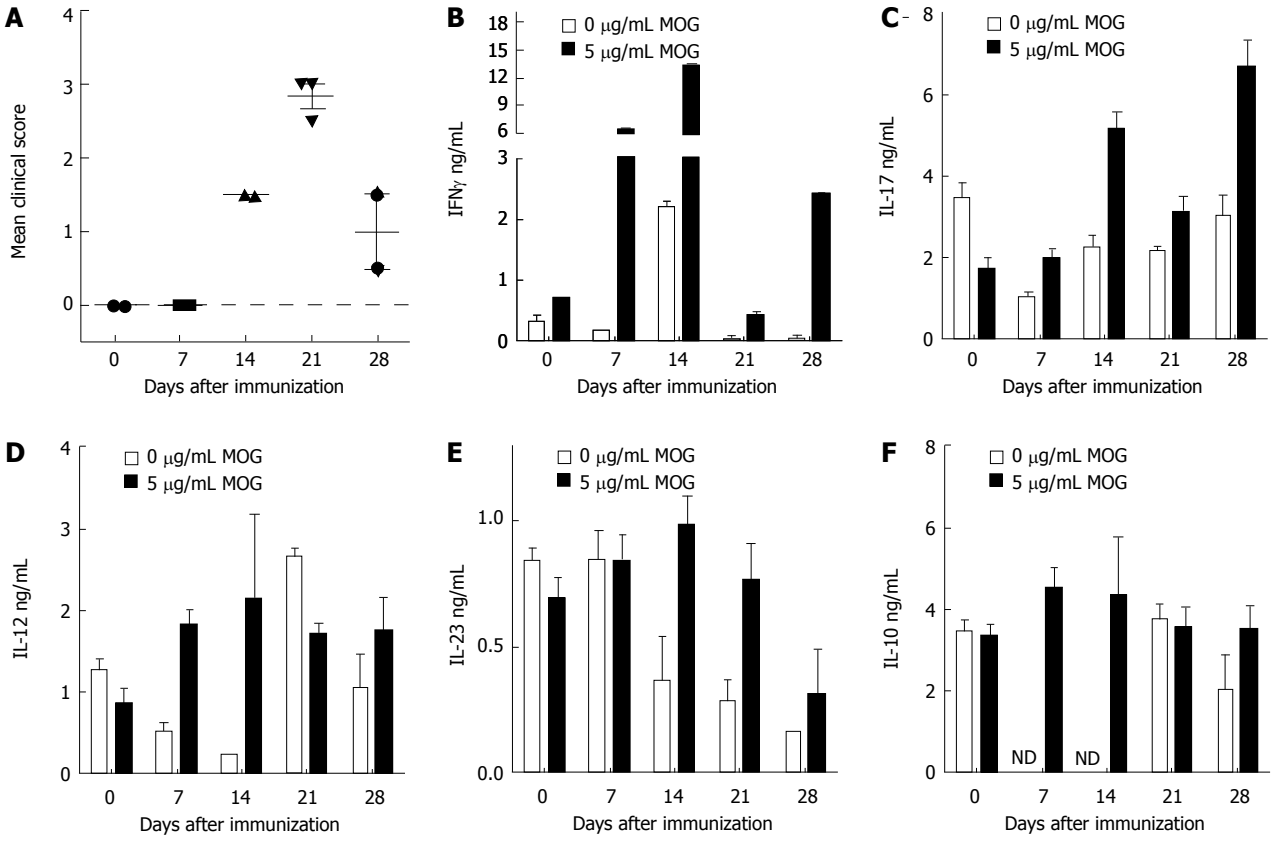

Figure 3 Secretion of cytokines from neural antigen sensitized spleen cells in culture. C57BL/6 male mice were induced to develop experimental autoimmune encephalomyelitis (EAE) by immunization with MOGp35-55. A: The clinical signs were scored on the day of sacrifice and presented as the mean ± SE for each group. The data represent 2 separate experiments; B-F: Spleen cells were isolated on different days following induction of EAE and cultured in the presence of 0 or 5 μg/mL of MOGp35-55 for 48 h. Cytokine secretion was analyzed in the pooled culture supernatants from two mice per group by enzyme linked immunosorbant assay. The data shown are the mean ± SE of triplicates.

mice, indicating the expansion of neural antigen-specific Th1 cells at the onset and peak of the disease (Figure 3B). Similarly, MOGp35-55-induced secretion of IL-17 was elevatedon days 14 and 28 indicating the presence of neural antigen-specific Th17 cells during the peak and recovery phases of the disease (Figure 3C). We also examined the IL-12 family cytokines IL-12 and IL-23 and anti-inflammatory cytokines IL-4 and IL-10 by ELISA. We found that the secretion of IL-12and IL-10 from spleen cells in response to neural antigen *ex vivo* was elevated on day 7 and secretion of IL-12, IL-10 and IL-23 was elevated on day 14 compared to naïve controls, suggesting their association with the onset and peak of disease (Figure 3D-F). The secretion of IL-4 was undetectable in spleen cells from EAE mice following *ex vivo* stimulation with neural antigens at all the time points tested (data not shown). The analysis of mean clinical scores at different phases of EAE show the normal pattern of disease onset by day 14 with a peak disease by day 21 that declined by day 28 (Figure 3A). We have used this clinical profile to correlate with the effector, memory and regulatory T cell profiles at different phases of EAE.

