Cell Transplantation
2019, Vol. 28(9-10) 1155–1160

DOI: 10.1177/0963689719852354
journals.sagepub.com/home/cll
SAGE

TGF-β in Mice Ameliorates Experimental Autoimmune Encephalomyelitis in Regulating NK Cell Activity

J. Xu[1,2], Y. Wang[3], H. Jiang[3], M. Sun[2,*], J. Gao[3,*], and A. Xie[1,*]

Abstract

Multiple sclerosis is a disease characterized by inflammation and demyelination located in the central nervous system. Experimental autoimmune encephalomyelitis (EAE) is the most common animal model for multiple sclerosis (MS). Although the roles of T cells in MS/EAE have been well investigated, little is known about the functions of other immune cells in the neuroinflammation model. Here we found that an essential cytokine transforming growth factor β (TGF-β) which could mediate the differentiation of Th17/regulatory T cells was implicated in the natural killer (NK) cells' activity in EAE. In EAE mice, TGF-β expression was first increased at the onset and then decreased at the peak, but the expressions of TGF-β receptors and downstream molecules were not affected in EAE. When we immunized the mice with MOG antigen, it was revealed that TGF-β treatment reduced susceptibility to EAE with a lower clinical score than the control mice without TGF-β. Consistently, inflammatory cytokine production was reduced in the TGF-β treated group, especially with downregulated pathogenic interleukin-17 in the central nervous system tissue. Furthermore, TGF-β could increase the transcription level of NK cell marker NCR1 both in the spleen and in the CNS without changing other T cell markers. Meanwhile TGF-β promoted the proliferation of NK cell proliferation. Taken together, our data demonstrated that TGF-β could confer protection against EAE model in mice through NK cells, which would be useful for the clinical therapy of MS.

Keywords

MS, EAE, TGF-β, NCR1, NK cells

Introduction

Multiple sclerosis (MS) is a disease in which inflammation in the central nervous system (CNS) results in the destruction of myelin sheath[1,2]. Although inflammation combined with environmental and genetic factors plays an essential role in the pathogenesis of MS, the precise mechanisms of MS remain unclear. The breakdown of the blood–brain barrier is the onset symptom of MS, then the inflammatory cells and cytokines invade the CNS to destroy myelin, and the remyelination process determines the recovery after each exacerbation[3]. Acute relapses respond well to corticosteroid therapy, providing further evidence that inflammation is central to the disease process[4]. Experimental autoimmune encephalomyelitis (EAE) is a mouse or rat T cell-mediated autoimmune disease model in the CNS used to simulate the human MS condition[5]. The transforming growth factor β (TGF-β) family of growth factors controls the homeostasis and development in multiple organs. The TGF-β signal

[1] Department of Neurology, The Affiliated Hospital of Qingdao University, China
[2] Department of Clinical Lab, Weifang Maternal and Child Health Hospital, China
[3] Department of Pediatrics, Weifang Maternal and Child Health Hospital, China
[*] These authors contributted equally to this article.

Submitted: July 21, 2018. Revised: February 26, 2019. Accepted: April 16, 2019.

Corresponding Authors:
A. Xie, Department of Neurology, The Affiliated Hospital of Qingdao University, Qingdao, China.
Email: xieanmu@163.com
J. Gao, Department of Pediatrics, Weifang Maternal and Child Health Hospital, 407 Qingnian Road, Weifang, Shandong 261011, P.R. China.
Email: gaojian1650@126.com
M. Sun, Department of Clinical lab, Weifang Maternal and Child Health Hospital, Weifang, Shandong 261011, P.R. China.
Email: sunmq98@163.com

