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Analysis of TGF- β 1 and TGF- β 3 as Regulators of Encephalitogenic Th17 Cells: Implications for Multiple Sclerosis

Priscilla W. Lee^a, Yuhong Yang^b, Michael K. Racke^c, and Amy E. Lovett-Racke^d

Priscilla W. Lee: lee.4037@osu.edu; Yuhong Yang: yuhong.yang@osumc.edu; Michael K. Racke: michael.racke@osumc.edu

^aMolecular Cellular and Developmental Biology Graduate Program, Department of Microbial Infection and Immunity, The Ohio State University, 460 W 12th Ave, Room 660, Columbus, OH, USA

^bDepartment of Neurology, The Ohio State University Wexner Medical Center, 460 W 12th Ave, Room 660, Columbus, OH, USA

^cDepartment of Neurology, Department of Neuroscience, The Ohio State University Wexner Medical Center, 460 W 12th Ave, Room 686, Columbus, OH, USA

Abstract

The phenotype of the CD4⁺ T cells that mediate the CNS pathology in multiple sclerosis is still unclear, and yet a vital question for developing therapies. One of the conundrums is the role of TGF- β in the development of encephalitogenic Th17 cells. In the present study, TGF- β 1 and TGF- β 3 were directly compared in their capacity to promote the differentiation of myelin-specific Th17 cells that could induce experimental autoimmune encephalomyelitis (EAE). Myelin-specific CD4⁺ T cell receptor transgenic cells differentiated with antigen in the presence of IL-6+TGF- β 1 or IL-6+TGF- β 3 generated T cells that produced robust amounts of IL-17, but were incapable of inducing EAE when transferred into mice. Further analysis of these non-encephalitogenic Th17 cells found that they expressed lower amounts of GM-CSF or IL-23R, both molecules necessary for encephalitogenicity. Thus, TGF- β , irrespective of isoform, negatively regulates the differentiation of encephalitogenic Th17 cells.

Keywords

Multiple Sclerosis; experimental autoimmune encephalomyelitis; TGF- β 1; TGF- β 3; T cell encephalitogenicity; Th17 cells; IL-23R; GM-CSF

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^dCorresponding Author: Amy E. Lovett-Racke, Department of Microbial Infection and Immunity, Department of Neuroscience, The Ohio State University Wexner Medical Center, Biomedical Research Tower, 460 W 12th Ave, Room 684, Columbus, OH 43210, USA. Phone: (614) 688-5647; Fax: (614) 292-7544, amy.lovett-racke@osumc.edu.

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1. Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) thought to have a CD4⁺ T cell-mediated autoimmune etiology. One of the questions that is still being debated is the phenotype of the CD4⁺ T cells that mediate CNS pathology. Historically, it was believed that myelin-specific Th1 cells were the encephalitogenic population in MS and its animal model, Experimental Autoimmune Encephalomyelitis (EAE) (Ando et al., 1989; Lovett-Racke et al., 2004; Olsson et al., 1990; Pelfrey et al., 2000; Sun et al., 1991; Waldburger et al., 1996). The observation that Interferon- γ (IFN- γ)-deficient mice were susceptible to EAE (Ferber et al., 1996) led to the speculation that another CD4⁺ T cell population may be responsible for mediating the disease. In 2005, it was demonstrated that myelin-specific, IL-17-producing T cells could induce EAE (Langrish et al., 2005). Numerous studies have investigated how Th17 cells develop. IL-6+TGF- β 1 can efficiently drive the differentiation of Th17 cells *in vitro* (Bettelli et al., 2006; Mangan et al., 2006). However, we and others have shown that myelin-specific T cells differentiated with IL-6+TGF- β 1 failed to induce EAE (Das et al., 2009; Ghoreschi et al., 2010; McGeachy et al., 2007; Yang et al., 2009), while myelin-specific Th17 cells generated with IL-6 plus neutralization of the Th1/Th2 pathways were highly pathogenic, suggesting TGF- β 1 was negatively regulating the encephalitogenic capacity of CD4⁺ T cells. A recent publication suggests that IL-6+TGF- β 3 may be more effective than IL-6+TGF- β 1 at generating pathogenic myelin-specific T cells (Lee et al., 2012). In this study, we used a myelin-specific T cell receptor (TCR) transgenic system to directly compare TGF- β 3 and TGF- β 1-generated Th17 cells in their capacity of inducing EAE.