Effector T cell subsets in EAE

To determine whether the differential expression of cytokines and transcription factors in EAE was associated with changes in the proportion of effector T cell subsets we performed flow cytometry analysis of spleen cells. We found that the PMA/ionomycin-activated spleen cells from EAE mice *ex vivo* showed no significant difference in the percentage of IFN^+ Th1 cells throughout the course of the disease (Figure 4A). In contrast, the EAE mice showed a 2.3 fold increase in the percentage of $IL\text{-}17^+$ Th17 cells on day 21 when compared to naïve mice (Figure 4B) which returned to naïve levels by day 28 uponclinical recovery. To determine whether these changes were due to alterations in the proportion of T cell subtypes, we analyzed the expression of T cell activation markers in EAE by flow cytometry. Interestingly, the mice induced to develop EAE displayed a 58%, 64% and 87% reduction in $CD4^+$, $CD8^+$ and $CD127^+$ T cells, respectively, on day 14 as compared to naïve mice (Figure 4E-G). However the percentage of $CD4^+$, $CD8^+$ and $CD127^+$ T cells gradually increased through the peak and recovery phases of EAE which led to partial restoration of naïve levels on day 28 by clinical recovery (Figure 4E-G). Further analysis showed that 75%-90% of naïve spleen cells expressed the Th17 transcription factor RORγ which was not significantly altered during any clinical phase of EAE (Figure 4H).

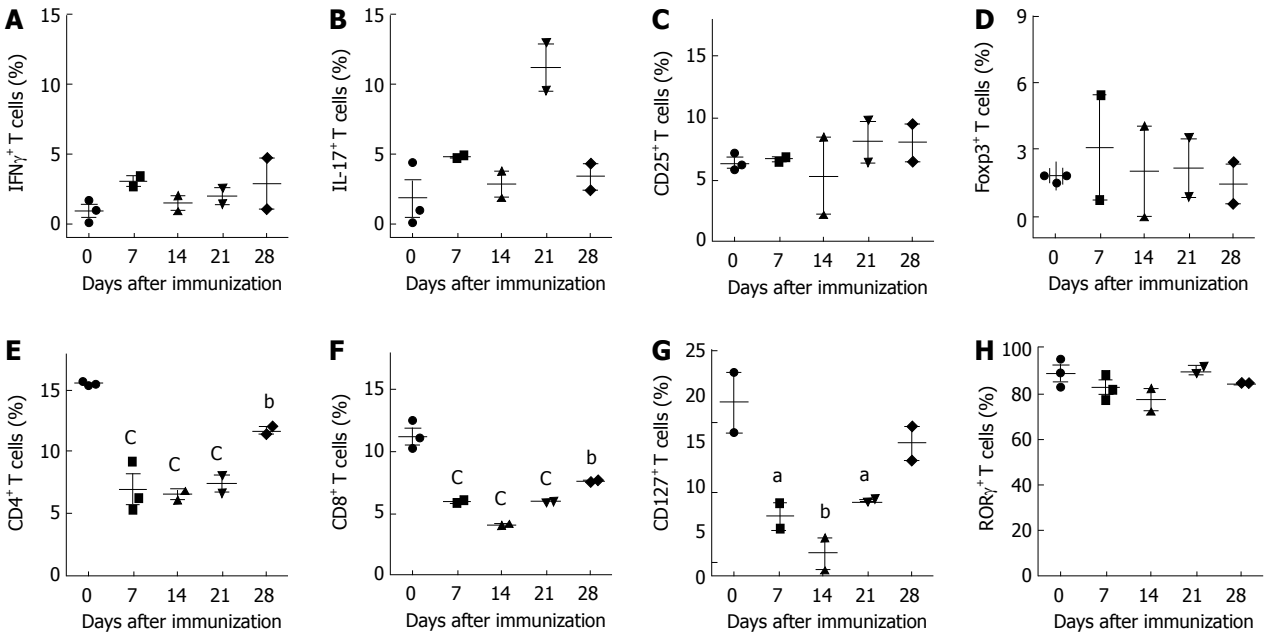

Figure 4 Analysis of T cell activation markers in mice with experimental autoimmune encephalomyelitis. Spleen cells were isolated from C57BL/6 mice on different days following induction of experimental autoimmune encephalomyelitis. A-B: Cells were cultured with phorbol 12-myristate 13-acetate and ionomycin for 6 h. GolgiStop (monensin) was added for the last 5 h of culture, and intracellular interleukin (IL)-17 and interferon gamma (IFNγ) were analyzed by flow cytometry; C: Fresh spleen cells were analyzed for expression of CD25; D: Foxp3; E: CD4; F: CD8; G: CD127; H: Related orphan receptor gamma (RORγ) by flow cytometry. The graphs show positive cells for selected single stain at each time point and represent the mean ± SE from at least two independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