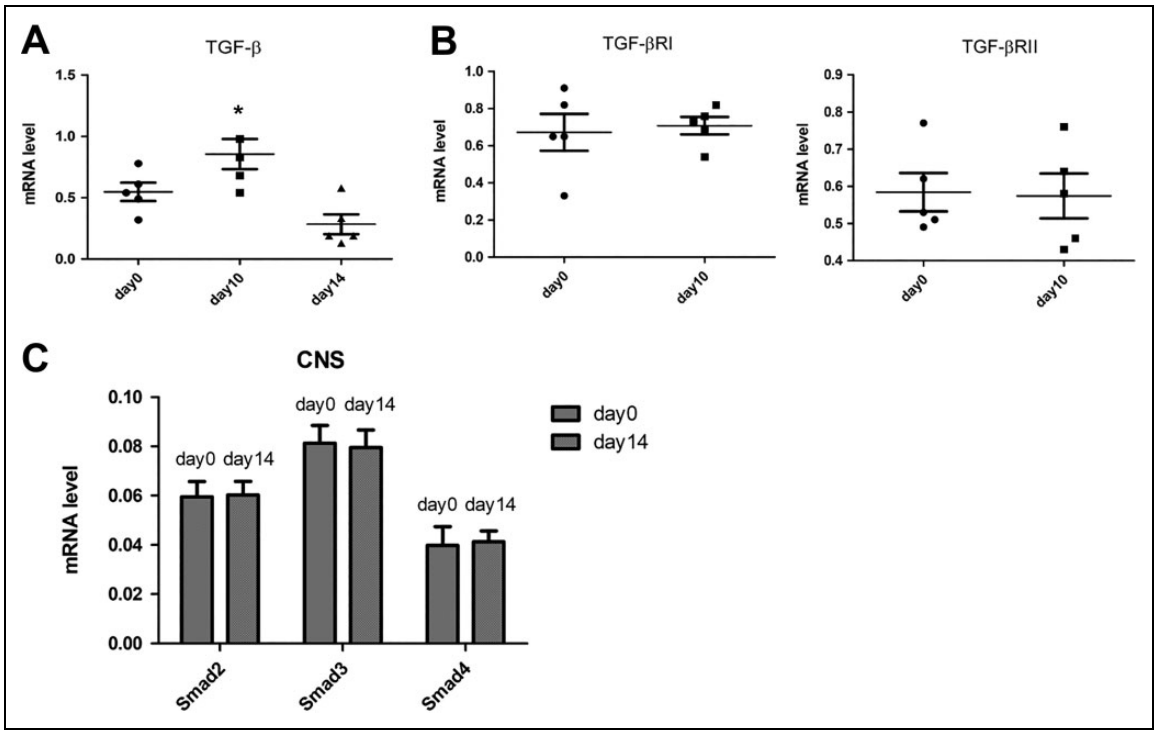

Figure 1. TGF-β expression in experimental autoimmune encephalomyelitis (EAE). (A) The expression of TGF-β on different days during EAE. (B) The expression of TGF-β receptors on different days during EAE. (C) The expression of TGF-β downstream SMAD molecules on day 14 during EAE.
*$p < 0.05$.
CNS: central nervous system; TGF-β: transforming growth factor β.

transduction network involving kinases and their substrates, the SMAD proteins, has been partly elucidated in the past few years[6,7]. After receiving the signaling from TGF-β via the receptors, SMAD can move into the nucleus to activate target gene transcription in association with DNA-binding partners such as transcriptional factors[8]. Therefore, either loss or specific mutations in these pathways could cause various forms of human immune disorders and cancer. It is also reported that TGF-β could regulate regulatory T (Treg) cells' function to inhibit autoimmune diseases mediated by Th17[9]. For the cell-specific mechanism, distinct cells display multifunctional characters in human MS and murine EAE. Although CD4 T cells play a central role in this process, other immune cells, including B cells[10] and natural killer (NK) cells[11], also contribute to the development and determine the severity of the disease. When mice were deprived of NK cells by antibody treatment before immunization, they developed a more serious form of EAE associated with relapse. This phenotype was supported by augmentation of T cell proliferation and production of Th1 cytokines in response to MOG antigen[12]. However, less is known about the mechanisms underlying NK cells in the control of MS/EAE. NK cells distinguish between normal healthy cells and abnormal cells by using a sophisticated repertoire of cell surface receptors that control their activation or inhibitory functions[13]. These receptors on NK cells, including rodent Ly49 receptors, human killer cell immunoglobulin-like receptors, and conserved CD94/NKG2 receptor family, could specifically recognize MHC class I molecules or related ligands or host encoded non-MHC ligands[14].

