Published in final edited form as:

J Neuroimmunol. 2011 January ; 230(1-2): 26–32. doi:10.1016/j.jneuroim.2010.08.007.

T CELLS THAT TRIGGER ACUTE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS ALSO MEDIATE SUBSEQUENT DISEASE RELAPSES AND PREDOMINANTLY PRODUCE IL-17

Jinzhua Li, Xiaoqing Zhao, Hui-Wen Hao, Michael K. Shaw, and Harley Y. Tse

Department of Immunology and Microbiology, Wayne State University School of Medicine,
Detroit, MI 48201, U.S.A.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is one of the most extensively studied autoimmune diseases and serves as an animal model for the human demyelinating disease, multiple sclerosis (MS). The disease is caused by autoimmune inflammation in the central nervous system (CNS) and can be induced in animals by immunization with myelin antigens or by adoptive transfer of myelin antigen-primed donor T cells into naive recipients (Traugott et al., 1985; Mokhtarian et al. 1984). SJL is a highly susceptible mouse strain that is often used as a prototype of this disease model. After developing an acute phase of the disease, certain mouse strains spontaneously undergo remission. Different mouse strains may recover to different extent. In this laboratory, clinical recovery in SJL mice is often complete such that these mice appear physically normal during remission (Kim and Tse, 1993). However, these mice are also prone to spontaneous disease relapses and the remitting/relapsing cycles become a recurring feature of the disease. Over the years, while major efforts have been spent elucidating the induction, the antigen specificity and the regulation of the initial phase of acute EAE, relatively little is known about the factors that trigger the subsequent relapsing episodes or the cytokine milieu at each stage of the disease course. In this respect, the nature and antigen specificity of the T cells mediating the recurring attacks are still open questions (McRae et al., 1995; Takacs et al., 1997; Jone et al., 2003; Kroenke and Segal, 2007). Some studies suggest that the relapsing cycles are triggered by newly primed T cells with specificities for secondary myelin antigens released from the damaged myelin sheath during the previous disease episode (Tuohy et al., 1999; Vanderlugt et al., 2000; Yu et al., 1996). This conclusion is supported by demonstration of the sequential appearance, during disease relapses, of dominant T cell populations with antigen specificities different from the priming epitopes such that each relapse cycle is associated with a new encephalitogenic epitope (Vanderlugt et al., 2000; Yu et al., 1996). On the other hand, more recent studies found that mice could still develop relapsing disease even under circumstances that determinant spread had not occurred (Jone et al., 2003). In addition, it was reported that cytokine responses during disease relapses were directed

© 2010 Elsevier B.V. All rights reserved.

Corresponding Author: Dr. Harley Y. Tse, Telephone: (313)577-1564, Fax: (313)577-1155, htse@wayne.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

predominantly against the priming epitope and not the secondary spread epitopes (Kroenke and Segal, 2007). This debate about the basic mechanism by which diseases relapses are triggered has important consequential implications for designs of therapeutic remedies. If the secondary spread determinants are indeed the triggers, therapy would have to chase a moving target throughout the relapsing cycles (Steinman, 1999; Vanderlugt and Miller, 2002). A corollary of this tenet is that T cells specific for the priming epitope associated with the initiation of the acute disease are lost along the way and they play no role in subsequent relapsing attacks. This is a testable concept and this report, using Thy-1 congenic mouse strains, aims at assessing this contention.

In previous studies using Thy-1 allelic markers to track the long-term trafficking of encephalitogenic T cells in the development of relapsing EAE, Skundric et al. (Skundric et al., 1993; Skundric et al., 1994) reported that donor T cells in an adoptive transfer system remained in the CNS of the recipients over several relapsing cycles. Although the mere presence of these primary antigen-primed donor T cells in the CNS does not *a priori* infer their involvement in disease relapses, their persistence in the CNS during the course of the relapsing disease warrants an in-depth study of their possible role as an alternative source of encephalitogenic T cells responsible for disease relapses. To this end, these donor cells were depleted with anti-Thy-1 antibodies and the effects of this depletion on subsequent development of the relapsing disease in the animals were observed. In addition, the cytokine profiles of the donor T cells in the hosts during the various stages of disease development were assessed. It was observed that spinal cord-infiltrating T cells shifted from a broad cytokine profile during the acute disease to one restricted to mainly IL-17 production during the relapsing phases. These results should impact on consideration of therapeutic approaches for MS.

2. Materials and Methods

2.1 Animals

SJL/J mice were purchased from the Frederick NCI (Frederick, MD) and the Jackson Laboratory (Bar Harbor, ME). SJL-Thy-1^a mice were supplied by the Jackson Laboratory on contract. Female donor mice were used between 10 to 12 weeks of age and recipient mice were of 6 weeks of age. All experimental procedures involving live animals had received prior approval from IACUC.

2.2 Antigen Peptides

Myelin antigen peptides were synthesized by Genemed Synthesis (South San Francisco, CA). The sequences for the peptides are: PLP_{139–151}: HSLGKWLGHDPKF; PLP_{178–191}: NTWTTCQSIAPFSK; MBP_{89–101}: VHFFKNIVTPRTP.