Regulatory T cell subsets in EAE

To determine whether the development of EAE was associated with changes in the proportion of regulatory $CD4^+$ T cell subsets we performed flow cytometry analyses of spleen cells. Interestingly, we found that the percentage of spleen cells expressing the Treg markers $CD25^+$ and $Foxp3^+$ were not significantly affected during thecourse of EAE as compared to naïve mice (Figure 4C and D). To further examine the modulation of Tregsin EAE, we analyzed $CD4^+CD25^+Foxp3^+$cells in fresh spleen. Interestingly, we found that the mice induced to develop EAE showed 45% decrease in the percentage of $CD4^+$ cells that were $CD25^+Foxp3^+$Tregs on day 7 compared to naïve, which gradually recovered through the onset and peak and returned to naïve levels by day 28 upon clinical recovery (Figure 5). These results show that the upregulation of IL-17^+Th17 effector cells and downregulation of $CD4^+CD25^+Foxp3^+$Treg cells associates with the pathogenesis of EAE.

Memory T cell subsets in EAE

To further explore the regulation of T cell subsets in autoimmune disease, we analyzed the changes in the memory T cell profile in EAE. We found that more than 90% of spleen T cells were $CD44^+$throughout the course of EAE (Figure 6F). Interestingly, the naïve mice showed 4.5% spleen cells expressing high levels of CD44 ($CD44^{high}$) that increased to 39.4%, 50.3% and 50.6% on days 7, 14 and 21, respectively, and then decreased to 22.3% by day 28 upon clinical recovery (Figure 6A-E). The percentage of $CD44^{high}$ population was inversely proportionate to $CD44^{low}$cells during the course of EAE. To further explore our results we analyzed the changes in $CD4^+CD44^+CD127^+$ memory T cells in EAE. We found that the percentage of $CD127^+$ cells that were $CD4^+CD44^{high}$memory T cells increased from 12.3% in naïve mice to 20.0% on day 14 during the disease onset (1.6 fold increase) or to 21.0% on day 28 during recovery (1.7 fold increase) (Figure 7G). Conversely, the percentage of $CD127^+$ cells that were $CD4^+CD44^{low}$effector T cells decreased from 32% in naïve mice to 22% on day 7 during pre-disease (1.5 fold decrease, $P < 0.05$), to 21% on day 14 during the disease onset (1.5 fold decrease, $P < 0.01$) or to 19.5% on day 28 during recovery (1.6 fold decrease, $P < 0.01$) (Figure 7F).

Further analysis of central (T_{CM}) and effector (T_{EM}) memory T cell subsets revealed that the percentage of $CD4^+$cells that were $CD44^{high}CD62L^+$ T_{CM} cells decreased slightly from 14% in wild-type naïve animals (Figure 8A) to 12% at disease onset (Figure 8C), whereas the percentage of $CD4^+$ cells that were $CD44^{high}CD62L^-$ T_{EM} cells increased from 19% in wild-type naïve animals (Figure 8A) to 35% at disease onset (Figure 8C). The expression level of CD62L is influenced by signaling through the IL-7Rα chain asnaïve IL-$7R\alpha^{-/-}$ mice have extremely low levels of $CD44^{low}CD62L^+$ effector cells (8%, Figure 8B) compared to wild-type naïve mice (54%, Figure 8A). However, in contrast to wild-type mice, IL-$7R\alpha^{-/-}$mice have high levels of $CD4^+CD44^{high}CD62L^-$ T_{EM} cells (72%, Figure 8B) which do not change following MOG_{p35-55} immunization (72%, Figure 8D). These results suggest that the changes in memory T cells associate with the clinical profile of autoimmune diseases.