Materials and Methods

Reagent

Purified TGF-β was purchased from R&D Systems China Co (Wuhan, China). The dose of TGF-β protein for in vivo administration was 200 μg with intraperitoneal (i.p.) injection and for in vitro treatment was 10 ng/ml.

Ethical Approval

The animal care and the experiments described here were carried out in agreement with the guidelines set by the Institutional Animal Investigation Committee of Animal Biosafety Level 3 Laboratory of Wuhan University (Wuhan, China). The protocol was approved by the Committee on the Ethics of Animal Experiments of Wuhan University. Animals were housed under a 12-h light/dark cycle, and were kept in the same animal care facility for the duration of the study. All efforts were made to minimize any suffering and to reduce the total number of animals used.

EAE Induction

To induce EAE in C57BL/6 mice, female mice were immunized subcutaneously in the flanks with 50 μg MOG35–55(MEVGWYRSPFSRVVHLYRNGK) in complete freund's adjuvant (CFA) containing 200 μg of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI, USA), followed by i.p. injection of 100 ng of pertussis toxin (List Biochemicals, Campbell, CA, USA)[15]. Clinical assessment of EAE was performed according to the following criteria from a previous study[16].

Reverse Transcription-Quantitative Polymerase Chain Reaction

Total RNA was extracted from the cells using the RNeasy mini kit (QIAGEN China Co., Shanghai, China), followed by complementary DNA synthesis using the Superscript III first strand synthesis kit (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) at 25°C for 10 min, 50°C for 30 min, and 85°C for 5 min. Quantitative polymerase chain reaction was performed on a Bio-Rad amplifier using the Bio-Rad real time polymerase chain reaction (PCR) mix, including SYBR Green dye (both Bio-Rad, Hercules, CA, USA). The following thermocycling conditions were used for the PCR: 50°C for 2 min, 10 min at 95°C; 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were analyzed using the Cq value normalized to the endogenous reference gene GAPDH[17].

Enzyme-linked immunosorbent assay

Mouse enzyme-linked immunosorbent assay (ELISA) kits for cytokine detection in the sera or homogenate were obtained from R&D Systems China Co. To detect low levels of cytokines in the samples, a standard curve was obtained by diluted standard reagents.

MTT Assay

Cells were incubated with 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reagent (5 μg/ml final concentration) at 37°C for 4 h. Formazan was solubilized by adding 100 μl dimethyl sulfoxide into each well. The extent of formazan production was determined by an ELISA reader at a wavelength of 550 nm, while 630 nm served as the reference wavelength. The results were calculated according to the manufacturer's instructions (Vybrant™ MTT Cell Proliferation Assay Kit, Thermo Fisher Scientific).

Results

EAE Model TGF-β Expression was Declined Without Affecting its Receptors and Downstream

Consistent with the clinical MS data[18], we found significant changes of TGF-β 1 expression with an increased level in early EAE but reduced level in the later stage, in both spinal cord and brain (Fig. 1A). However, the other molecules associated with the TGF-β RI and TGF-β RII (Fig. 1B) in spinal cord; meanwhile, the downstream SMAD subtype expression was not influenced in the EAE model (Fig. 1C).

