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Deficiency of Thrombospondin-1 Reduces Th17 Differentiation and Attenuates Experimental Autoimmune Encephalomyelitis

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Abstract

Transforming growth factor β (TGF- β) plays a role both in the induction of Treg and in the differentiation of the IL-17-secreting T cells (Th17) which drive inflammation in experimental autoimmune encephalomyelitis (EAE). We investigated the role that thrombospondin-1 (TSP-1) dependent activation of TGF- β played in the generation of an encephalitic Th17 response in EAE. Upon immunization with myelin oligodendrocyte glycoprotein peptide (MOG₃₅₋₅₅), TSP-1 deficient (TSP-1^{null}) mice and MOG₃₅₋₅₅ TCR transgenic mice that lack of TSP-1 (2D2-TSP-1^{null}) exhibited an attenuated form of EAE, and secreted lower levels of IL-17. Adoptive transfer of *in vitro* activated 2D2-TSP-1^{null} T cells induced a milder form of EAE, independent of TSP-1 expression in the recipient mice. Furthermore, *in vitro* studies demonstrated that anti-CD3/anti-CD28 pre-activated CD4⁺ T cells transiently upregulate latent TGF- β in a TSP-1 dependent way, and such activation of latent TGF- β is required for the differentiation of Th17 cells. These results demonstrate that TSP-1 participates the differentiation of Th17 cells through its ability to activate latent TGF- β , and enhances the inflammatory response in EAE.

Introduction

Experimental allergic encephalomyelitis (EAE) is a well described animal model for the study of multiple sclerosis, a demyelinating disease of the human central nervous system (CNS). It was previously thought that EAE was primarily mediated by a Th1 immune response characterized by the production of IFN γ (1,2). However, a primary role of IFN γ in EAE was not consistent with the observation that IFN γ -/- mice develop a form of EAE that carries an unusually high morbidity and mortality (3,4). More recent data indicates that IL-17-producing (Th17) cells are central to the inflammation in CNS in EAE as these cells are found at peak numbers during the height of the disease (5), and in their absence, mice develop a mild form of the disease (6). An unexpected participant in generating this encephalitogenic Th17 response during EAE development is TGF- β , a cytokine previously known for its immunosuppressive functions (7-9). It is now known that in conjunction with IL-6, TGF- β induces ROR γ t expression and suppresses Foxp3 expression, thus driving naïve T cells to differentiate into

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Th17 cells *in vitro* (10,11). In mice whose TGF β R signaling in T cells is blocked by the expression of dominant negative TGF β RII, there is reduction of Th17 cells and inhibition of EAE development (12). Moreover, over-expression of TGF- β in the T cell compartment results in an elevated Th17 cell infiltration in CNS after MOG₃₅₋₅₅ immunization and worsens the disease (10).

The majority of TGF- β produced *in vivo* is present in latent TGF- β complexes, in which latency-associated protein (LAP) is tightly bound to an active TGF- β core, preventing TGF- β and its cognitive receptor binding (13). Little is known about the mechanism by which TGF- β is activated during the generation of the Th17 response which is central to EAE pathogenesis. One candidate to mediate this function is thrombospondin-1 (TSP-1), a multifaceted extracellular matrix protein which is able to interact with TGF- β , and multiple other ligands including heparin sulfate proteoglycans, protein glycosaminoglycans, acathepsin G, elastase, multiple integrins, CD36, CD47, and calreticulin (14-17). By virtue of its ability to bind these different ligands, TSP-1 modulates cellular adhesion, migration, proliferation, survival and differentiation (14,15,18,19). Importantly, TSP-1 activates TGF- β by inducing the dissociation of LAP from latent TGF- β complex (20). This mechanism is thought to be of major importance *in vivo*, as suggested by the findings that TSP-1^{null} mice display a phenotype that partially resembles that of TGF- β 1-knockout (TGF- β _{ko}) mice (20). TSP-1 also mediates immune privilege in the eye through this mechanism (21). Furthermore, we have found that LAP+ T cells also express TSP-1 on their surface, a finding which suggests that TSP-1 might confer T cells with the ability to activate TGF- β (unpublished data).

In the present study we investigated the role that TSP-1 plays in the generation of Th17 cells in EAE. Our results demonstrate that TSP-1 activates TGF- β during the formation of Th17 responses, a function that plays an important role in the development of EAE.