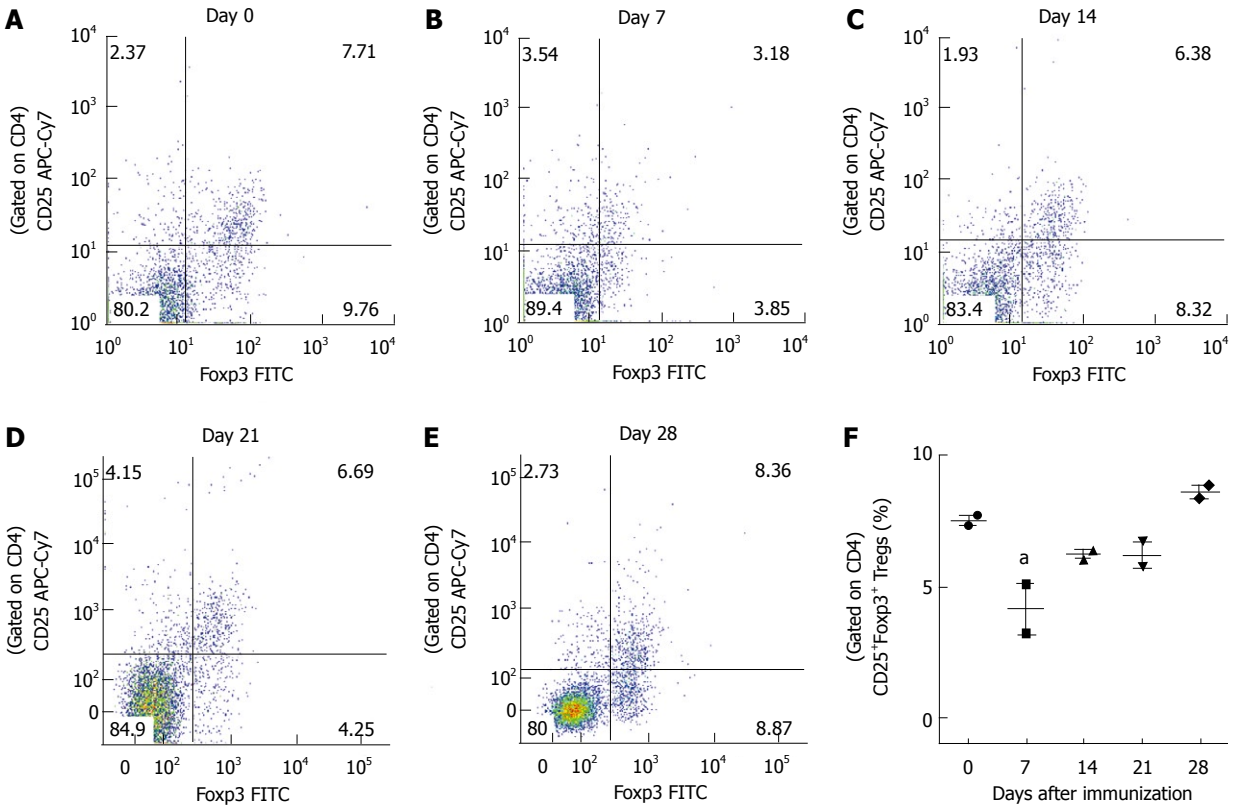

Figure 5 Analysis of regulatory T cells in mice with experimental autoimmune encephalomyelitis. Spleen cells were isolated from C57BL/6 mice on different days following induction of experimental autoimmune encephalomyelitis. Fresh spleen cells were analyzed for surface antigens CD4 and CD25 and intracellular Foxp3 by flow cytometry. A-E: Representative dot plots show CD25 and Foxp3 expression in $CD4^+$ T cells at different time points; F: The percentages of $CD4^+CD25^+Foxp3^+$Tregs are shown for each time point and the graph represents the mean ± SE from two independent experiments. The statistical significance is shown as $^aP < 0.05$.

DISCUSSION

EAE is a T cell-mediated autoimmune disease model of MS which has been commonly used to study the mechanism of MS pathogenesis and to test the efficacy of potential therapeutic agents for the treatment of MS[6,15-17]. The pathogenesis of EAE involves activation of immune cells, differentiation of neural Ag-induced Th1 and Th17 cells and secretion of myelinotoxic inflammatory cytokines in the CNS. The involvement of memory and regulatory $CD4^+$T cells has been demonstrated in EAE and other autoimmune diseases[18-22]. In this study we examinedthe differential regulation of effector, memory, and regulatory $CD4^+$ T cells inthe EAE model of MS. We have demonstrated the temporal onset of pro- and anti-inflammatory cytokines and associated transcription factorsduring different clinical phases of EAE. We used RT-PCR analysis to follow the expression of nine cytokines or cytokine subunits and three transcription factors in the CNS and lymphoid tissue throughout the course of EAE. We found thatthe naïve mice did not express mRNA for IFNgIL-17, IL-4 or IL-10 in the brain that is consistent with a previous report[23]. However, well-defined gene expression patterns were observed during the pre-clinical, onset, peak and recovery phases of EAE. The development of EAE was characterized by a reduction in gene expression for Th1 and Th17 transcripts in the spleen at pre-disease with a significant increase at the peak followed by a rapid decline during recovery phase. The pattern of increased Foxp3 expression in the brain during the peak of disease followed by a rapid decrease in the recovery phase was also described in the spinal cord of Lewis rats with EAE[24]. Analysis of brain lesions from MS patients showed elevated levels of mRNA for IFNgand IL-17, while elevated IL-17 and IL-10 were detected in PBMCs from MS patients[25-30]. Although we did not identify specific cell types expressing inflammatory genes in the brain, our findings indicate the differential regulation of pro- and anti-inflammatory pathways at different phases of EAE.

Further analysis revealed that the pattern of mRNA expression in the peripheral lymphoid organs was different from the brain in EAE. Analysis of spleen tissue revealed that during pre-disease the expression of nearly all the transcripts examined were downregulated, except IL-17, IL-27p28, and IL-23p19. At the recovery phase, some of these downregulated genes recovered to or exceeded naïve levels, while others gradually returning to naïve levels but never fully recovered. Interestingly, mRNA levels of IL-4, IFNγ, T-bet, EBI3 and Foxp3 remained suppressed even during clinical recovery. This gene expression profile is different from the one reported in MBP-immunized SJL/J mice where pro-and anti-inflammatory cytokines, including IFNγ, increased