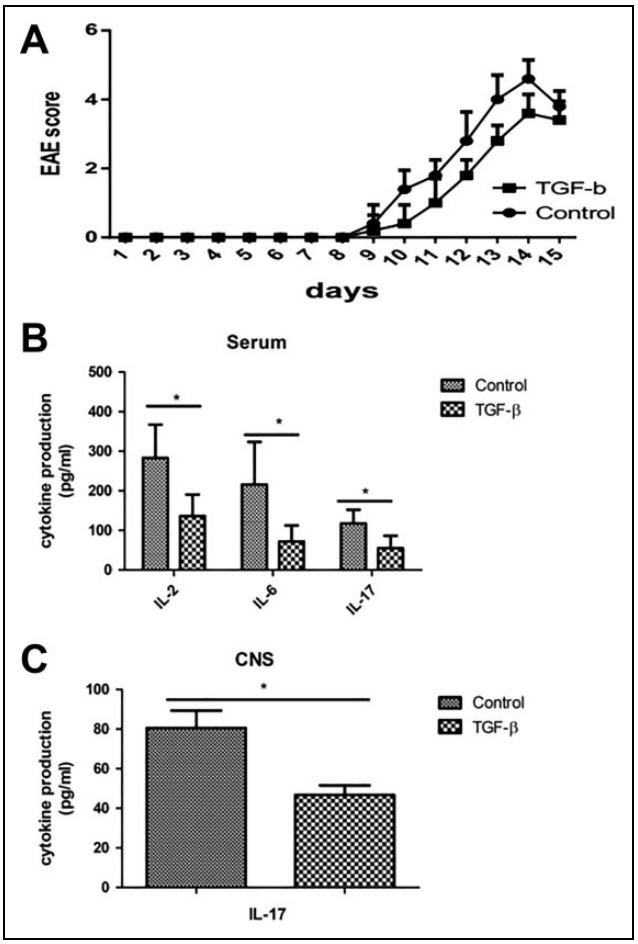

Figure 2. The effect of TGF-β on the experimental autoimmune encephalomyelitis (EAE) model. (A) Clinical score of EAE model following treatment with TGF-β. EAE clinical score shown on Y axis means: 0, no clinical signs; 1, partially limp tail; 2, paralyzed tail; 3, hind limb paresis, uncoordinated movement; 4, one hind limb paralyzed; 5, both hind limbs paralyzed; 6, hind limbs paralyzed, weakness in forelimbs; 7, hind limbs paralyzed, one forelimb paralyzed; 8, hind limbs paralyzed, both forelimbs paralyzed; 9, moribund; 10, death. (B) Serum cytokine production in each group was measured by enzyme-linked immunosorbent assay (ELISA). (C) CNS IL-17 production in each group was measured by ELISA.
*$p < 0.05$.
CNS: central nervous system; IL: interleukin; TGF-β: transforming growth factor β.

TGF-β Treatment Attenuated EAE

Next we tested the function of TGF-β in the EAE model and detected that TGF-β treated mice were resistant to neuroinflammation in EAE with reduced clinical score (Fig. 2A) in the i.p. injection. During a 15-day observation, both the