Materials and Methods

Animals

Female C57BL/6 (B6) mice were obtained from The Jackson Laboratory. MOG₃₅₋₅₅ specific TCR transgenic mice (2D2 mice) were kindly provided by Dr. Vijay Kuchroo at the Center for Neurological Diseases, Brigham and Women's Hospital (22). TSP-1^{null} mice were kindly provided by Dr. Jack Lawler from the Department of Pathology at the Beth Israel Deaconess Hospital, Boston. All mice used in this study were bred on the C57BL6 background. To generate TSP-1^{null} MOG₃₅₋₅₅ specific mice, male 2D2 mice were initially bred with female TSP-1^{null} mice, and their F1 progenitors were selected for the presence of the 2D2 T cell receptor (TCR) transgene by staining peripheral blood cells using specific antibodies against CD4, TCR V β 11 and/or V α 3.2 (22), and heterozygosity of TSP-1 mutation by PCR using 40 cycles of 94° C, 30 sec; 65° C, 1 min; 72° C, 2 min and the following primers: MGR: GAGTTTGCTTGT GGTGAACGCTCAG; MGF: AGGGCTATGTGGAATTAATATCGG; NEO MGF: TGCTG TCCATCTGCACGAGACTAG. The male F1 progenies that carried 2D2 TCR transgene (2D2⁺TSP1^{+/-}) were back-crossed with female TSP-1^{null} mice, and the F2 progenitors were selected for the presence of 2D2 TCR transgene by antibody staining and homozygosity of TSP-1 mutation by PCR. The F2 progenitor mice that carried 2D2 TCR transgene and homozygous deletion of TSP-1 gene were designated as 2D2·TSP-1^{null} mice. The 2D2·TSP-1^{null} mice were maintained by interbreeding male 2D2·TSP-1^{null} with female TSP-1^{null} mice followed by selection for the presence of 2D2 TCR transgene by antibody staining. The homozygosity of TSP-1 mutation was confirmed by PCR. All mice were housed in a pathogen/viral-free animal facility at the Harvard Institutes of Medicine, and used for experiments between the ages of 8-12 weeks. All breeding and experiments were performed in accordance with the guidelines of the Animal Use and Care Committee of the Harvard Medical School.

Antibodies and reagents

The MOG₃₅₋₅₅ peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Dr. Teplow from the Biopolymer Facility at the Center for Neurologic Diseases, Brigham and Women's Hospital. The following antibodies were obtained from BD Biosciences (San Diego, CA): purified anti-mouse CD3, CD28 and CD16/CD32; FITC-conjugated anti-mouse CD4 and TCR V α 3.2; PE-conjugated anti-mouse V β 11 and biotinylated anti-mouse B220, CD8, CD25. The cytokines IL-2, IL-6, IL-7, IL-12, active and latent TGF β , and the DuoSet ELISA Development System specific for mouse TGF β -1 were purchased from R&D Systems (Minneapolis, MN). The lipopolysaccharide (LPS) and *Mycobacterium tuberculosis* were purchased from Sigma-Aldrich (St. Louis, MO). Foxp3 staining was performed using a kit purchased from e-bioscience (San Diego, CA).

Purification of naïve CD4+ T cells

Single cell suspensions were prepared by mincing spleens through a 70 μ cell strainer. After lysis of red blood cells with ACK buffer (Sigma; St Louis, MO), total splenocytes were incubated on ice with biotinylated anti-B220, anti-CD8, anti-Gr-I, anti-Mac-I, anti-CD25, and anti-MHC-II antibodies, followed by a new incubation with streptavidin microbeads (Miltenyi Biotech, Auburn, CA). Total cells were washed, and loaded onto LD columns in a magnetic field. The unbound CD4+ T cells were eluted and used in different experiments as described below.

T cell proliferation assay

Purified CD4+ T cells or whole splenocytes were cultured in DMEM medium supplemented with 10% FCS, 20mM HEPES, 5×10^{-5} M 2- β -mercaptoethanol, 2 μ M L-glutamine, 1 μ M non-essential amino acids, 1 μ M sodium pyruvate and 100 U/ μ g penicillin/streptomycin. Incubations with MOG₃₅₋₅₅ were performed at 37°C between 48 and 96h as described in the figure legends. Cells were pulsed with 1 μ Ci [³H] thymidine for the last 16 hr of incubation, and thymidine incorporation was measured using a microbeta liquid scintillation and luminescence counter (PerkinElmer, Waltham, Massachusetts). Data are presented as mean counts per minute (CPM) $\pm$ SEM.

Measurement of cytokine production

Total splenocytes or purified T cells (2.5×10^5 cells/well) were stimulated with 0.1-100 μ g/ml MOG₃₅₋₅₅ or 0.1-1 μ g/ml plate-bound anti-CD3/anti-CD28 in complete DMEM medium at 37°C. Culture supernatants were harvested 48h later and the levels of IFN γ , IL-2, IL-4, IL-5, IL-6, IL-10, TGF- β were determined via sandwich ELISAs using antibodies obtained from BD Biosciences using methods previously described (23). IL-17 was quantified using an IL-17 ELISA kit obtained from R&D Systems (Minneapolis, MN) and following the manufacturer's instructions. Briefly, plates were coated with capture antibodies in bicarbonate buffer overnight at 4°C. They were then blocked with 1% BSA and subsequently incubated with culture supernatants for 2h at room temperature (IL-17), or overnight at 4°C (all other cytokines). This was followed by washing and a 2h incubation period with biotinylated cytokine-specific antibodies and a 1hr incubation with HRP-conjugated streptavidin. After extensive washing, TMB substrate (Pierce Biotechnology, Rockford, IL) was added to detect cytokine of interest.