2.3 Induction of adoptive EAE

PLP_{139–151} at 100 µg/mouse was emulsified with an equal volume of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI). 0.05 ml was injected at four sites in the flanks draining the inguinal and axillary lymph nodes. For adoptive transfer, lymph node cells from donor mice 10 to 14 days after immunization were placed in cultures at 4×10^6 /ml in RPMI culture media (Shaw et al., 1996) supplemented with 10% fetal bovine serum and 100 µg/ml PLP peptides. Cells were cultured in 2-ml volumes in 24-well plates for 4 days. Cells were harvested, washed and 5×10^7 viable cells in 0.2 ml were transferred into recipient mice through the tail veins. The mice were monitored daily and graded for clinical signs of disease: 0 = no abnormality; 1 = loss of tail tone; 2 = weakness of one hind limb; 3 = total paralysis of both hind limbs; 4 = total paralysis of all four limbs; 5 = premoribund state.

2.4 Isolation of CNS-infiltrating mononuclear cells

Mice were perfused with saline and spinal cords were removed. A single cell suspension was prepared and passed through nylon mesh to remove clumps. The cells were washed twice, resuspended in 70% percoll and loaded to the bottom of a 25-ml tube. This was overlaid with a 37% and then a 30% Percoll solution at the top. The Percoll gradient was centrifuged at 500g for 20 minutes. Lymphoid cells banded at the 37%/70% interface were collected.

2.5 T cell proliferative responses

2×10^4 FACS sorted Thy-1.2⁺ donor T cells were cultured in 96-well plates with 5×10^5 irradiated spleen cells and various antigens. 1 μ Ci of ³H thymidine was added 16 hours before the termination of the 4-day culture. Cells were harvested in an automatic cell harvester and incorporation of isotopes was determined in a scintillation counter.

2.6 Detection of cytokines

Sorted Thy-1.2⁺ CNS-infiltrating donor T cells and Thy-1.1⁺ host cells were cultured in 96-well round-bottom plates (5×10^4 cells/well for Thy-1.2⁺ and 2×10^5 cells/well for Thy-1.1⁺ cells) with 2×10^5 irradiated spleen cells and with or without PLP₁₃₉₋₁₅₁ peptides for 24 or 48 h. The supernatants were collected and stored at -80°C until analyzed. Culture supernatants were analyzed using SABiosciences (Federick, MD) Cytokine Multi-Analyte ELISArray kit according to the manufacturer's instructions.

2.7 Depletion of Thy-1.2⁺ Donor Cell in vivo

SJL.Thy-1^a recipient mice were given three doses of anti-Thy1.2 ascites i.p. over a six-day period as described for each experimental design. Anti-Thy-1.2 ascites were prepared previously and were thawed and diluted 1:2 with PBS. One mg in 1 ml was given for each injection.

3. Results

3.1 Proliferative responses of FACS-sorted CNS-infiltrating donor T Cells and host cells to antigens during disease relapses

In previous studies using immunohistochemical techniques and adoptive transfer of MBP-primed SJL donor cells (Thy-1.2⁺) into Thy-1 congenic SJL.Thy-1^a recipients (Thy-1.1⁺) to study the trafficking of encephalitogenic T cells in EAE, Skundric et. al. (Skundric et al., 1993) showed that donor T cells persisted in the CNS of the recipients through many cycles of remitting and relapsing disease. The significance of maintaining these cells in the recipients was not known. Over the years, techniques were developed to recover infiltrating T cells from the CNS of EAE animals and to study their antigen specificity and cytokine secretion. In these experiments, PLP₁₃₉₋₁₅₁-primed and in vitro activated donor SJL lymph node cells (Thy-1.2⁺) were transferred into naïve SJL.Thy-1^a (Thy-1.1⁺) recipients (Kim and Tse, 1993). The recipient mice developed a normal course of acute disease, remission and relapse. At or near the peak of the first relapse (disease grades 2 to 3), groups of mice were sacrificed and perfused with saline. Spinal cords were isolated and teased into single cell suspension. Spinal cord cells were subjected to Percoll gradient centrifugation for lymphocyte enrichment. Purified cells were labeled with anti-Thy-1.2 antibodies and donor T cells (Thy-1.2⁺) were sorted by FACS. Sorted cells were over 95% purity. The cells were cultured with various antigens and proliferative responses by incorporation of tritiated thymidine were measured four days later. As shown in Figure 1A, donor T cells recovered from the CNS of recipients during the first EAE relapse proliferated in response to the priming peptide PLP₁₃₉₋₁₅₁, but not to PLP₁₇₈₋₁₉₁, MPB₈₉₋₁₀₁ or PPD. These results

indicate that the donor T cells that initiated the acute disease in the recipients maintained their antigen specificity throughout the acute and first relapsing phases. This cell population did not develop responses to other neuroantigenic peptides. These conclusions are further supported by a follow-up experiment in which mice were kept through the second relapsing cycle. At or near the peak of disease (disease grades 2 to 3), groups of mice were similarly labeled with anti-Thy-1.2 antibodies and FACS-sorted to isolate Thy-1.2⁺ donor T cells for tests of proliferative responses. The results closely resembled those of the first relapsing disease as shown in Figure 1B, confirming a lack of determinant spread from PLP_{139–151} to other myelin epitopes in this cell population during the relapsing course. In addition, Thy-1.1⁺ host cells at both disease stages were also FACS-sorted. It is important to note that these cells exhibited no proliferative responses to any of the antigens and peptides tested (Fig. 1). Responses to PLP_{139–151}, PLP_{178–191}, MPB_{89–101} and PPD were all at media background level. Taken together, these results support the contention that donor T cells in passive EAE remain in the CNS of the recipients and are immunologically active during subsequent relapsing episodes. Furthermore, there is no evidence that determinant spread has occurred in this T cell population.