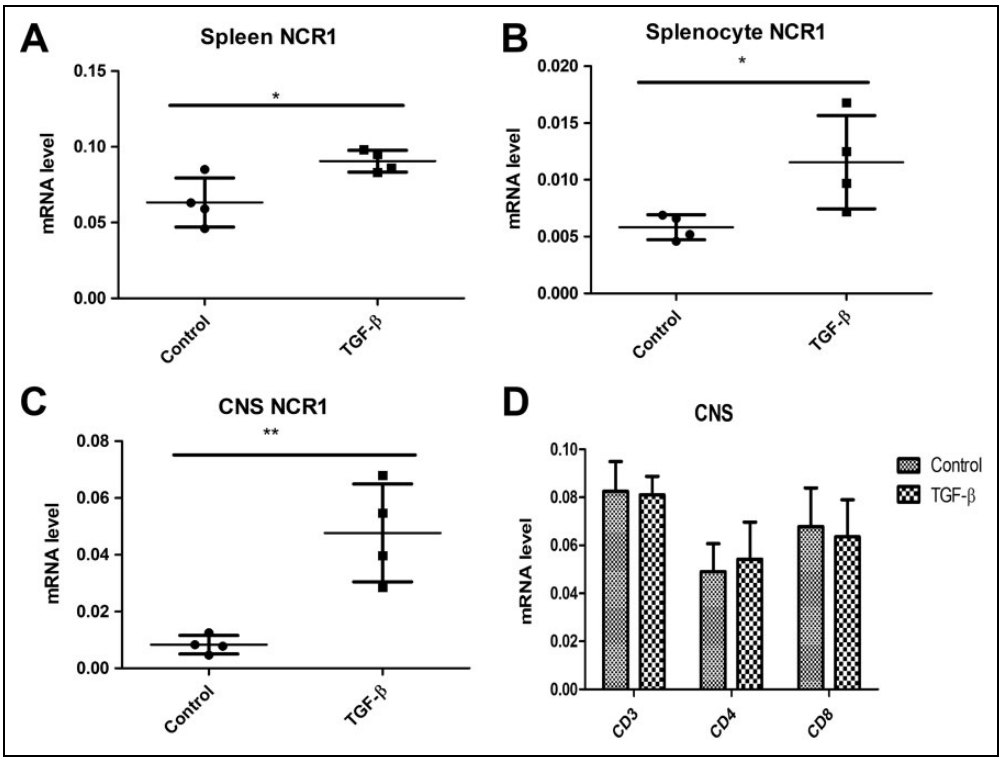

Figure 3. TGF-β regulated natural killer (NK) cell marker NCR1 transcription in vivo. (A) Spleen NCR1 mRNA expression was determined by quantitative polymerase chain reaction (qPCR). (B) Splenocyte NCR1 mRNA expression was determined by qPCR. (C) CNS NCR1 mRNA expression was determined by qPCR. (D) CNS CD molecules' mRNA expression was determined by qPCR.
*$p < 0.05$.
**$p < 0.01$.
CNS: central nervous system; TGF-β: transforming growth factor β.

TGF-β cytokine treated mice and the control group without TGF-β intervention displayed the symptoms of EAE after model establishment from day 8, but TGF-β treatment in vivo could attenuate disease severity, displayed by lower clinical score, suggesting TGF-β suppresses EAE development with certain mechanisms. Considering MS/EAE is a type of autoimmune disease with increased inflammatory cytokine production, we next detected the cytokine profiles in the EAE mice in the presence of TGF-β. Consistent with the phenotype demonstrated by clinical scores, TGF-β inhibited inflammation of EAE with decreased serum levels of interleukin (IL)-2/IL-6/IL-17 (Fig. 2B). Furthermore, as Th17 cells and its cytokine IL-17 play central roles in the EAE model, we observed decreased IL-17 production in the CNS homogenate (spinal cord and brain) from EAE mice with TGF-β treatment, suggesting TGF-β specifically prevents neuroinflammation in the disease development (Fig. 2C).

The NK Marker NCR1 Was Increased by the TGF-β In Vivo

When we treated the mice with high dose TGF-β recombinant protein, we found that the mouse NK cell marker NCR1 was increased not only in spleen and splenocyte (Fig. 3A and B), but also in the CNS tissue (spinal cord in Fig. 3C and brain in Fig. 3D). However, TGF-β failed to alter the T cell markers' expression in CNS such as CD3, CD4 and CD8 (Fig. 3C), suggesting the specific cytokine could selectively promote the proliferation and infiltration of NK cells without affecting infiltrated T cells in the CNS of EAE mice.

TGF-β Increases the NK Cell Proliferation In Vitro

To further confirm the effect of TGF-β in vitro, we evaluated the proliferation of NK cells after TGF-β pretreatment. We found that TGF-β enhanced NK cell proliferation in a dose dependent manner (Fig. 4A). Taken together, our data demonstrated that TGF-β confers protection on the murine EAE model through specific NK cell activity.