Isolation of APCs

Intact mice were sacrificed and their spleens harvested and single cell suspensions were prepared. Cells were re-suspended and cultured at 37°C for 2h in order to allow APCs to adhere to the culture plates. At the end of this period, the culture medium was removed along with all non-adherent cells. To ensure high purity of APCs, the cultured flasks were struck forcefully against a hard surface and washed twice with fresh medium.

Quantification of active and latent TGF β

The levels of active and latent TGF β were measured using a sandwich ELISA assay from R&D Systems. To avoid the interference by exogenous TGF- β from fetal calf serum, cells were cultured in the HL-1 serum free medium supplemented with 20mM HEPES, 5×10^{-5} M 2-beta mercaptoethanol, 2 μ M L-glutamine, 1 μ M non-essential amino acid, 1 μ M sodium pyruvate and 100 U/ μ g penicillin/streptomycin for our assays. To quantify active TGF- β levels, CD4+ T cells were stimulated for 72 hrs with plate bound anti-CD3/anti-CD28 and their culture supernatants were incubated on a plate pre-coated with a TGF- β capture antibody specific for active TGF- β , at room temperature for 3 hrs. This was followed by a 2h incubation with a HRP-conjugated TGF- β detection antibody. After extensive washing TMB substrate (Pierce Biotechnology, Rockford, IL) was added in order to visualize the amount of bound active TGF- β . The optical density was read at 450 nm with correction at 570 nm. To determine the levels of total TGF- β , culture supernatants were treated with 1N HCl for 30 min followed by 1.2N NaOH/0.5M HEPES, and immediately assayed for TGF- β using ELISA as described above.

EAE induction

Mice were given a flank *s.c.* injection of 10-150 μ g of MOG₃₅₋₅₅ emulsified with complete Freund's adjuvant (MOG/CFA) which contained 0.3 mg of heat-inactivated *Mycobacterium tuberculosis* (H37RA; Difco Laboratories). Each animal also received pertussis toxin (200ng *i.p.*; List Biological Laboratories) on day 0 and day 2 post-immunization. Mice were monitored up to 30 days for signs of disease. The EAE clinical score was determined as follows: 0, no disease; 1, limp tail or isolated weakness of gait without limp tail; 2, partial hind limb paralysis; 3, total hind limb or partial hind and front limb paralysis; 4, total hind leg and partial front leg paralysis; 5, moribund or dead animal. To induce EAE by adoptive transfer of polarized MOG-specific T cells, splenocytes from 2D2 mice or from 2D2-TSP-1_{null} mice were cultured and stimulated with 20 μ g/ml MOG₃₅₋₅₅ in the presence of 5 μ g/ml IL-2/IL-7 for 2 days. At the end of this incubation period cells were expanded with IL-2 and IL-7 for 4 days followed by a 24h re-activation period with plate-bound anti-CD3 and anti-CD28 in the presence of 20ng/ml IL-12 and IL-18. Activated T cells were then collected, washed and counted. $0.5-1 \times 10^6$ cells were transferred to recipient mice via *i.p.* injections.

Flow cytometry analysis

Cells were suspended in HBSS containing 2% BSA, and treated with anti-CD16/anti-CD32 on ice for 20 min in order to prevent non-specific binding of staining antibodies through their Fc receptors. Staining was performed with FITC-, PE-, or APC-conjugated antibodies for 30 min on ice followed by two washes with cold HBSS and analysis using FACScalibur cytometer (BD Bioscience, San Diego, CA).

Histological analysis

Wt (wt) and TSP-1_{null} mice were sacrificed on day 12, 16 and 30 post-MOG/CFA immunization, and rapidly perfused with saline solution. Brains and spinal cords were removed from the encasing bones and placed in 0.32 M sucrose solution at 4°C overnight. The tissues were then dipped in fixative solution and embedded for sectioning through the frontal lobes, cerebellum, brainstem, and the cervical and lumbar enlargements of the spinal cord. Adjacent sections were stained with both luxol fast blue, and haematoxylin and eosin using standard staining protocols. Inflammatory foci from spinal cord sections were counted through a light microscope, and the average of 5 H&E sections was used for statistical analysis (n=3 group)

Statistical analysis

Statistical analysis was carried out using GraphPad Prism Version 4 (GraphPad Software Inc). All graphs show mean $\pm$ S.E.M. Differences between control and treated groups for EAE tests

were evaluated by repeated measures two way ANOVA tests. Where indicated, student's t-test was used. Significance was established at a p value of 0.05. Experiments shown in the figure sections are representative of at least 3 repeat experiments.