3.2 Cytokine profiles of FACS-sorted CNS-infiltrating donor T cells and host cells during disease relapses

Immune and autoimmune responses are often mediated through production of cytokines. In EAE, inflammatory IFN γ , TNF α , IL-2 and IL-17 are found to be the dominant cytokines produced by CD4⁺ T cells from lymph nodes, spleens or spinal cords of mice during acute disease (Begolka et al., 1998; Hofstetter et al., 2005). It is therefore of interest to investigate whether different cytokines are produced during the acute and relapsing phases of EAE. Initially, cytokine production by donor cells in the in vitro cultures before adoptive transfer was analyzed by intracellular staining. We found that by day two in culture, the frequencies of cells producing IFN γ and IL-17 were 5.96% and 7.94%, respectively. These probably represent separate populations of cells producing these cytokines. In the present study, FACS-sorted CNS-infiltrating PLP_{139–151}-primed donor T cells through the various stages of adoptive EAE as described in section 3.1 were also assessed for their cytokine profiles using a multiplex cytokine kit with capability to measure 12 cytokines in a single assay. Sorted Thy-1.2⁺ donor T cells and host Thy-1.1⁺ cells from the spinal cords of animals at the peak of the acute, the first relapse and the second relapse were cultured with or without the priming antigen PLP_{139–151} for 24 and 48 hours. Culture supernatants were collected and subjected to Multi-Protein ELISA assays. Representative results from an experiment are shown in Table 1 for the 24-hour cultures. The experiment was repeated twice with similar results. The concentrations of cytokines shown were calculated from a standard curve of known concentrations provided by the manufacturer. During acute EAE, donor T cells in the spinal cords produced a multitude of cytokines, dominant among which were IL-2 and IL-17A. There were also smaller amounts of IL-6, IL-13 and IFN γ produced. As the disease progressed through remission and then to the first relapse, these CNS-seeking donor T cells maintained high levels of IL-17A, but produced reduced amounts of other cytokines. In the second relapse, it was clear that IL-17A production was greatly increased and became the single dominant cytokine with relatively minute amount of IFN γ produced by this population of T cells. It is not clear from these data whether the same or separate T cells produced the two cytokines (Suryani and Sutton, 2007). In addition, when Thy-1.1⁺ host cells from the three phases of EAE were similarly FACS-sorted and analyzed, it was found that these cells produced none of the cytokines tested (Table 1). This total lack of cytokine activity is consistent with the lack of proliferative responses in the host cell population described earlier.

3.3 Effectiveness of anti-Thy-1.2 antibodies in depletion of donor SJL lymph node cells transferred into SJL-Thy-1^a recipients

The demonstration that the acute disease-initiating donor T cells isolated during the disease relapsing phases were antigen-specific and functionally intact does not a priori imply that these cells are involved in the development of the relapsing disease. One approach to answer this question is to deplete these cells and test how their absence affects the subsequent disease relapses. To this end, recipient mice were treated with anti-Thy-1.2 antibodies in vivo at the onset of the recovery phase after the acute disease and after each of the subsequent relapsing episodes. Initial experiments were conducted to assure that the anti-Thy-1.2 antibodies were effective in depleting donor T cells and that such treatment did not interfere with the ability of the animals to subsequently mount an EAE response. In the first of a series of three experiments, donor SJL mice were primed with PLP_{139–151} in CFA. Ten days later, draining lymph node cells were isolated and put into culture with the priming antigen for four days. At the end of the culture period, cells were washed and 5×10^7 viable cells were transferred i.v. into the tail veins of SJL-Thy-1^a recipient mice. At day -3, at the time of and at day +3 after cell transfer, each recipient mouse also received 1 mg of anti-Thy-1.2 ascites intraperitoneally. If the antibodies were effective in depleting the donor cells, it was expected that no acute EAE should develop. Results in Table 2 show that this was indeed the case. Data are pooled from two experiments. Thirteen of 14 untreated mice and 100% of the mice treated with saline or with isotype-matched antibodies exhibited normal development of EAE while none of the anti-Thy-1.2-treated mice developed any clinical signs of disease.