Discussion

Th17 and anti-inflammatory Treg cells often keep a balance in the homeostasis of a healthy state, while in the EAE environment, the proinflammatory cytokine IL-17 produced by Th17 cells driven by IL-23 could induce the tissue lesions in the CNS, whereas the IL-17 deficient mice were not sensitive to the EAE model[19–21]. In these two major subsets of

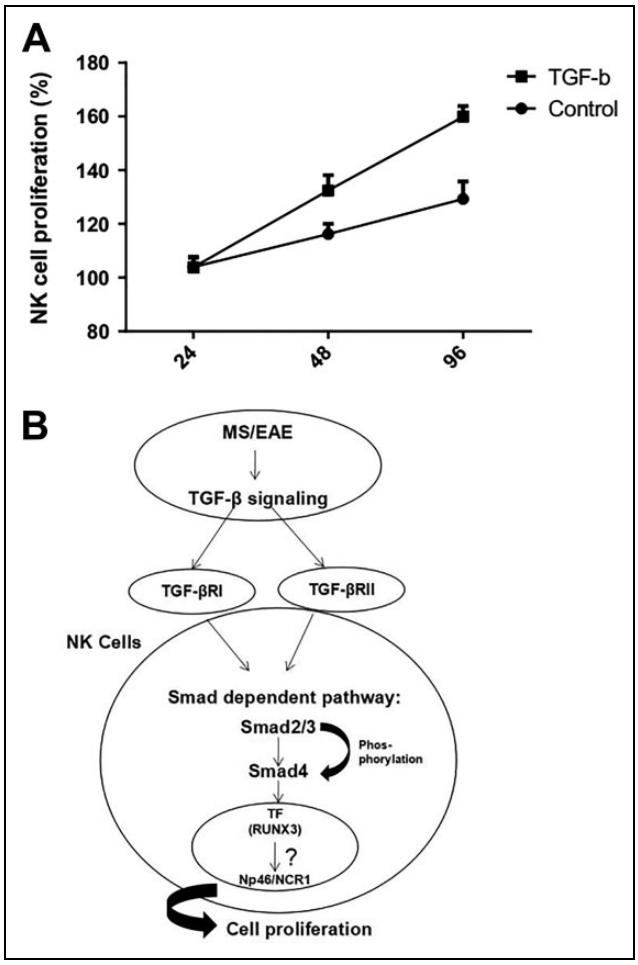

Figure 4. TGF-β regulated NK cell proliferation in vitro. (A) MTT assay of proliferation of NK cells isolated from the control mice treated with TGF-β in vitro. (B) A model of the biological function of TGF-β in the experimental autoimmune encephalomyelitis targeting NK cells.
EAE: experimental autoimmune encephalomyelitis; MS: multiple sclerosis; MTT: 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetra zolium; NK: natural killer; RUNX3: runt-related transcription factor 3; TF: transcription factors; TGF-β: transforming growth factor β.

CD4 T cells, TGF-β played an important role in the in vitro differentiation: TGF-β plus IL-6 as the factors responsible for differentiation of Th17 cells (IL-6 is added to suppress FOXP3 expression but upregulates the Th17 related RORA and RORC genes). However, in the absence of IL-6, Treg cells can be induced by the TGF-β alone in vitro[22]. Signalings through TGF-β and its receptors, including TGF-β RI/ TGF-β RII are essential for both Th17 and Treg development[23,24]. However, the roles of TGF-β and NK cells remain uncertain in the EAE model. In most clinical MS patients, increased pro-inflammatory cytokines were combined with decreased production of TGF-β, which plays an important role as anti-inflammatory cytokine in the manifestation of MS. Defective production of anti-inflammatory cytokine TGF-β by T cell lines was detected in patients with active MS[25]. In this current study we also found that TGF-β confers protection against the mouse model of MS. TGF-β was downregulated in the peak of the disease, and the administration of TGF-β protein can rescue the severity of EAE in a dose dependent manner.