Results

TSP-1 deficiency in mice results in an attenuated form of EAE

To study the role of TSP-1 in EAE, we immunized wt and TSP-1^{null} mice with MOG/CFA. When mice were immunized with 10 µg of MOG₃₅₋₅₅ the majority of wt mice developed signs of EAE (8/12, EAE score ≥1) while the majority of TSP-1^{null} mice were disease free (TSP-1 deficient, 1/12, EAE score >1) (Table 1, Figure 1A). Similar results were obtained when EAE was induced with 50µg of MOG₃₅₋₅₅ (Table 1, Figure 1B). The EAE in TSP-1^{null} mice immunized with 50µg was associated with a reduction of inflammatory foci in the spinal cord as compared with wt mice (13±3.7 vs 0.5±0.5; p<0.0001). Representative luxol fast blue and H&E stained sections of each group (n=3) are shown in figure 1D-E.

To further study the role of TSP-1 in EAE we crossed 2D2 transgenic mice with TSP-1^{null} mice. The 2D2 mouse strain was chosen because CD4⁺ T cells express the TCR Vα3.2 and Vβ11 genes, which recognize MOG₃₅₋₅₅. 2D2 animals were highly susceptible to EAE compared to wt C57BL/6 mice. When immunized with 10µg MOG₃₅₋₅₅ in CFA, most 2D2 mice developed signs of severe disease by the 9th day post-immunization, and 6 out of 11 died (Table 1, Figure 1C). By contrast, 2D2-TSP-1^{null} mice immunized in an identical fashion developed a very mild EAE, showing no signs of disease until the 11th day after immunization, and reaching a mean maximum score that was significantly lower than that observed in its TSP-1 expressing 2D2 counterparts (4.1 vs 1.8 p<0.001; Figure 1C). Only one out of 9 immunized animals from this group died as a result of severe EAE.

Absence of TSP-1 does not affect the immune compartment

To investigate whether the attenuation in EAE seen in TSP-1^{null} mice was due to a developmental abnormality in the immune system, we compared the distribution of cells throughout the immune compartment of TSP-1^{null} mice with that of wt controls. Total splenocytes of each group (n=3 mice per group) were stained with markers specific for the following cellular populations: T cells (CD4, CD8), B cells (CD19), macrophages and monocytes (CD11b), dendritic cells (CD11c), regulatory T cells (Foxp3). In all cases, wt and TSP-1^{null} mice demonstrated similar distributions of these markers. The cellular distribution in the lymph nodes was similarly normal in TSP-1^{null} mice (data not shown).

We also analyzed TSP-1^{null} DCs for cytokine production. Upon stimulation of different TLR ligands, TSP-1^{null} DCs produced slightly higher amounts of pro-inflammatory cytokines than wt DCs (*i.e.* CpG stimulation, B6 DCs vs TSP-1^{null} DCs, IL-6, 1056±72 pg/ml vs 1446±132 pg/ml; MCP-1, 1149±642 pg/ml vs 1579±520 pg/ml; IL-12, 5.8±3.8 pg/ml vs 6.0±0.9 pg/ml). When pulsed with MOG₃₅₋₅₅ peptide and mixed with purified 2D2 T cells, TSP-1^{null} DCs support 2D2 T-cell proliferation as efficiently as wt DCs as determined by ³H-thymidine incorporation (37502±3097 CPM vs 39495±2075 CPM in a culture of equal amounts of 2D2 T cells with wt DCs vs TSP-1^{null} DCs).

These findings suggested that TSP-1^{null} mice possess a normal cellular immune compartment, and led us to search further for functional deficiencies that could explain the observed discrepancy in EAE severity.

T-cell derived TSP-1 plays a critical role in EAE

Multiple steps of T-cell activation in peripheral lymphoid tissues and in CNS are required for the induction of inflammation in CNS. To investigate whether a T-cell deficiency could account for the attenuated form of EAE observed in TSP-1_{null} animals, we transferred *in vitro* polarized 2D2-TSP-1_{null} T cells into wt and TSP-1_{null} recipients and followed EAE induction in these recipient mice. As control, we also transferred *in vitro* polarized 2D2-T cells into wt and TSP-1_{null} recipients. While 2D2 T cells induced a severe form of EAE in both wt and TSP-1_{null} recipients, 2D2-TSP-1_{null} T cells induced a mild form of EAE, regardless of TSP-1 expression in the recipient mice (Figure 2). These results demonstrate that T-cell derived TSP-1 plays an important role in EAE.

Absence of TSP-1 leads to decreased IL-17 and IFN γ responses

We then tested whether TSP-1_{null} mice exhibit a functional deficiency in T-cell proliferation or cytokine production. To this end, cohorts of wt and TSP-1_{null} mice were immunized with different doses of MOG₃₅₋₅₅, and 10 days later their spleens were harvested and stimulated *in vitro* with 20 μ g/ml MOG₃₅₋₅₅. Both groups exhibited similar patterns of T-cell proliferation both at 10 and 50 μ g of MOG₃₅₋₅₅ tested (Figure 3A and 3B), suggesting that induction of antigen specific T cells is normal in the absence of TSP-1.