2. Materials and Methods

2.1 Animals and adoptive transfer of EAE

MBP Ac1-11-specific T cell receptor (TCR) transgenic B10.PL mice have been previously described (Goverman et al., 1993). Wild-type B10.PL mice were purchased from The Jackson Laboratory. All mice were bred and maintained in a specific pathogen-free animal facility at The Ohio State University Wexner Medical Center. The Institutional Animal Care and Use Committee at The Ohio State University approved all animal protocols.

EAE was induced by intraperitoneal injection of 5×10^6 cells/mouse in 200 μ l PBS. EAE disease course was scored on a scale of 0–6: 0, no clinical disease; 1, limp tail; 2, moderate hind limb weakness; 3, severe hind limb weakness; 4, complete hind limb paralysis; 5, quadriplegia or premoribund state; 6, death due to EAE.

2.2 In vitro T cell differentiation

Splenocytes were isolated from naive 6–10-week-old MBP Ac1-11-specific TCR transgenic mice and cultured in 24-well plates at 2×10^6 cells/well with 6×10^6 cells/well of feeder cells (irradiated B10.PL splenocytes). T effector cells were generated with MBP Ac1-11 peptide (10 μ g/ml) and different combinations of cytokines and neutralizing antibodies. The combinations were as follows: mouse IL-12 (0.5 ng/ml); mouse IL-6 (25 ng/ml) plus anti-IFN- γ (2 μ g/ml), anti-IL-12 (0.5 μ g/ml) and anti-IL-4 (1 μ g/ml); human TGF- β 1 (1 μ g/ml) plus mouse IL-6 (25 ng/ml); human TGF- β 3 (1 μ g/ml) plus mouse IL-6 (25 ng/ml). IL-6 was

purchased from Miltenyi Biotec. The other cytokines and antibodies were obtained from R&D Systems.

2.3 ELISA

Supernatants from each cultured condition were collected and analyzed for IFN- γ , IL-17A and GM-CSF. ELISA was performed using purified capture antibodies and biotinylated detection antibodies (IFN- γ and GM-CSF, R&D Systems; IL-17A, eBioscience). Cytokine concentrations were calculated by generating a standard curve using recombinant proteins (R&D Systems) and analyzed using SoftMax Pro Software.

2.4 Flow cytometric analysis

Flow cytometric analysis was performed to evaluate IL-23R expression in CD4⁺ T cells. Cells were collected, washed and resuspended in staining buffer (1% BSA in PBS) and then stained for cell-surface markers. Approximately 100,000 cell events were acquired on a FACSCanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.). PerCP-anti-CD4 and FITC-anti-CD44 were obtained from BD BioScience. APC-anti-IL-23R was obtained from R&D systems.

2.5 RNA isolation and Real-time PCR

RNA was isolated from T cells with TRIzol Reagent (Ambion). cDNA was generated with 1 μ g of RNA per reaction by using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol.

Real-time PCR was performed using the Taqman Gene Expression assay (Applied Biosystems). Results were analyzed with the comparative threshold cycle (Ct) method, by which data were normalized with internal control gene, *Hprt1*. Relative fold change was calculated relative to the average of IL-6+Ab* condition.

2.6 Statistical analysis

Prism software (GraphPad) was used to perform all statistical analyses. A single comparison was analyzed by two-tailed unpaired Student *t* test ELISA data. A *p*-value<0.05 was considered significant. EAE scores are graphed as means $\pm$ SEM.

3. Results

In order to compare the encephalitogenicity of Th17 cells generated with IL-6 in the presence or absence of TGF- β 1 or TGF- β 3, we used splenocytes from naïve MBP Ac1-11-specific TCR transgenic mice. The splenocytes were cultured with MBP Ac1-11 and IL-12 (Th1), IL-6 plus anti-IL-4/IL-12/IFN- γ (Th17), IL-6+TGF- β 1 (Th17), or IL-6+TGF- β 3 (Th17). Antibodies to neutralize IL-4, IL-12 and IFN- γ were added to the IL-6 condition to prevent the dominant effects of these cytokines on the differentiation of the CD4⁺ T cells, favoring Th17 differentiation. TGF- β blocks the signaling pathways that promote Th1 and Th2 differentiation (Gorelik et al., 2002; 2000), reducing the necessity of the neutralizing antibodies. The cells were transferred into wild type mice after 72 hours in culture. Mice receiving Th1 cells and Th17 cells generated in the absence of TGF- β developed EAE (Fig.