The effectiveness of anti-Thy-1.2 antibodies in depleting donor T cells was explored in a second experiment. In this case, spleens and lymph nodes of SJL-Thy-1^a mice that received SJL donor lymph node cells and treated with anti-Thy-1.2 antibodies as described were isolated from individual mice two days after the last treatment. Control mice were treated with PBS only. Cells were stained with FITC-anti-CD4 and PE-anti-Thy-1.2 antibodies for flow cytometric analysis. The results of spleen cells from three mouse pairs (PBS- or anti-Thy-1.2-treated) are displayed in Figure 2 to document the levels of individual variations. We did not perform staining of CNS lymphocytes. It was noted that the percentages of donor Thy-1.2⁺ T cell in the spleens were lower than those in the lymph nodes and that depletion by the antibodies was more complete in the spleen than in the lymph nodes. In the PBS-treated mice, the percentage of CD4⁺Thy-1.2⁺ T cells in the spleens ranged from 0.55 to 0.71. Anti-Thy-1.2 antibody treatment reduced these percentages to 0.01 to 0.11. In the lymph nodes, the range in PBS-treated mice was 0.74 to 0.94 and in the anti-Thy-1.2-treated mice was 0.02 to 0.27 (data not shown). These results do not by themselves indicate depletion of donor cells as the reduction in donor cell numbers in the flow cytometry charts can be explained as being due to blocking of staining by the anti-Thy-1.2 antibodies. However, together with the results of Table 2 showing loss of donor cell function after antibody treatment, these results confirm the efficacy of the antibodies in depleting the transferred SJL donor cells in the spleen and lymph nodes. Nevertheless, to show that the antibody treatment did not hamper the ability of the recipient mice to mount an EAE response, the asymptomatic mice in Table 2 were kept for two months and then another batch of freshly prepared PLP-primed and in vitro activated SJL lymph nodes cells (5×10^7 viable cells) were transferred into these mice. The bottom half of Table II shows the results of two pooled experiments. All 6 of the control mice which did not receive any new donor cells remained asymptomatic while 6 of 6 mice which received 5×10^7 new donor lymph node cells developed normal levels of acute EAE.

3.4 Effects of depleting donor SJL T cells on subsequent development of relapsing disease in SJL-Thy1^a recipients

To determine the effect of donor cell depletion on subsequent development of relapsing disease, acute EAE was induced in large cohorts of SJL-Thy-1^a recipient mice, often more than 40, by transfer of PLP_{139–151}-primed and in vitro activated donor SJL lymph node cells. These mice were allowed to undergo the full acute disease course and into the recovery phase. It was noted that recovery was always complete with the mice returning to normal state of disease grade 0 (Kim and Tse, 1993). At this stage, most of the mice were fairly well synchronized in the development of acute disease and recovery. Immediately before full recovery from the acute phase disease (1st remission), groups of mice were treated with three doses of anti-Thy-1.2 or isotype-matched antibodies over the next six days. These mice were observed for subsequent development of relapsing EAE (1st remission, Table 3). Disease relapse is defined as upward change of at least one disease grade. Of the remaining untreated mice in the experiments, many proceeded to develop the first relapse. Those mice that followed a similar disease course and had similar disease severity were chosen and, when they began to enter the second remission phase, were treated with anti-Thy-1.2 and isotype-matched antibodies. Three doses of the antibodies were given over the next six days and the mice were kept for observation of post-treatment relapses (2nd remission, Table 3). At this stage of the experiments, many of the remaining untreated mice were less synchronized in their disease course, with some having longer relapse or remission periods than others. This necessitated reduction in the group size for the rest of the experiments. In one experiment, it was possible to identify a small number of animals that developed a third and a fourth cycle of relapse and remission. These mice were likewise treated with anti-Thy-1.2 and isotype-matched antibodies when they began to enter the third and fourth remission phases. These mice were also kept for observation of post-treatment disease relapses. Composite results pooled from three separate experiments are shown in Table 3. Not all experiments had the same number of groups or group size. In addition, because of the small number of mice treated during the third and fourth remission, results for these two groups are combined to achieve a larger group size (3rd/4th remissions, Table 3). It is clear from the results that irrespective of whether the mice were treated with anti-Thy1.2 antibodies during the first, second, or third/fourth remission, the majority of the mice were disease-free for the duration of the experiment. Mice treated with isotype-matched antibodies during the first remission had a 89% (16/18) relapse rate while 95% of those treated with anti-Thy1.2 antibodies were relapse-free. One mouse (1/8) treated with anti-Thy1.2 antibodies during the second remission and one (1/7) during the third/fourth remission also broke through and developed a mild degree of EAE (disease grade 1). Most of the treatments with anti-Thy1.2 antibodies were effective. These experiments were repeated several times although some with smaller animal group sizes. The results were very similar. It is concluded that encephalitogenic T cells that induce development of acute EAE also participate in the relapsing phase of the disease. Depleting these cells after the acute disease phase completely prevented the recurrence of the disease.