Despite normal T-cell proliferation, levels of IL-17 and IFN γ were significantly decreased in TSP-1_{null} mice (Figure 3C and 3D). These findings were consistent across five independent experiments. The reduction of pro-inflammatory cytokines did not skew TSP-1_{null} T cells towards Th2 lineage development. In fact, both IL-4 and IL-10 levels were not detectable.

We also analyzed the cytokine patterns of naïve 2D2 and 2D2-TSP-1_{null} mice. Again, levels of IL-17 and IFN γ were significantly decreased in 2D2-TSP-1_{null} mice without skewing towards Th2 cytokines (Figure 4A). Consistent with inefficiency of 2D2-TSP-1_{null} T cells to induce EAE after adoptive transfer, levels of IL-17 and IFN γ by 2D2-TSP-1_{null} T cells remained decreased by 83% and 63% (2D2 vs 2D2-TSP-1_{null}, IL-17, 22877 $\pm$ 3883 pg/ml vs 3639 $\pm$ 714 pg/ml; IFN γ , 336820 $\pm$ 820 pg/ml vs 124025 $\pm$ 69 pg/ml) after re-activation *in vitro* with plate-bound anti-CD3/anti-CD28 for 48hrs (Figure 4B). Because IL-17 (6) appears to be more central for EAE than IFN γ (24), we focused our investigation on the role that TSP-1 might play in the development of the Th17 immune response.

T-cell receptor stimulated CD4+ T cells activate TGF- β

Naïve and active T cells express TSP-1 (25), and TSP-1 is modulated between the surface membrane and intracellular compartments in T cells. However, internalization of TSP-1 is inhibited by TCR engagement and surface TSP-1 accumulates (26-28). Because TSP-1 is known to activate TGF- β (20), a molecule that along with IL-6 drives Th17 responses in EAE (10,29), we hypothesized that the reduction of IL-17 by TSP-1_{null} mice results from a deficiency in TGF- β activation. To test this hypothesis naïve, non-stimulated and anti-CD3/anti-CD28-stimulated wt and TSP-1_{null} CD4+ T cells were treated with latent TGF- β .

Quantification of active TGF- β at 2, 6 and 24 hours showed that neither wt nor TSP-1_{null} non-stimulated CD4+ T cells converted latent TGF- β , as levels of active TGF- β continue to decline during the test period (Figure 5B, comparing B6/CD4+latent TGF- β vs TSP-1_{null}/CD4+latent TGF- β). However, 6h after the addition of exogenous latent TGF- β into the culture system, a significant increase in active TGF- β was detected in the supernatants of anti-CD3/anti-CD28-stimulated wt CD4+ T cells, but not in the supernatants of anti-CD3/anti-CD28-stimulated TSP-1_{null} CD4+ T cells (Figure 5C, comparing B6/CD4+latent TGF- β vs TSP-1_{null}/CD4+latent TGF- β). Interestingly, such increase of active TGF- β in pre-activated wt CD4+ T-cell culture was short-lived, as the level of active TGF- β quickly declined to basal level by 24hr.

These results provided evidence demonstrating that endogenous TSP-1 confers T cells with the ability to transiently activate TGF- β in an activation dependent manner.

While CD4⁺ T cells from wt and TSP-1^{null} mice did not produce any detectable total TGF- β by their own (Figure 5D, B6/CD4 alone and TSP-1^{null}/CD4 alone), we observed the levels of total TGF- β in the cultures of CD4⁺ T cells exceed that of control culture (Figure 5D, latent TGF- β alone) after addition of exogenous latent TGF- β (Figure 5D, comparing B6/CD4+latent TGF- β and TSP-1^{null}/CD4 +latent TGF- β vs latent TGF- β alone). These results suggest that CD4⁺ T cells from wt and TSP-1^{null} mice produce latent TGF- β after the addition of exogenous latent TGF- β . Such increases of total TGF- β were obvious in the cultures of non-stimulated and anti-CD3/anti-CD28-stimulated CD4⁺ T cells from wt and TSP-1^{null} mice (Figure 5D and 5E), suggesting that the production of latent TGF- β by CD4⁺ T cells is independent of T-cell activation and TSP-1 expression. Moreover, the increases of total TGF- β appeared rapidly, and were detected as early as 2 hrs after addition of exogenous latent TGF- β , suggesting that the source of latent TGF- β by CD4⁺ T cells may come from a pre-made storage of latent TGF- β in CD4⁺ T cells, instead of *de novo* production by CD4⁺ T cells.

T-cell derived TSP-1 activates TGF- β and drives Th17 responses

To test the relevance of a TSP-1-mediated activation of TGF- β during the generation of a Th17 response, we activated wt and TSP-1^{null} CD4⁺ T cells in the presence of a constant dose of IL-6 (25ng/ml), and three different doses latent TGF- β (14, 43, and 130 ng/ml). Forty-eight and 96h later, we assayed the cultured supernatants for IL-17. As controls, we tested the levels of IL-17 produced by both cell populations when they were activated in the presence of IL-6 (25 ng/ml) and *active* TGF- β (5 ng/ml). At both times tested, TSP-1^{null} CD4⁺ T cells secreted significantly lower levels of IL-17 at all concentrations of latent TGF- β by comparison with wt controls (Figure 6). Importantly, IL-17 secretion was restored to normal levels if TSP-1^{null} CD4⁺ T cells were activated in the presence of IL-6 and *active* TGF- β (Figure 6). These results provide direct evidence that TSP-1 mediated TGF- β activation by T cells is critical for the generation of a Th17 response.