1A). In contrast, mice that received Th17 cells generated with IL-6+TGF- β 1 or TGF- β 3 did not develop disease, suggesting that TGF- β irrespective of isoforms does not promote the differentiation of encephalitogenic Th17 cells.

To phenotype the Th17 populations differentiated under the various conditions, we used ELISA to measure cytokine expression. Th17 cells generated with IL-6+TGF- β 1 and IL-6+TGF- β 3 produced comparable amounts of IL-17 (Fig. 1B). The encephalitogenic Th17 cells produced by IL-6+neutralizing antibodies had similar amount of IL-17 compared to the Th17 cells generated with TGF- β . As expected, Th1 cells express high levels of IFN- γ and low IL-17.

Several studies have shown that GM-CSF is a critical cytokine produced by encephalitogenic T cells (Codarri et al., 2011; El-Behi et al., 2011; Ponomarev et al., 2007). Therefore, we analyzed the level of GM-CSF produced by the myelin-specific T cells differentiated under the various conditions. The T cells that induced EAE expressed GM-CSF, whereas the non-encephalitogenic Th17 cells did not (Fig. 1B), suggesting TGF- β suppressed GM-CSF production.

It has been shown that IL-23 signaling was a critical component of generating encephalitogenic Th17 cells (Cua et al., 2003). Thus, we investigated IL-23R expression on the myelin-specific T cells differentiated with IL-6 in the presence or absence of TGF- β 1 or TGF- β 3. The percentage of IL-23R⁺ cells in IL-6+TGF- β 1 and IL-6+TGF- β 3 conditions were comparable (7.1% and 8.7%), but substantially lower than the percentage of IL-23R⁺ cells differentiated with IL-6+neutralizing antibodies (23.4%) (Fig. 1C). This suggested that TGF- β dampened the expression of IL-23R and diminished the encephalitogenic capacity of Th17 cells.

To control for the neutralizing antibodies used in the encephalitogenic Th17 culture conditions, as well as address the contribution of IL-23 to the encephalitogenicity of Th17 cells, the experiments were repeated with these additional parameters. Addition of anti-IL-4/IL-12/IFN- γ to T cells differentiated with IL-6+TGF- β 1 or IL-6+TGF- β 3 resulted in Th17 cells that failed to induce EAE (Fig. 2A). The addition of IL-23 to the cultures did not restore encephalitogenicity. Analysis of cytokine expression by the Th17 cells prior to transfer indicates that all conditions produced abundant IL-17 and minimal IFN- γ (Fig. 2B). Consistent with the prior experiment, GM-CSF was significantly higher in the IL-6+Ab condition. In a previous study (Lee et al., 2012), it was demonstrated that *Tgfb3* mRNA was significantly elevated in CD4⁺ T cells differentiated with IL-6+TGF- β 1+IL-23, and this may be responsible for promoting highly encephalitogenic Th17 cells. However, *Tgfb3* mRNA levels were very similar in all our culture conditions, irrespective of IL-23 (Fig. 2C). *Il23r* mRNA levels were significantly lower in the presence of TGF- β . In addition, *Tbx21*, the gene that encodes T-bet, expression was significantly higher in the encephalitogenic Th17 cells, consistent with our previous observation that T-bet can regulate *Il23r* expression and this is associated with encephalitogenicity (Ghoreschi et al., 2010; Gocke et al., 2007; Yang et al., 2009).