4. Discussion

According to a report defining the clinical course of multiple sclerosis (Lublin and Reingold, 1996), 85–90% of individuals with MS suffer from the remitting-relapsing form of the disease. It is essential to understand what triggers the relapsing disease course. One popular explanation is based on the concept of "epitope spreading". Vanderlugt et al. pre-tolerized SJL mice to PLP_{139–151} with ECDI-coupled spleen cells and induced EAE in these mice with MBP_{84–104}. Tolerization did not affect the induction of acute disease, but abrogated the subsequent development of relapsing EAE (Vanderlugt et al., 2000). In addition, Yu et al. demonstrated, in (SWR × SJL)F1 mice, a sequential appearance, during disease relapses, of

dominant T cell populations with antigen specificities different from the priming epitopes such that each relapse cycle was associated with a new encephalitogenic epitope (Yu et al., 1996). It was concluded that, as disease progressed, responses to the initial priming antigen diminished and became undetectable. In its place was an orderly and definable set of new immune reactivity involved in disease relapses. Contrasting these studies, however, were reports citing evidence inconsistent with these conclusions. Talacs et al. conducted a systematic study of relapsing EAE using SJL mice responding to PLP_{139–151}, PLP_{178–191} and MBP_{87–106} and found that the priming epitope remained the major epitope recognized during the course of disease (Takacs et al., 1997). These results were further strengthened by the studies of Jones et al. who created an experimental milieu in which epitope spreading was not likely to occur. These investigators generated a SCID mouse line expressing a single H-2^d-restricted/myelin-specific T cell receptor (TCR). Donor TCR transgenic T cells from these mice were transferred into non-TCR-transgenic SCID littermates and the recipient mice were immunized with MBP peptides. It was shown that these mice developed progressive and remitting-relapsing EAE in the apparent absence of epitope spreading. More recently (Jones et al., 2003), Kroenke and Segal (2007) studied the cytokine profiles of spleen and lymph node cells and showed that production of Th1 and Th17 cytokines throughout the course of relapsing EAE was directed against the immunizing epitope PLP_{139–151} and not against putative secondary epitopes. Furthermore, tolerance induced against a putative secondary epitope did not affect the development of relapsing disease.

The present investigation adopts a more direct approach by targeting the T cells specific for the priming antigen. Taking advantage of the unique allelic expression of the T cell surface antigen, Thy-1, in an adoptive transfer system (Kim and Tse, 1993), it was possible to distinguish T cells that recognize the priming epitope versus T cells that recognize the spread epitopes. In this system, PLP_{139–151}-primed and in vitro-expanded donor lymph node cells from Thy-1.2⁺ SJL mice were transferred into Thy-1.1⁺ SJL-Thy-1^a (Thy-1.1⁺) recipients. If epitope spreading develops, it is expected that T cells recognizing the spread epitopes would be derived from the host T cell pool. Depletion of the Thy-1.2⁺ T cells would allow assessment of the roles of these PLP_{139–151}-primed T cells in the development of relapsing EAE. The results (Table 3) are unambiguous and show that treatment of the Thy-1.1⁺ recipient mice with anti-Thy-1.2 antibodies abrogated subsequent development of relapsing disease. The antigen-specificity of the Thy-1.2⁺ donor T cells in the recipients mice was verified by flow cytometry sorting of T cells infiltrating the spinal cords and tested in proliferative assays. These cells responded to PLP_{139–151} only, and not to any one of the possible spread epitopes such as PLP_{178–191} or MBP_{89–101} (Figure 1). When the CNS-seeking Thy-1.1⁺ host T cells were sorted and tested in a similar manner, it was found that these cells did not respond to any of the antigen epitopes tested (Figure 1). Thus, epitope spreading did not occur in these populations of T cells that infiltrated the CNS during relapsing EAE. The efficacy of anti-Thy-1.2 antibody depletion of donor encephalomyelitis T cells was also stringently tested. First, Figure 2 shows that administration of anti-Thy-1.2 antibodies into SJL-Thy-1^a recipient mice significantly reduced the number of Thy-1.2⁺ donor T cells in the spleens and lymph nodes of these mice. Second, treatment of SJL-Thy-1^a recipient mice with anti-Thy-1.2 antibodies immediately after adoptive transfer of PLP_{139–151}-primed and in vitro expanded SJL Thy-1.2⁺ lymph node cells prevented the induction of acute passive EAE in the recipients (Table 2). That treatment with the anti-Thy-1.2 antibodies did not affect the inherent ability of the mice to develop EAE was shown by subsequent transfer, 60 days later, of new donor cells into the recipients and observation of EAE development. The results of these experiments reveal new insights into alternative mechanisms in the development of relapsing EAE. In contrast to previous conclusion that encephalitogenic T cells initiating the symptoms of acute clinical EAE fade away during the development of clinical relapsing disease (Tuohy et al., 1999; Vanderlugt et al., 2000; Yu et al., 1996), it is now shown directly that these cells persist in the lymphoid tissues as well as

the CNS and their depletion abrogates development of subsequent EAE relapses. These conclusions have significant implications on the concepts of therapeutic designs for relapsing EAE and MS. Concerns have been expressed whether therapies should target T cells with specificities for the priming epitopes or the spread epitopes (Steinman, 1999; Vanderlugt and Miller, 2002; Steinman, 2000). The latter approach is obviously difficult as the spread epitopes vary from disease episode to episode. The results of this report and those of Kroenke and Segal (2007), on the other hand, support targeting of the priming epitopes. These factors should be considered when designing therapeutic approaches.