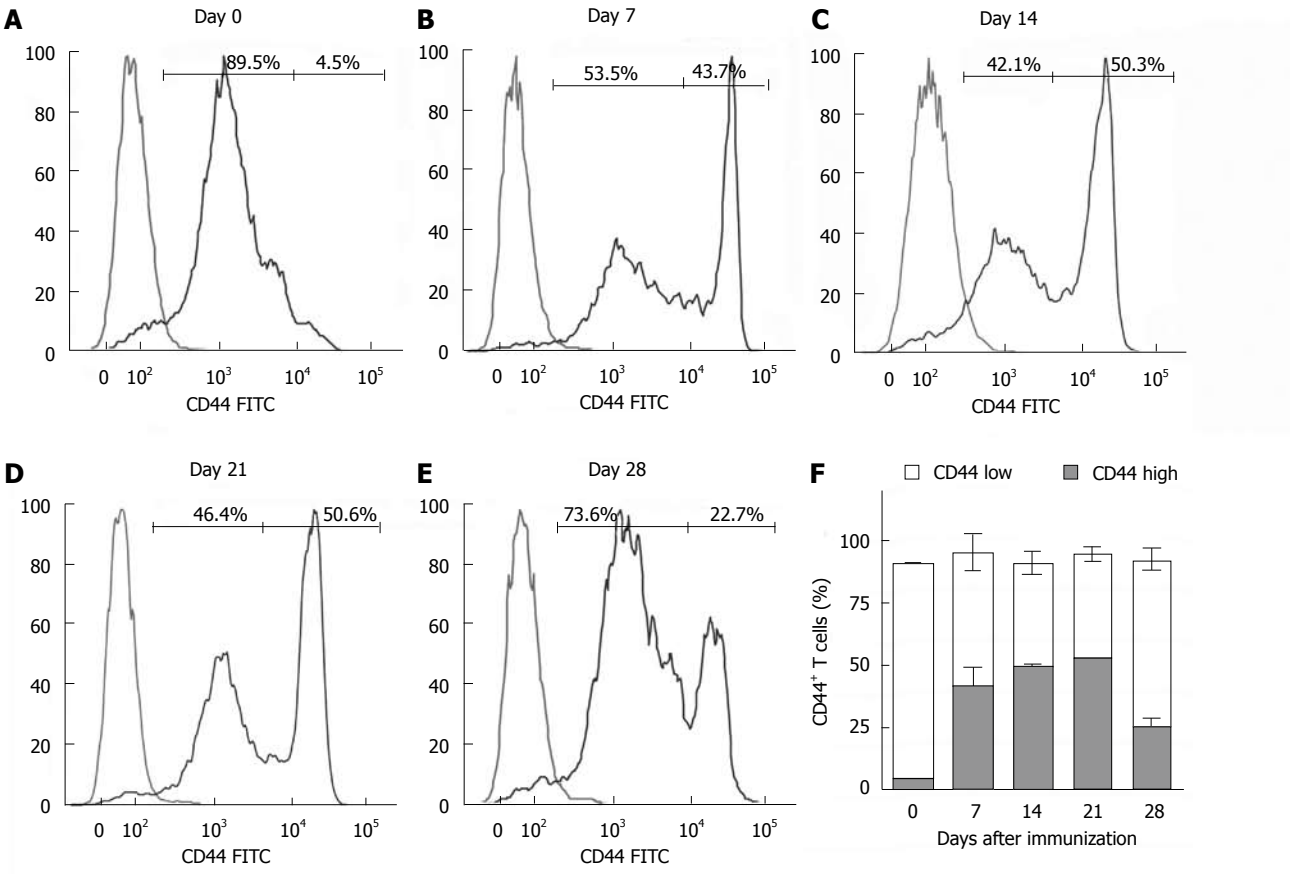

Figure 6 Analysis of memory T cells in mice with experimental autoimmune encephalomyelitis. Spleen cells were isolated from C57BL/6 mice on different days following induction of experimental autoimmune encephalomyelitis. Fresh spleen cells were analyzed for the surface antigen CD44 by flow cytometry. A-E: Representative histograms of $CD44^{low}$ and $CD44^{high}$ expression for each time point are shown; F: The percentage of total $CD44^{+}$ cells for each time point is shown as the combination of $CD44^{low}$ (white bar) and $CD44^{low}$ (gray bar). The graph represents the mean ± SE from two independent experiments.

during early disease and then returned to normal levels during remission[31]. Although IFNγ has been considered responsible for proinflammatory responses in the CNS, the development of exacerbated EAE in $IFN\gamma^{-/-}$ and $IFN\gamma R^{-/-}$ mice suggests its indirect role to suppress the disease[32,33]. Therefore, the downregulation of IFNγ, T-bet, IL-12p35 and IL-12p40 mRNA observed in the lymphoid organs may be necessary for the onset of EAE. Furthermore, the reduction in Foxp3, EBI3 and IL-12p35 mRNA and $CD4^{+}CD25^{+}Foxp3^{+}$ cells in the spleen following induction of EAE and the increase in Foxp3, EBI3 and IL-12p35 mRNA in the brain at the peak of disease suggests that Tregs generated in the lymphoid organs during preclinical and clinicalstages migrate to the CNS to induce disease remission.This concept has been controversial as treatment with *ex-vivo*-generated Tregs failed to control EAE despite accumulating in the CNS while others have shown amelioration of EAE by adoptive transfer of Tregcells[34,35]. Interestingly, Tregsaccumulated in the CNS of $IL\text{-}10^{-/-}$ mice failed to suppress EAE, suggesting the requirement of IL-10 for the disease ameliorating effects of Tregs[35]. This supports our findings on the concurrent increase in IL-10 and Foxp3 expression in the brain during the peak of disease. Future studies of CNS-infiltrating effector, memory and regulatory T cells isolated from EAE mice will further define their distinct roles in target organs.