Discussion

It has been reported that IL-17 plays a role in human autoimmune diseases such as arthritis (30), psoriasis (31), inflammatory bowel disease (32), and multiple sclerosis (33). IL-17 also appears to be critical for the development of EAE (10,34), as IL-17-producing cells are found at peak numbers during the height of inflammation in EAE (5), and IL-17^{-/-} mice only develop a very mild form of EAE, with delay of disease onset and reduction of disease severity (6). In mice, the differentiation of Th17 cells is driven by a concerted action between TGF- β and IL-6 (10,11). However, few studies have addressed the role of TGF- β activators in EAE. Multiple lines of evidence support a role for TSP-1 as an important activator of TGF- β in the context of inflammation. First, TSP-1^{null} mice exhibit phenotypes that partially resemble that of TGF- β 1^{ko} mice (20); second, TSP-1 expression during wound healing is associated with TGF- β -mediated tissue repair (35). Also through its ability to mediate TGF- β activation, TSP-1 is thought to play an important role in the establishment and maintenance of ocular immune privilege (21). Finally, both mouse (36) and human (25) T cells have been shown to express TSP-1 on cell surface, suggesting that TSP-1 might confer T cells with the ability to activate TGF- β .

Despite multiple cellular sources of TSP-1 *in vivo*, our studies suggest that T cells serve as the source of TSP-1 for the development of Th17 response in the EAE model. This is evident from adoptive transfer of *in vitro* polarized 2D2-TSP-1^{null} T cells which failed to induce disease in either TSP-1^{null} or wt recipients. These results suggest that despite the multiple cellular sources of TSP-1 in recipient wt mice, it is T-cell derived TSP-1 that is critical for the development of

EAE. Furthermore, *in vitro* polarized TSP-1 expressing 2D2 T cells caused severe disease in both TSP-1_{null} and wt recipient mice, indicating that TSP-1 expression on effector T cells is sufficient to drive a full inflammatory EAE response regardless of TSP-1 expression in the recipient mice. A similar result was reported by Li et al (37) using mice in which TGF- β was specifically deleted in the CD4⁺ T-cell compartment. Despite other cellular sources of TGF- β production *in vivo*, Li et al (37) found that T-cell specific deletion of TGF- β was sufficient to inhibit the generation of Th17 cells, and reduce susceptibility to EAE induction. Similar observations were found in our studies in mice lacking TSP-1, a molecule that binds to and activates TGF- β . These results suggest that T cells promote their own Th17 differentiation *in vivo* by regulating their own TGF- β production and/or TSP-1 dependent latent TGF- β activation.

We also investigated the ability of CD4⁺ T cells to activate latent TGF- β *in vitro*. Only CD4⁺ T cells pre-activated by CD3/CD28 convert latent TGF- β ; no conversion was detected by naïve, non-stimulated CD4⁺ T cells. Activation of latent TGF- β by CD4⁺ T cells was TSP-1 dependent and was rapid and transient, peaking by 6 hrs after addition of latent TGF- β , then returning to background by 24 hrs. The features of rapid and transient activation of latent TGF- β suggest that the functions of active TGF- β *in vivo* are limited, most likely to the sites of inflammation. Consistent with this idea, local injection of anti-TGF- β antibody to sites of immunization interferes with TGF- β signaling, prevents Th17 cell differentiation, and blocks EAE induction (12).

It was unexpected that levels of total TGF- β in the culture supernatant also increased, exceeding the levels of exogenously added latent TGF- β . The increases of total TGF- β were neither dependent on the activation status of CD4⁺ T cells, nor dependent on the expression of TSP-1. In addition, the increases of latent TGF- β in cell culture appeared as early as 2 hrs after latent TGF- β addition, making it unlikely that latent TGF- β produced by CD4⁺ T cells was synthesized *de novo*. On the contrary, we favor the hypothesis that CD4⁺ T cells rapidly release their own intracellular latent TGF- β upon addition of exogenous latent TGF- β . The significance of T-cell produced latent TGF- β is unknown. In light of Li et al's study (37), we believe that that T-cell specific production of latent TGF- β production may also contribute to inducing Th17 generation and EAE induction. The nature of the signal that induces CD4⁺ T cells to release latent TGF- β is unknown. Clearly, activation of CD4⁺ T cells through TCR is not sufficient to induce latent TGF- β release since we did not detect latent TGF- β in the supernatant when we simply stimulated CD4⁺ T cells with plate bound anti-CD3/anti-CD28 for 24hrs.