4. Discussion

This study was designed to determine if TGF- β 3 was capable of promoting the differentiation of encephalitogenic Th17 cells. Using a myelin-specific TCR transgenic mouse system, we had previously demonstrated that TGF- β 1 plus IL-6 was very efficient at generating myelin-specific Th17 cells, but these Th17 cells were not encephalitogenic and did not induce EAE (Yang et al., 2009). The fact that TGF- β 1 was not necessary for EAE induction via Th17 cells, and may actually negatively regulate pathways necessary for the development of encephalitogenic Th17 cells was validated in additional studies (Das et al., 2009; Ghoreschi et al., 2010). Yet, a recent study demonstrated that deletion of IRF8 made mice resistant to EAE and suggested that this was due to the inability of these mice to produce active TGF- β and lack of Th17 cells. However, these mice also had a global defect in CD4 T cell differentiation which may have made them EAE resistant (Yoshida et al., 2014). A study comparing TGF- β 1 and TGF- β 3 in their ability to generate encephalitogenic Th17 cells concluded that TGF- β 3 promoted the development of highly pathogenic Th17 cells, whereas TGF- β 1 generated less pathogenic Th17 cells (Lee et al., 2012). Using an antigen-driven myelin-specific TCR transgenic mouse system, we found that TGF- β 3, like TGF- β 1, did not promote the differentiation of encephalitogenic Th17 cells. Both TGF- β 1 and TGF- β 3 in the presence of IL-6 generated CD4⁺ T cells that produced abundant IL-17 (Fig. 1B and 2B), but these cells failed to induce EAE when transferred into recipient mice (Fig. 1A and 2A).

It had previously been demonstrated that GM-CSF was a critical cytokine produced by encephalitogenic T cells (Codarri et al., 2011; El-Behi et al., 2011; McQualter et al., 2001; Ponomarev et al., 2007), and thus, GM-CSF levels were evaluated in the myelin-specific CD4⁺ T cells differentiated with TGF- β 1 and TGF- β 3. Interestingly, Th17 cells generated with TGF- β 1 or TGF- β 3 did not produce GM-CSF, whereas myelin-specific Th1 cells and encephalitogenic Th17 cells differentiated in the absence of TGF- β readily produced GM-CSF (Fig. 1B and 2B). IL-23 had also been implicated in the development of encephalitogenic Th17 cells (Becher et al., 2003; Cua et al., 2003; Langrish et al., 2005; Lee et al., 2012). However, Th17 cells differentiated with TGF- β 1 or TGF- β 3 had a dramatically lower expression level of IL-23R in comparison to Th17 cells differentiated with IL-6 in the absence of TGF- β 1 or TGF- β 3 (Fig. 1C and 2C), suggesting that the Th17 cells differentiated with TGF- β 1 or TGF- β 3 would be less sensitive to IL-23-mediated signaling. This contradicts the findings of Lee *et al* (Lee et al., 2012) that IL-23R mRNA and protein were increased in IL-6+TGF- β 3 generated Th17 cells compared to IL-6+TGF- β 1 generated Th17 cells, but this study did not compare IL-23R levels to cells differentiated with IL-6 in the absence of TGF- β . The reduced levels of GM-CSF and IL-23R signaling may at least partially explain why myelin-specific Th17 cells generated in the presence of TGF- β may not be encephalitogenic. Since TGF- β 1 and TGF- β 3 function through the same receptor complex, it is not surprising that they would generate Th17 cells with a similar phenotype. We had previously found that Th17 cells generated with IL-6+TGF- β 1 can revert to a pathogenic phenotype when restimulated with antigen in the absence of TGF- β 1; however, these CD4⁺ T cells now produced robust amounts of IFN γ (Yang et al., 2009). The methodology used in the *in vitro* differentiation of Th17 cells differs between this study and

the study that found TGF- β 3 promotes the development of highly encephalitogenic Th17 cells (Lee et al., 2012), notable is the fact that this study used antigen-driven differentiation which may at least partially explain the difference. In addition to the use of a different T cell receptor transgenic model, the previous study transferred the Th17 cells after a secondary stimulation with anti-CD3/CD28 in the absence of TGF- β as described in Jäger et al (Jäger et al., 2009; Lee et al., 2012), not after the primary differentiation as performed in this study. Thus, the absence of TGF- β may restore encephalitogenicity, and implies that the CD4⁺ T cells maintain plasticity. This is consistent with the observation that TGF- β promotes the epigenetic modifications of the *Il17a-Il17f* locus that are reversible during restimulation of Th17 cells in the absence of TGF- β (Mukasa et al., 2010).