Memory cells are long-lived antigen-experienced T cells that upon re-exposure can quickly mount a stronger anamnestic response than the first time the antigen was presented. The persistence of human autoimmune disease is believed to be mediated by memory T cells. Memory cells appear to derive fromTh1 effector, Treg and other T cell subsets[21,22,36]. Memory cells can also acquire additional functional capabilities during the secondary response. Recently, a unique study examined homeostatic and autoimmune conditions in mice and found that Tregs that had lost Foxp3 expression acquired an activated-memory phenotype and produced inflammatory cytokines[22]. Furthermore, T cell activation of human $CD4^{+}CD45RO^{+}$ T_{EM} cells resulted in IL-17 secretion and Foxp3 expression that was dependent on the cytokine milieu[37]. Interestingly, the link between memory cells and Tregs is not restricted to autoimmune disease models. A recent study has demonstrated that the parallel increases of $CD4^{+}Foxp3^{+}$Tregs and $CD4^{+}CD44^{+}$ memory T cells was associated with aging and provided evidence that $Foxp3^{+}$ cells originate as $CD44^{+}$ cells. Furthermore, activated $CD4^{+}CD25^{+}Foxp3^{+}$ T cells from healthy individuals also showed an increase

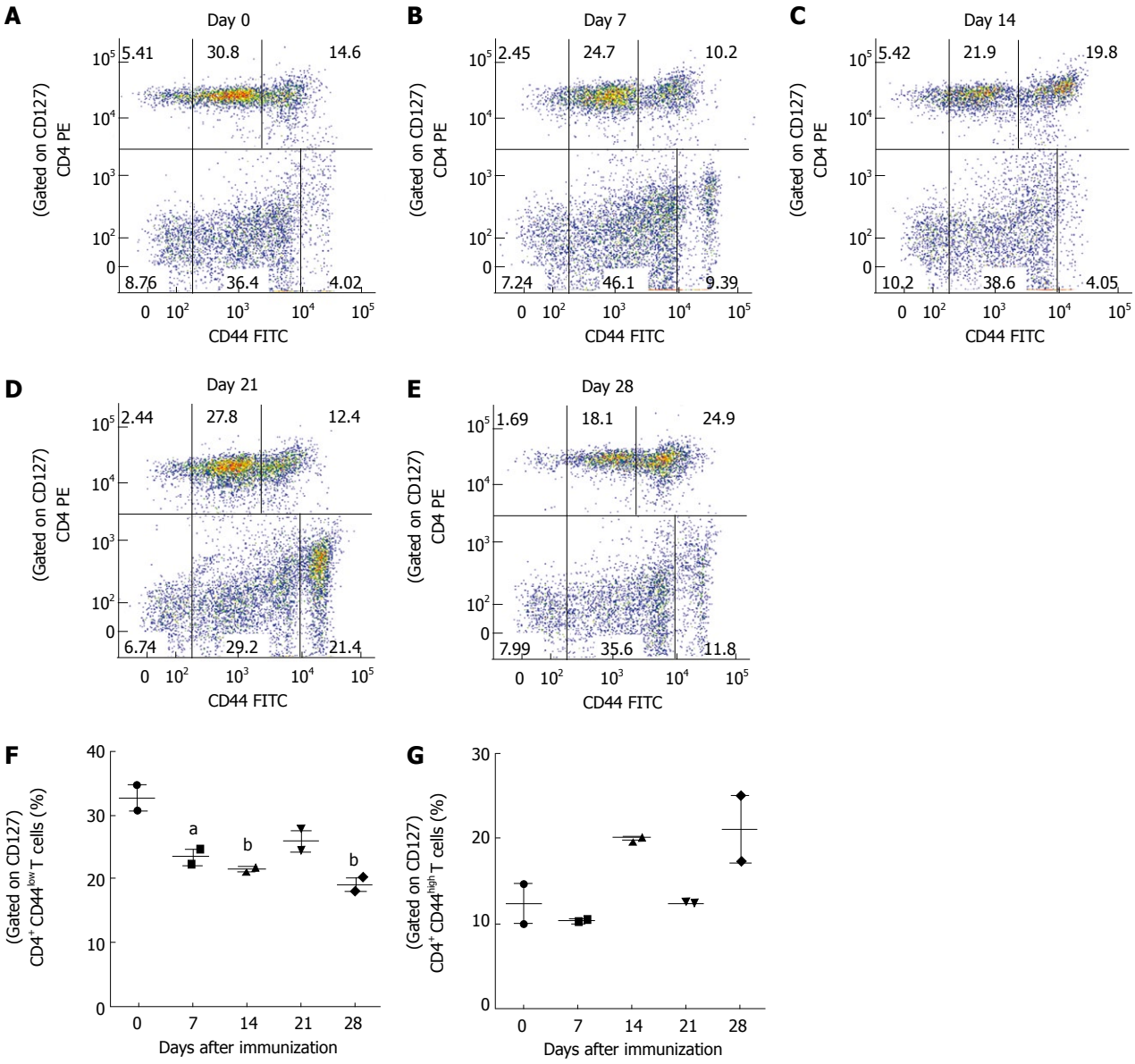

Figure 7 Analysis of effector and memory T cells in mice with experimental autoimmune encephalomyelitis. Spleen cells were isolated from C57BL/6 mice on different days following induction of experimental autoimmune encephalomyelitis. Fresh spleen cells were analyzed for surface antigens CD4, CD44 and CD127 by flow cytometry. A-E: Representative dot plots show CD44 and CD4 expression in $CD127^+$ T cells at different time points; F: The percentages of $CD4^+CD44^{low}CD127^+$ effector cells or G, $CD4^+CD44^{high}CD127^+$ memory T cells are shown for each time point. The graphs represent the mean ± SE from two independent experiments and the statistical significance is shown as $^aP < 0.05$, $^bP < 0.01$ *vs* naïve.

in the expression of CD44 and CD62L, indicating an effector/memory phenotype[38]. Therefore, the transient decrease in Tregs during preclinical disease, followed by increased memory cells at disease onset suggests the possible conversion of Tregs to memory cells in the autoimmune environment in EAE. Further studies using Foxp3 genetic lineage tracing would be necessary to confirm this finding[22].