In EAE, inflammatory IFN γ , TNF α , IL-2 and IL-17 are found to be the dominant cytokines produced by CD4⁺ T cells from lymph nodes, spleens or spinal cords of mice during acute disease (Begolka et al., 1998; Hofstetter et al., 2005). It is still controversial whether Th2 cytokines are responsible for disease remission (DiRosa et al., 1998). Neither settled is the role between Th1 and Th17 cytokines in the induction of EAE (Luger et al., 2008; Suryani and Sutton, 2007; Segal, 2003; Xiao et al., 2008; Cua et al., 2003). Th1 cytokines have been championed as the important cytokine in the induction of EAE for many years (Xiao et al., 2008) before the tide turned towards IL-17, in recent years, as the essential EAE inducer (Cua et al., 2003). In an EAU system, it has also been suggested that IL-17 serves in the maintenance of autoimmune inflammation (Shi et al., 2009). In the present investigation, the cytokine profiles of CNS-infiltrating T cells were followed throughout the different stages of disease development. Acute disease was typically characterized by a broad spectrum of inflammatory cytokine production (Table 1), dominant among which were IL-2 and IL-17A. There were also smaller amount of IL-6, IL-13 and IFN γ produced. Interestingly, as disease progressed through the first and second remissions and relapses, the cytokine profiles regressed towards the production of predominantly IL-17 (Table 1). Only trace amount of IFN γ was detected at this stage. The observation that IL-17 becomes the only major inflammatory cytokine produced during relapsing disease supports the proposal that Th1 cytokines are important during the early stage of EAE and Th-17 cytokines are involved in the longer term maintenance of encephalitogenic responses (Shi et al., 2009).

Acknowledgments

Supported in part by grants from the National Institutes of Health (R01 NS37253 and R01 NS055167) and the National Multiple Sclerosis Society (RG 3288-A6).

References

- Begolka WS, Vanderlugt CL, Rahbe SM, Miller SD. Differential expression of inflammatory cytokines parallels progression of central nervous system pathology in two clinically distinct models of multiple sclerosis. *J. Immunol* 1998;161:4437–4446. [PubMed: 9780223]
- Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, Lucian L, To W, Kwan S, Churakova T, Zurawski S, Wiekowski M, Lira SA, Gorman D, Kastelein RA, Sedgwick JD. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 2003;342:744–748. [PubMed: 12610626]
- Di Rosa F, Francesconi A, Di Virgilio A, Finocchi L, Santilio I, Barnaba V. Lack of Th2 cytokine increase during spontaneous remission of experimental allergic encephalomyelitis. *Eur. J. Immunol* 1998;28:3893–3903. [PubMed: 9862326]
- Hofstetter HH, Karulin AY, Forsthuber TG, Ott PA, Tary-Lehmann M, Lehmann PV. The cytokine signature of MOG-specific CD4 cells in the EAE of C57BL/6 mice. *J. Neuroimmunol* 2005;170:105–114. [PubMed: 16257061]
- Jone RE, Bourdette D, Moes N, Vandenbark A, Zamora A, Offner H. Epitope spreading is not required for relapses in experimental autoimmune encephalomyelitis. *J. Immunol* 2003;170:1690–1698. [PubMed: 12574332]