In the aggregate, our results suggest that T-cell derived TSP-1 is a key activator of TGF- β which in turn plays a critical role during the generation of the Th17 response which drives inflammation in CNS.

Aside from producing lower levels of IL-17, splenocytes from MOG-immunized TSP-1_{null} mice also produced lower levels of IFN γ , by comparison with controls. The significance of this finding is uncertain. Previous reports showed that IFN γ ^{-/-} mice develop severe EAE (24) and put into question the role that this cytokine had been given as the main cytokine driving this disease. Since then, the role that IFN γ plays in EAE remains uncertain, and some have proposed that this cytokine might have a protective role in EAE (4,38). Therefore, we believe that the attenuated EAE seen with TSP-1_{null} mice is best explained by the decreased Th17 response, and not the decreased IFN γ response observed in these mice.

An important question raised by this study is whether T-cell-derived TSP-1 might also play a supporting role in other autoimmune diseases in which TGF- β drives Th17 mediated inflammation. For example, TSP-1 is found at high levels in the joints of rheumatoid arthritis patients, (39) where it is thought to provide co-stimulatory signals for autoreactive T cell

activation (40). In this disease, TGF- β , IL-6 and IL-17 are also produced at high levels, and independent studies have associated each of these cytokines with worse clinical outcomes. Therefore, it is possible that instead of, or in addition to, providing a costimulatory signal for autoreactive T cells in this disease, TSP-1 activates TGF- β in the context of a Th17 immune response. However, further investigation is needed in order to assess the role that TSP-1 might play in this and other Th17-mediated autoimmune diseases.

Our results in the EAE model are different than what has been observed in experimental autoimmune uveoretinitis (EAU). In EAU, TSP-1_{null} mice develop a severe and unrelenting form of disease by comparison with wt controls (41). This may relate to mechanisms of pathogenesis of EAU as IL-17^{-/-} mice develop EAU of equal severity to wt controls (42), whereas IL-17^{-/-} mice develop a milder form of EAE by comparison with wt controls (6). In EAU, other pro-inflammatory molecules such as IFN- γ appear to play a more significant role in mediating disease, even in the absence of IL-17 (6,42).

In summary, our results suggest that T-cell derived TSP-1 is a key activator of TGF- β during the generation of the Th17 response which drives inflammation in EAE. Whether TSP-1 plays a similar role in other autoimmune diseases mediated by IL-17 deserves further investigation.

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Figure 1. TSP-1 plays an important role in EAE

TSP-1^{null} (A, B) and 2D2 TSP-1^{null} mice (C) and their control groups were immunized with different doses of MOG₃₅₋₅₅/CFA. Clinical EAE was followed over time as described in *Methods*. **A**, TSP-1^{null} mice, 10 μ g MOG₃₅₋₅₅ per mouse. **B**, TSP-1^{null} mice, 50 μ g MOG₃₅₋₅₅ per mouse. **C**, 2D2-TSP-1^{null} mice, 10 μ g MOG₃₅₋₅₅ per mouse. **D-E**. Histological analysis of spinal cord sections from immunized TSP-1^{null} and wt control mice sacrificed on d16 post-immunization with 150 μ g MOG₃₅₋₅₅/CFA (150 μ g). The black arrows point to areas of cellular infiltration in the wt sections. **LFB**= Luxol fast blue staining; **H&E**= hematoxylin and eosin staining. Top panel magnification = $\times 10$; bottom panel = $\times 20$.

Figure 2. T-cell derived TSP-1 plays a key role in EAE induced by adoptive transfer

A. Scheme of T-cell expansion, activation and transfer. splenocytes of 2D2 mice or 2D2-TSP-1_{null} mice were stimulated with 20 μ g/ml MOG₃₅₋₅₅ in the presence of 5 μ g/ml IL-2/IL-7 for 2 days. Cells were expanded continuously in the presence of IL-2/IL-7 for four more days, and were re-activated by plate bound anti-CD3/anti-CD28 in the presence of 20ng/ml IL-12 and IL-18. Activated T cells were collected, washed, transferred into recipient mice *i.p.* in the following day. B. EAE course of wt and TSP-1_{null} recipients given *in vitro* polarized 2D2 T cells or 2D2-TSP-1_{null} T cells. Each group contains 5-8 recipients. Data shown are representative of two independent experiments with identical results.