$CD4^+$ memory T cells express specific cell surface markers and distinct immune responses that distinguish them from naïve or effector T cells. CD127 (IL-7Rα) expression is highest on resting naïve and memory T cells and in combination with CD4, CD44 and CD62L is a useful marker to identify memory T cells[36,39-41]. Two subsets of memory T cells called central-memory (TCM) and effector-memory (TEM) can be determined by their organ residence, cell surface marker expression and effector functions[36]. TCM cells express CD62L and CCR7 and reside in lymph nodes, whereas TEM cells lack these markers and reside in non-lymphoid tissues[20]. However, both subsets can be found in the blood and spleen. Interestingly, we found that $CD4^+CD44^{high}CD127^+$ memory T cells peaked during disease onset and again during late/remitting disease. Further analysis revealed that at disease onset, TCMcells decrease while TEM increase in wild-type mice. Male mice that are deficient in IL-7Rα/CD127 ($IL\text{-}7R\alpha^{-/-}$) are completely resistant to developing EAE (unpublished data) and surprisingly have very low levels of $CD4^+CD44^{low}$ effector cells and high levels of TEM cells. Unlike wild-type mice, the levels

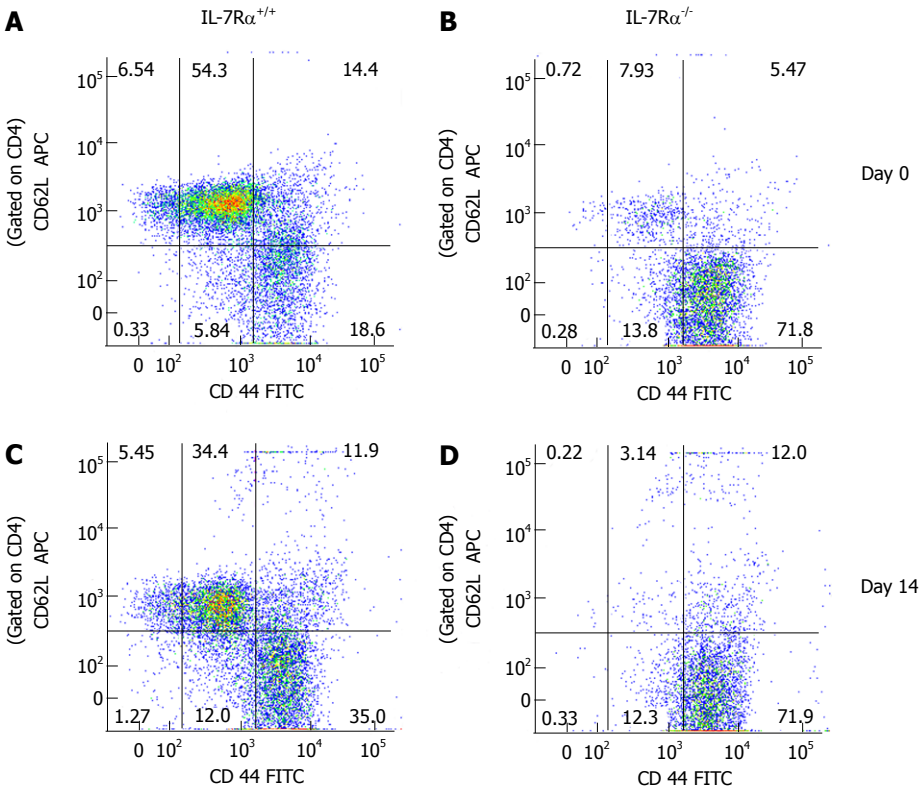

Figure 8 Analysis of central and effector memory T cells in mice induced to develop experimental autoimmune encephalomyelitis. Spleen cells were isolated from C57BL/6 wild type and interleukin (IL)-7R$^{-/-}$ naïve mice or on day 14 following immunization with MOGp35-55. Fresh spleen cells were analyzed for surface antigens CD4, CD44 and CD62L by flow cytometry. Representative dot plots for CD62L and CD44 expression in CD4^{+} spleen cells from wild-type or IL-7R$^{-/-}$ mice are shown. The figure is representative of two independent experiments.

of TEM cells did not change as a result of immunization. This suggests that signaling through the IL-7R may be important for the transition of naïve/effector cells to memory cells, a step that may be required for the pathogenesis of EAE. A limitation of this study was that wild-type memory cells in Figure 8 could not be further distinguished by CD127 expression as in Figure 7 so that a direct comparison could be made with the CD127/IL-7Rα$^{-/-}$ mice.