- Kim C, Tse HY. Adoptive transfer of murine experimental autoimmune encephalomyelitis in SJL/Thy-1 congenic mouse strains. *J. Neuroimmunol* 1993;46:129–136. [PubMed: 7689583]
- Kroenke MA, Segal BM. Th17 and Th1 responses directed against the immunizing epitope, as opposed to secondary epitopes, dominate the autoimmune repertoire during relapses of experimental autoimmune encephalomyelitis. *J. Neurosci. Res* 2007;85:1685–1693. [PubMed: 17465017]
- Lublin F, Reingold SC. Defining the clinical course of multiple sclerosis: results of an international survey. *Neurology* 1996;46:907–911. [PubMed: 8780061]
- Luger D, Silver PB, Tang J, Cua D, Chen Z, Iwakura Y, Bowman EP, Sgambellone NM, Chan C-C, Caspi RR. Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. *J. Exp. Med* 2008;205:799–810. [PubMed: 18391061]
- McRae BL, Vanderlugt CL, Dal Canto MC, Miller SD. Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. *J. Exp. Med* 1995;182:75–85. [PubMed: 7540658]
- Mokhtarian F, McFarlin DE, Raine CS. adoptive transfer of myelin basic protein-sensitized T cells produces chronic relapsing demyelinating disease in mice. *Nature* 1984;309:356–358. [PubMed: 6203039]
- Segal BM. Experimental autoimmune encephalomyelitis: cytokines, effector T cells, and antigen-presenting cells in a prototypical Th1-mediated autoimmune disease. *Curr. Allergy Asthma Rep* 2003;3:86–93. [PubMed: 12543000]
- Shaw MK, Kim C, Hao HW, Chen F, Tse HY. Induction of myelin basic protein-specific experimental autoimmune encephalitis in C57BL/6 mice. Mapping of T cell epitopes and T cell receptor V β gene segment usage. *J. Neurosci. Res* 1996;45:690–699. [PubMed: 8892080]
- Shi G, Ramaswamy M, Vistica BP, Cox CA, Tan C, Wawrousek EF, Siegel RM, Gery I. Unlike Th1, Th17 cells mediate sustained autoimmune inflammation and are highly resistant to restimulation-induced cell death. *J. Immunol* 2009;183:7547–7556. [PubMed: 19890052]
- Skundric DS, Kim C, Tse HY, Raine CS. Homing of T cells to the central nervous system throughout the course of relapsing experimental autoimmune encephalomyelitis in Thy-1 congenic mice. *J. Neuroimmunol* 1993;46:113–122. [PubMed: 7689581]
- Skundric DS, Huston K, Shaw K, Tse HY, Raine CS. Experimental allergic encephalomyelitis: T cell trafficking to the central nervous system in a resistant Thy-1 congenic mouse strain. *Lab. Invest* 1994;71:671–679. [PubMed: 7526038]
- Steinman L. Absence of “Original Antigenic Sin” in autoimmunity provides an unforeseen platform for immune therapy. *J. Exp. Med* 1999;189:1021–1024. [PubMed: 10190892]
- Steinman L. Despite epitope spreading in the pathogenesis of autoimmune disease, highly restricted approaches to immune therapy may still succeed [with a hedge on this bet]. *J. Autoimm* 2000;14:278–282.
- Suryani S, Sutton I. An interferon- γ -producing Th1 subset is the major source of IL-17 in experimental autoimmune encephalitis. *J. Neuroimmunol* 2007;183:96–103. [PubMed: 17240458]
- Takacs K, Chandler P, Altmann DM. Relapsing and remitting experimental allergic encephalomyelitis: a focused response to the encephalitogenic peptide rather than epitope spread. *Eur. J. Immunol* 1997;27:2927–2934. [PubMed: 9394820]
- Traugott U, Raine CS, McFarlin DE. Acute experimental allergic encephalomyelitis in the mouse: immunopathology of the developing lesion. *Cell. Immunol* 1985;91:240–254. [PubMed: 2578895]
- Tuohy VK, Yu M, Yin L, Kawczak JA, Kinkel RP. Spontaneous regression of primary autoreactivity during chronic progression of experimental autoimmune encephalomyelitis and multiple sclerosis. *J. Exp. Med* 1999;189:1033–1042. [PubMed: 10190894]
- Vanderlugt CL, Neville KL, Nikcevic KM, Eager TN, Bluestone JA, Miller SD. Pathologic role and temporal appearance of newly emerging autoepitopes in relapsing experimental autoimmune encephalomyelitis. *J. Immunol* 2000;164:670–678. [PubMed: 10623809]
- Vanderlugt CL, Miller SD. Epitope spreading in immune-mediated diseases: Implications for immunotherapy. *Nat. Rev* 2002;2:85–95.
- Xiao BG, Ma CG, Xu LY, Link H, Lu CZ. IL-12/IFN- γ /NO axis plays critical role in development of Th1-mediated experimental autoimmune encephalomyelitis. *Mol. Immunol* 2008;45:1191–1196. [PubMed: 17697713]

Yu M, Johnson JM, Tuohy VK. A predictable sequential determinant spreading cascade invariably accompanies progression of experimental autoimmune encephalomyelitis: A basis for peptide-specific therapy after onset of clinical disease. *J. Exp. Med* 1996;183:1777–1788. [PubMed: 8666934]

Figure 1. Proliferative responses of FACS-sorted CNS-infiltrating donor T Cells and host cells to antigens during disease relapses

Adoptive EAE was induced in SJL-Thy-1^a recipients by the transfer of PLP₁₃₉₋₁₅₁-primed and in vitro activated SJL donor lymph node cells. At the first disease relapse (A), recipient mice were sacrificed and spinal cord cells were purified on a Percoll gradient to enrich for lymphocytes. The cells were labeled and FACS sorted for Thy-1.2⁺ and Thy-1.1⁺ cells. Sorted cells were over 95% purity. 2×10^4 sorted cells were cultured with various antigens and irradiated spleen cells as a source of antigen presenting cells. Proliferative responses were assessed. In (B), the spinal cords cells were collected at or near the peak of the second disease relapse. All error bars are standard deviation of the means. Each experiment was repeated once with similar results. Comparing PLP₁₃₉₋₁₅₁ with medium, t test p=0.0001 in A and p<0.0001 in B. The d- designations, such as d-medium, in the x-axis denote donor T cell (Thy-1.2⁺) responses and the h- designations denote responses of host cells (Thy-1.1⁺).

Figure 2. Effectiveness of anti-Thy-1.2 antibodies in depletion of donor SJL lymph node cells transferred into SJL.Thy-1^a recipients

Adoptive EAE was induced in naïve SJL-Thy-1^a recipients by the transfer of PLP_{139–151}-primed and in vitro activated SJL donor lymph node cells. At days –3, 0 and +3 relative to cell transfer, recipient mice were treated with anti-Thy-1.2 antibodies or PBS as control. Two days after the last antibody treatment, spleens of individual SJL-Thy-1^a mice were isolated and stained with FITC-anti-CD4 and PE-anti-Thy-1.2 antibodies for flow cytometric analysis. Numbers in each quadrant represent percentage of gated spleen cells stained positive with the antibodies. This experiment was performed with six mice in each group. Individual results of three of the six mice in each group are shown here to indicate the range of variability.