Figure 3. TSP-1_{null} mice mount attenuated IL-17 and IFN γ responses

TSP-1_{null} and wt mice were immunized with MOG (10-50 μ g)/CFA as described in *Methods*. Ten days later their spleens were harvested and re-challenged in culture with MOG₃₅₋₅₅ (20 μ g/ml). Cultures were treated with 1 μ Ci ³H-thymidine for the last 16hrs to measure the T-cell proliferation (**A, 10 μ g/mouse; B, 50 μ g/mouse**) Culture supernatants were collect at the end of a 72hr culture period and cytokine production (**C, 10 μ g/mouse; D, 50 μ g/mouse**), were measured as described in *Methods*. Data shown is representative of 3 independent experiments (n=3 mice per group)

Figure 4. 2D2 TSP-1_{null} mice produce decreased levels of IL-17 and IFN γ

A, cytokine patterns of 2D2 and 2D2-TSP-1_{null} mice. Total splenocytes of naïve, non-treated 2D2 and 2D2-TSP-1_{null} mice were stimulated with 20 μ g/ml MOG₃₅₋₅₅. Supernatants were harvested 2 days after stimulation, and cytokine levels were detected by CBA and ELISA. **B, cytokine patterns of re-stimulated 2D2 and 2D2-TSP-1_{null} CD4⁺ T cells.** 2D2 and 2D2-TSP-1_{null} T cells were activated, expanded as described in Figure 3. Six day after expansion, cells were re-activated by plate bound anti-CD3/anti-CD28 in the presence of IL-12/IL-18 for 48hrs, and cytokine levels were detected by CBA and ELISA.

Figure 5. T-cell derived TSP-1 activates latent TGF- β

A. Assay for latent TGF- β activation. To determine if naïve CD4⁺ T cells can activate latent TGF- β , naïve CD4⁺ T cells were isolated from TSP-1^{null} mice and cultured with or without 10 ng/ml latent TGF- β . Two, six and 24 hrs later, total and active TGF- β in the culture supernatants were quantified via ELISA. As control, CD4⁺ T cells were isolated from wt mice and subjected to same treatments of latent TGF- β and quantifications. A culture that only contains latent TGF- β but no CD4⁺ T cells (latent TGF- β alone) was also included. The results of this experiment were summarized in **B** (active TGF- β) and **D** (total TGF- β). To determine if *activated* CD4⁺ T cells can transform latent TGF- β , CD4⁺ T cells isolated from TSP-1^{null} and wt mice were pre-activated with plate-bound anti-CD3/anti-CD28 for 24hrs before subjected to same treatments of latent TGF- β and quantifications. The results of this experiment were summarized in **C** (active TGF- β) and **E** (total TGF- β). **B.** Naïve, non-stimulated CD4⁺ T cells from wt and TSP-1^{null} mice fail to activate latent TGF- β . **C.** Anti-CD3/anti-CD28

stimulated CD4⁺ T cells activate latent TGF- β in a TSP-1 dependent way. D. Naïve CD4⁺ T cells from wt and TSP-1_{null} mice produce excessive total TGF- β after exogenous latent TGF- β treatment. E. Excessive latent TGF- β production by activated CD4⁺ T cells from wt and TSP-1_{null} mice. The symbols are listed as following: Latent TGF- β alone, culture medium containing 10 ng/ml latent TGF- β but no CD4⁺ T cells; B6/CD4 alone, B6 CD4⁺ T cells alone, not treated with latent TGF- β ; B6/CD4+latent TGF- β , B6 CD4⁺ T cells treated with 10ng/ml latent TGF- β ; TSP-1_{null}/CD4 alone, TSP-1_{null} CD4⁺ T cells alone, not treated with latent TGF- β ; TSP-1_{null}/CD4+latent TGF- β , TSP-1_{null} CD4⁺ T cells treated with 10ng/ml latent TGF- β . For D and E, Please note that the levels of total TGF- β in the CD4⁺ T-cell cultures treated with latent TGF- β were significantly higher than that of control treatment (Latent TGF- β alone, which contains NO CD4⁺ T cell).

Figure 6. T-cell derived TSP-1 supports Th17 differentiation *in vitro*

Purified wt and TSP-1_{null} CD4⁺ T cells were activated via plate-bound anti-CD3/anti-CD28 in the presence of a constant dose of IL-6 (25 ng/ml), and three different doses latent TGF- β (LTGF- β , 14, 43, and 130 ng/ml). Control cultures were treated with 25 ng/ml of IL-6 and 5 ng/ml of active TGF- β . Culture supernatants were assayed 48hr and 96hr later for IL-17.

Table 1
EAE in MOG35-55 Immunized wt and TSP-1_{null} mice

Group	Immunizing Dose	Incidence *	Mortality (%)	Onset day (mean±sem)	Max score (mean±sem)
B6	10µg	8/12 (67%)	0	9.5±0.2	1.2±0.2
TSP-1 _{null}	10µg	1/12 (8%)	0	12.8±0.7 ***	0.6±0.1 ***
B6	50µg	10/10 (100%)	0	9.2±0.	3.1±0.1
TSP-1 _{null}	50µg	10/10 (100%)	0	12.7±0.7 ***	2.6±0.1 **
2D2	10µg	10/11 (91%)	55	8.5±0.2	4.1±0.4
2D2 TSP-1 _{null}	10µg	4/9 (44%)	11	10.8±0.5	1.9±0.6 ***

Mice were immunized with MOG35-55 peptide emulsified in complete Freund's Adjuvant and monitored for the development of EAE. Statistical analysis was performed by comparing two groups using *t*-test

* EAE Clinical score equals or greater than 1.

** p<0.001

*** p<0.001