Next, we wondered whether TGF ameliorates EAE through T cell signaling, in the TGF-SMAD signaling; the downstream transcriptional factors could be activated by the TGF through the receptors for gene transcription. However, no T cell markers' (CD3, CD4,CD8) mRNA expression was affected in the CNS region by TGF treatment, suggesting that TGF may regulate a unique pathway in other immune cells. Considering the reported data that NCR1 in NK cells was regulated in the MS white matter lesions by regulating innate immunity[26], we attempted to clarify that NCR1 and NK cells are functionally activated in the EAE model associated with the TGF-β signaling. Natural cytotoxicity receptors (NCRs) are activating receptors expressed on the NK cells. The human NCR family includes NKp30, NKp44, and NKp46, but mice express only the homologue protein NKp46, named NCR1[27]. In fact, pathogenic or regulatory roles of NK cells with the cell marker NCR1 are implicated in many diseases, for example, it is reported that NCR1 plays an essential role in type 1 diabetes. Targeting NK cells' reactivity by employing NCR1 antibody could lower the incidence of diabetes in both the non-obese diabetes model and the low-dose streptozotocin induced diabetes model[28]. In another study, TGF-β was found to be associated with NK cell activity in cancer. In the murine model of head and neck cancer, TGF-β could downregulate the NKG2D, which is the NK-activating receptor[29]. In our study we first evaluated the phenotype of TGF-β in the EAE model and then measured the effect of TGF-β on the NK cells' activating receptor NCR1. In vitro data also supported that TGF-β promoted NK cell proliferation, and we speculate that TGF-β might trigger NCR1 expression as well as NK cell proliferation through the TGF-R-SMAD pathway (Fig. 4B). At the transcriptional level, NCR1/NKp46 expression could be directly regulated by the runt-related transcription factor 3 (RUNX3) in vitro[30], while the TGF-β regulating NCR1 in NK through the RUNX3 still needs further study (Fig. 4B). Taken together, our findings could provide an important therapeutic approach based on immune regulation for the murine EAE model and clinical MS.

Ethical Approval

The study was approved by the Institutional Animal Investigation Committee of Animal Biosafety Level 3 Laboratory of Wuhan University (Wuhan, China).

Statement of Human and Animal Rights

All of the experimental procedures involving animals were conducted in accordance with the Institutional Animal Investigation Committee of Animal Biosafety Level 3 Laboratory of Wuhan University (Wuhan, China).