Table 1

Cytokine profiles of FACS-sorted CNS-infiltrating donor T cells and host cells during disease relapses

Spinal Cord Cell Populations	Disease Stages	Cytokines produced (pg/10 ⁴ cells)									
		IL-2	IL-4	IL-6	IL-10	IL-13	IL-17A	IL-23	IFN γ	TNF α	TGF β
Donor T Cells	Acute	17.9 $\pm$ 0.2	0.9 $\pm$ 0.2	4.7 $\pm$ 0.65	0.0	9.1 $\pm$ 2.2	70.5 $\pm$ 7.2	0.0	4.3 $\pm$ 2.1	0.0	0.0
	1 st Relapse	5.2 $\pm$ 0.15	0.0	0.0	0.0	0.0	73.2 $\pm$ 3.75	0.0	1.3 $\pm$ 0.2	0.3 $\pm$ 0.15	0.0
	2 nd Relapse	0.0	0.0	0.0	0.0	0.0	166.3 $\pm$ 11.15	0.0	3.6 $\pm$ 1.8	0.0	0.0
Host Cells	Acute	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	1 st Relapse	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	2 nd Relapse	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

FACS-sorted CNS-infiltrating donor T cells and host cells (over 95% purity) as described in Figure 1 were cultured with PLP₁₃₉₋₁₅₁ peptides and irradiated spleen cells as a source of antigen presenting cells for 24 hours (5×10^4 cells/well for donor Thy-1.2⁺ and 2×10^5 cells/well for host Thy-1.1⁺ cells). Culture supernatants were collected and analyzed with SABiosciences' Multi-Protein ELISA Kits. Values are normalized to pg/10⁴ cells. Data are mean $\pm$ SEM of two experiments.

Table 2Effects of depleting donor SJL T cells after cell transfer into SJL-Thy1^a recipients

Clinical Signs of Acute EAE				
Treatment	Day of treatment	Disease incidence	Av. max. dis. grade (mean ± SEM)	Av. day dis. onset (mean ± SEM)
No treatment	-	13/14	3.5 ± 0.18	10.0 ± 0.30
Saline	-3, 0, +3	4/4	4.0 ± 0	8.5 ± 0.29
Isotype-matched Ig	-3, 0, +3	6/6	3.0 ± 0.26	9.5 ± 0.42
Anti-Thy-1.2	-3, 0, +3	0/12	--	--

Development of acute EAE after 2 nd transfer of donor cells 60 days post anti-Thy-1.2 treatment			
Cell transfer	Disease incidence	Av. max. dis. grade (mean ± SEM)	Av. day dis onset (mean ± SEM)
No cell transfer	0/6	--	--
With cell transfer	6/6	3.1 ± 0.27	8.3 ± 0.42

SJL mice were immunized with PLP_{139–151} peptides emulsified in CFA. Ten days later, draining lymph nodes were removed and the cells were restimulated in vitro with the priming antigen for four days. Cells were harvested and 5×10^7 viable cells were transferred into naïve SJL-Thy-1^a recipients through the tail vein. At days -3, 0 and +3 relative to cell transfer, recipient mice were treated with isotype-matched and anti-Thy-1.2 antibodies. Disease was monitored for 30 days. Anti-Thy-1.2 antibody-treated mice failed to develop acute adoptive EAE. 60 days later, these mice received a new batch of PLP_{139–151}-primed and in vitro-stimulated SJL donor lymph nodes cells i.v. Disease was monitored for 30 days.

Table 3

Effects of depleting donor SJL T cells on subsequent development of relapsing disease in SJL-Thy1^a recipients

Time of Antibody Treatment	Treatment	Incidence of relapsing disease after treatment	Av. max. relapsing disease grade after treatment * (Mean $\pm$ SEM)
During 1 st Remission	Isotype-matched Ab	16/18 [#]	2.8 $\pm$ 0.19
During 1 st Remission	Anti-Thy1.2	1/18 [#]	1.0
During 2 nd Remission	Isotype-matched Ab	9/10 [^]	2.8 $\pm$ 0.22
During 2 nd Remission	Anti-Thy1.2	1/8 [^]	1.0
During 3 rd /4 th Remissions	Isotype-matched Ab	4/5 ^{&}	3.3 $\pm$ 0.25
During 3 rd /4 th Remissions	Anti-Thy1.2	1/7 ^{&}	1.0

PLP139–151-primed and in vitro activated donor SJL lymph node cells were transferred into SJL-Thy-1^a recipient mice. These mice developed acute EAE and then entered the remission cycle. At this time (1st remission), groups of mice were treated with three doses of anti-Thy-1.2 or isotype-matched antibodies over the next six days. These mice were observed for subsequent development of relapsing EAE. Of the remaining untreated mice that proceeded to the first relapse and then second remission, groups of mice were similarly treated with anti-Thy-1.2 antibodies and observed for subsequently development of relapsing EAE. The remaining untreated mice went through the third and some through the 4th remission. These mice were treated similarly. Because of the small number of mice in these groups, the mice were pooled for analysis. Disease was monitored for 60 days after antibody treatment. Data were combined from multiple experiments with varied group sizes.

* only include mice showing relapsing disease after administration of antibodies.

Fisher's Exact Tests:

[#] two-tailed *p* value < 0.0001

[^] two-tailed *p* value = 0.0029

[&] two-tailed *p* value = 0.072

[&] one-tailed *p* value = 0.044