It is also important to note that systemic administration of TGF- β 1 or TGF- β 2 to mice with EAE resulted in amelioration of disease (Racke et al., 1991; 1993), and administration of anti-TGF- β worsens EAE (Johns and Sriram, 1993). If TGF- β signaling promoted the generation of encephalitogenic Th17 cells, one would have anticipated that TGF- β therapy would have exacerbated EAE. In addition, numerous studies on human CD4⁺ T cells demonstrated that Th17 cells are readily differentiated without exogenous TGF- β (Acosta-Rodriguez et al., 2007; Evans et al., 2007; Santarlasci et al., 2009; Wilson et al., 2007), suggesting that TGF- β is likely not a vital player in the development of encephalitogenic CD4⁺ Th17 cells in humans. However, TGF- β clearly plays a critical role in the development of CD4⁺ regulatory cells, which would be consistent with the observation that administration of TGF- β results in amelioration of disease. It has also been shown that TGF- β has a regulatory effect on encephalitogenic Th1 cells (Huss et al., 2011; 2010), further supporting the dominant immunosuppressive role of TGF- β in autoimmune demyelinating disease.

TGF- β is a pleiotrophic cytokine that has numerous functions. Its role in the differentiation of Th17 cells that can induce central nervous system pathology has been controversial. In this study, we found that both TGF- β 1 and TGF- β 3 in the presence of IL-6 promoted the development of myelin-specific Th17 cells, but these Th17 cells were not encephalitogenic and had reduced expression key molecules associated with encephalitogenic T cells.

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Abbreviations used in this paper

MS	Multiple Sclerosis
EAE	experimental autoimmune encephalomyelitis
Th	T helper
IL-6	interleukin-6
IL-17	interleukin-17

IL-23	interleukin-23
IL-23R	interleukin-23 receptor
TCR	T cell receptor
TGF-β	Transforming growth factor- β
MBP	myelin basic protein
IFN-γ	interferon- γ
GM-CSF	Granulocyte-macrophage colony-stimulating factor

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Highlights

1. Both TGF β 1 and TGF β 3 promote the differentiation of myelin-specific Th17 cells, but fail to generate encephalitogenic Th17 cells capable of inducing experimental autoimmune encephalomyelitis.
2. Myelin-specific Th17 cells generated with TGF β 1 or TGF β 3 have similar expression of IL-17, but lack expression of GM-CSF, a cytokine highly associated with encephalitogenicity.
3. Th17 cells generated with TGF β 1 or TGF β 3 express lower levels of IL-23R, a critical signaling component of encephalitogenic T cells.

Figure 1. Neither IL-6+TGF-β1 nor IL-6+TGF-β3 generate encephalitogenic Th17 cells
Splenocytes from naïve MBP Ac1-11-specific TCR transgenic mice were differentiated *in vitro* with MBP Ac1-11 peptide in the presence of IL-12, IL-6+anti-IFN-γ/IL-12/IL-4 (Ab*), IL-6+TGF-β1, and IL-6+TGF-β3. (A) After 72 hours, cells were harvested and adoptively transferred into naïve B10.PL mice (means ± SEM). (B) Supernatants were analyzed by ELISA for IFN-γ, IL-17A, and GM-CSF (means ± SEM). (C) After 72 hours, cells were analyzed by flow cytometry. Cells were gated on CD4⁺CD44^{hi}, and percentages of IL-23R⁺ cells are shown. Data is representative of three independent experiments.

Figure 2. Additional IL-23 does not restore the encephalitogenicity of Th17 cells generated with IL-6+TGF- β 1 or IL-6+TGF- β 3

Splenocytes from naïve MBP Ac1-11-specific TCR transgenic mice were differentiated *in vitro* with MBP Ac1-11 peptide in the presence of various combinations of cytokines and anti-IFN- γ /IL-12/IL-4 antibodies (Ab*). (A) After 72 hours, cells were harvested and adoptively transferred into naïve B10.PL mice (means $\pm$ SEM). (B) Supernatants were analyzed by ELISA for IFN- γ , IL-17A, and GM-CSF (means $\pm$ SEM). The level of GM-CSF in IL-6+Ab* condition was significantly higher than other conditions (Student *t* test, $p < 0.0001$) (C) RNA was extracted at the 48 hours time point. *Tgfb3*, *Il23r*, and *Tbx21* mRNA were determined by RT-PCR. Data were expressed as fold change relative to IL-6+Ab* condition (means $\pm$ SEM). The mRNA expressions of *Tgfb3*, *Il23r*, and *Tbx21* in IL-6+Ab* condition were significantly higher comparing to other conditions (Student *t* test, $p < 0.0005$).