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

References

1. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mörk S, Bö L. Axonal transection in the lesions of multiple sclerosis. New Engl J Med.1998;338(5):278–285.
2. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, Fujihara K, Havrdova E, Hutchinson M, Kappos L, Lublin FD, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann Neurol. 2011; 69(2):292–302.
3. Minagar A, Alexander JS. Blood–brain barrier disruption in multiple sclerosis. Mult Scler. 2003;9(6):540–549.
4. Hoogstraten MC, Minderhoud JM. A double-blind controlled trial of high dose methylprednisolone in patients with multiple sclerosis. J Neurol Neurosurg Psychiatry. 1988;51(4):597–8.
5. Kurschus FC. T cell mediated pathogenesis in EAE: molecular mechanisms. Biomed J. 2015;38(3):183–193.
6. Li MO, Flavell RA. TGF-β: a master of all T cell trades. Cell. 2008;134(3):392–404.
7. Wang G, Yu Y, Sun C, Liu T, Liang T, Zhan L, Lin X, Feng XH. STAT3 selectively interacts with Smad3 to antagonize TGF-β signaling. Oncogene. 2016;35(33):4388–4398.
8. Annes JP, Munger JS, Rifkin DB. Making sense of latent TGF beta activation. J Cell Sci. 2003;116(2):217.
9. Huter EN, Stummvoll GH, DiPaolo RJ, Glass DD, Shevach EM. Cutting edge: antigen-specific TGF beta-induced regulatory T cells suppress Th17-mediated autoimmune disease. J Immunol. 2008;181(12):8209–8213.
10. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J Clin Invest. 2008;118(10): 3420–3430.
11. Xu W, Tabira T. The role of natural killer (NK) cells in experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis (MS). Adv Neuro Biol. 2011;1(1):87–94.
12. Zhang B, Yamamura T, Kondo T, Fujiwara M, Tabira T. Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. J Exp Med. 1997;186(10):1677–1687.
13. Spits H, Blom B, Jaleco AC, Weijer K, Verschuren MC, van Dongen JJ, Heemskerk MH, Res PC. Early stages in the development of human T, natural killer and thymic dendritic cells. Immunol Rev. 1998;165:75–86.
14. Lanier LL. NK cell recognition. Annu Rev of Immunol. 2005; 23(1):225–274.
15. Kuerten S, Kostova-Bales DA, Frenzel LP, Tigno JT, Tary-Lehmann M, Angelov DN, Lehmann PV. MP4- and MOG:35-55-induced EAE in C57BL/6 mice differentially targets brain, spinal cord and cerebellum. J Neuroimmunol. 2007;189(1–2):31–40.
16. Bittner S, Afzali AM, Wiendl H, Meuth SG. Myelin oligodendrocyte glycoprotein (MOG35-55) induced experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice. J Vis Exp. 2014;86(86):e51275.
17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402–408.
18. Carrieri PB, Provitera V, Bruno R, Perrella M, Tartaglia G, Busto A, Perrella O. Possible role of transforming growth factor-beta in relapsing–remitting multiple sclerosis. Neurol Res. 1997;19(6):599–600.
19. Aranami T, Yamamura T. Th17 cells and autoimmune encephalomyelitis (EAE/MS). Allergol Int. 2008;57(2):115–120.
20. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. The orphan nuclear receptor ROR gammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell. 2006;126(6):1121–1133.
21. Kang Z, Wang C, Zepp J, Wu L, Sun K, Zhao J, Chandrasekharan U, DiCorleto PE, Trapp BD, Ransohoff RM, Li X. Act1 mediates IL-17-induced EAE pathogenesis selectively in NG2+ glial cells. Nat Neurosci. 2013;16(10):1401–1408.
22. Weaver CT, Hatton RD. Interplay between the TH17 and TReg cell lineages: a (co-)evolutionary perspective. Nat Rev Immunol.2009;9(12):883–889.
23. Gorelik L, Flavell RA. Abrogation of TGF beta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity. 2000;12(2):171–181.
24. Massague J. TGF-β signal transduction. Adv Cement Based Material.1998;2(95):30–38.
25. Navikas V, Link H. Cytokines and the pathogenesis of MS. J Neurosci Res. 1996;45(4):322–333.
26. Durrenberger PF, Ettorre A, Kamel F, Webb LV, Sim M, Nicholas RS, Malik O, Reynolds R, Boyton RJ, Altmann DM. Innate Immunity in multiple sclerosis white matter lesions: expression of natural cytotoxicity triggering receptor 1 (NCR1). J Neuroinflamm. 2012;9(1):1.
27. Hecht ML, Rosental B, Horlacher T, Hershkovitz O, De Paz JL, Noti C, Schauer S, Porgador A, Seeberger PH. Natural cytotoxicity receptors NKp30, NKp44 and NKp46 bind to different heparan sulfate/heparin sequences. J Proteome Res. 2009;8(2):712–720.
28. Yossef R, Gur C, Shemesh A, Guttman O, Hadad U, Nedvetzki S, Miletić A, Nalbandyan K, Cerwenka A, Jonjic S, Mandelboim O, Porgador A. Targeting natural killer cell reactivity by employing antibody to NKp46: implications for type 1 diabetes. PLoS One. 2015;10(2):e0118936.
29. Dasgupta S, Bhattacharya-Chatterjee M, O'Malley BWJr, Chatterjee SK. Inhibition of NK cell activity through TGF-βeta 1 by down-regulation of NKG2D in a murine model of head and neck cancer. J Immunol. 2005;175(8):5541–5550.
30. Lai CB, Mager DL. Role of runt-related transcription factor 3 (RUNX3) in transcription regulation of natural cytotoxicity receptor 1 (NCR1/NKp46), an activating natural killer (NK) cell receptor. J Bio Chem. 2012;287(10):7324–7334.