A characteristic response of T cells to TCR activation is the rapid, and often transient, down-regulation of CD127 (IL-7Rα) in animal models of infection[14,39,42]. Our data indicated that during the course of EAE, the transient down-regulation of CD127 was mirrored by a transient down-regulation of CD4 and CD8, although the magnitude of CD127 down-regulation was larger (CD127, 8fold decrease; CD4, 2.4fold decrease; CD8, 2.8fold decrease). Since CD127 expression is highest on naïve and memory T cells, the transient expression of CD127 suggests the transition of naïve cells (pre/early disease) to effector cells (middle disease) to memory cells (late disease) (Figure 4G). Although the expression of CD25 and Foxp3 was not significantly altered during the course of EAE, expression levels became more variable between animals despite the animals having the same or similar EAE score within a group (Figure 3A, Figure 4C and D). CD4^{+}CD25^{+}Foxp3^{+}Tregs decreased during preclinical disease, began to recoverby disease onset and gradually surpassed naïve levels by day 28 (Figure 5). This is consistent with previous reports showing decreased CD4^{+}CD25^{+}or CD4^{+}CD25^{+}Foxp3^{+} spleen cells from EAE mice at day 10 or 12, respectively[43,44]. Taken together, our findings highlight that CD4^{+} Th1 and Th17 effector, memory and regulatory T cells are temporally regulated during different phases of EAE with an increase in effector/memory T cells during the onset and peak of the disease, while Treg response associates with recovery and remission in EAE. Understanding the dynamic interplay or conversion of these T cell subsets at different phases of EAE may reveal the pathogenic mechanisms of autoimmune diseases. Novel therapies targeting these regulatory mechanisms may be useful in treating MS and other autoimmune diseases.

COMMENTS

Background

The immune system has evolved to discriminate self from non-self, thereby protecting the host from infection and malignancy. Nevertheless, a breakdown of this system often results in the pathogenesis of infection, cancer and autoimmune diseases. Multiple sclerosis is a neurological disorder induced by myelin reactive CD4^{+} T cells that affects more than a million people in the United States and other parts of the world. Experimental autoimmune encephalomyelitis (EAE) is a CD4^{+} Th1/Th17 cell-mediated autoimmune disease model of multiple sclerosis (MS). Earlier studies have shown that the activation, differentiation and expansion of myelin antigen-specific effector, memory and regulatory T cells with distinct cytokine profiles and biological functions are critical events associated with the pathogenesis of EAE/MS.

Research frontiers

Neural antigen-specific CD4$^+$ Th1 and Th17 cells function as effectors, while Th2 and Treg cells confer resistance and recovery in EAE. However, the regulation of different T cell subsets and their distinct functional significance to clinical outcome in EAE is not well defined.

Innovations and breakthroughs

The authors used innovative approaches to analyze the differential regulation of T helper cell subsets in the EAE model of MS. Quantitative real-time polymerase chain reaction analysis showed that EAE mice express elevated levels of *Th1* [interferon gamma (*IFNγ*), interleukin (*IL*)-*12p40*], *Th17* [*IL-17*, related orphan receptor gamma (*RORγ*), *IL-12p40*] and *Treg* (*Foxp3*, Epstein-Barr virus induced gene 3 (*EBI3*), *IL-10*) genes in the central nervous system at the peak of the disease. Whereas, the expression of *Th1* (*IFNγ*, *T-bet*, *IL-12p35*, *IL-12p40*), *Th17* (*RORγ*, *IL-12p40*), *Th2* (*IL-4*) and *Treg* (*Foxp3*, *EBI3*) response genes was reduced in the spleen during pre-disease but gradually recovered at the later phases of EAE. Enzyme linked immunosorbant assay and flow cytometry analyses showed an increase in Th17 response in the periphery, while Th1 response remained unchanged at the peak of disease. CD4$^+$CD25$^+$Foxp3$^+$Treg response was reduced at pre-disease but recovered to naïve levels by disease onset. Moreover, the CD4$^+$CD127$^+$CD44^{high} memory T cell response was increased during the onset and recovery phases of EAE. The wild type C57BL/6 mice with EAE showed elevated levels of effector-memory T cells (T_{EM}) with concomitant reduction in central-memory T cells (T_{CM}), but the EAE-resistant IL-7R deficient mice showed an elevated T_{CM} with no effect on T_{EM} cells in EAE.

Applications

The study results suggest the temporal onset and dynamic interplay of effector, memory and regulatory CD4$^+$ T cell subsets and its significance to clinical outcome in EAE and other autoimmune diseases.

Peer review

This is an important study.

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