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ORIGINAL ARTICLE

CD226 attenuates Treg suppressive capacity via CTLA-4 and TIGIT during EAE

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Abstract

The Cluster of differentiation 226(CD226)/T cell immunoglobulin and immune receptor tyrosine-based inhibitory motif domain (TIGIT) axis plays an important role in the balance of the immune response. A previous study showed that CD226 is involved in $CD4^+$ T cell differentiation and that blocking CD226 may attenuate experimental autoimmune encephalomyelitis (EAE) development. However, the molecular mechanisms underlying this process remain incompletely understood. In this study, it was found that *Cd226*$^{-/-}$ mice were less susceptible to EAE and that there was less T helper 17(Th17) cell infiltration with higher levels of regulatory cells (Tregs) infiltration in the *Cd226*$^{-/-}$ EAE mouse central nervous system (CNS) compared with that in the WT EAE mouse CNS. Moreover, the suppressive function of *Cd226*$^{-/-}$ Tregs was upregulated compared with that of WT Tregs. Furthermore, it was observed that the expression levels of CTLA-4 and TIGIT on *Cd226*$^{-/-}$ Tregs were higher than those on WT Tregs during EAE in the spleen and CNS. Our results demonstrate a pivotal role for CD226 in attenuating Treg function in EAE that was associated with downregulating the expression levels of CTLA-4 and TIGIT.

Keywords CD226 · EAE · Treg · Th17 cell · CTLA-4 · TIGIT

Introduction

Cluster of differentiation 226 (CD226) is an adhesion and costimulatory molecule that is mainly expressed on immune cells [1]. A series of reports have suggested that CD226 is a multifunctional molecule in autoimmune diseases and tumours that competes with T cell immunoglobulin and immune receptor tyrosine-based inhibitory motif domain (TIGIT) to bind to their common ligands CD155 and CD112 [2]. CD226 is involved in the pathogenesis of several autoimmune diseases because of differential regulation of the pro-inflammatory and anti-inflammatory balance in $CD4^+$ T cell subsets [3, 4]. Multiple sclerosis (MS) is a chronic autoimmune disease that is characterized by the over-activation of pathogenic $CD4^+$ T cells such as T helper 1 (Th1) and Th17 cells and widespread inflammatory processes in the central nervous system (CNS) [5]. Previous studies have reported that the TIGIT/CD226 axis is associated with susceptibility to EAE, a typical mouse model for studying the pathogenesis of MS, because TIGIT antagonizes CD226 and suppresses T cell responses indirectly by the induction of tolerogenic antigen-presenting cells [6].

It is generally known that Th17 cells and their effector, the cytokine interleukin (IL)-17, are important mediators implicated in the pathology of EAE [7]. In addition, decreased expression of transcription factor forkhead box P3 (Foxp3) and the dysregulation of Treg suppression capacity have been linked to the pathogenesis of EAE [8]. Published results demonstrated that mice treated with anti-CD226 pAb were markedly resistant to the development and progression of EAE [9]. In addition, $CD226^+$ $TIGIT^-$ Tregs exhibited decreased suppressive function following expansion in vitro [10]. However, the molecular mechanisms by which CD226 modulates Treg functions to mediate EAE pathogenesis remain unknown. Tregs express multiple checkpoint molecules, including cytotoxic T lymphocyte antigen 4 (CTLA-4), TIGIT and

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programmed cell death 1 (PD-1), which promote their development, stability and suppressive capacity [11–13]. To better elucidate the molecular mechanisms of CD226 in Treg functions during EAE, *Cd226*$^{-/-}$ mice were generated, and the expression levels of CTLA-4, TIGIT and PD-1 on Tregs were assessed.

In this study, we observed that CD226 knockout attenuated Th17-mediated EAE pathogenesis and that there were increased infiltration of Tregs and enhanced production of IL-10 in the mouse CNS. We next confirmed that CD226 deficiency promoted EAE-associated Treg suppressive capacity in vitro. Furthermore, the expression levels of CTLA-4 and TIGIT on *Cd226*$^{-/-}$ Tregs were upregulated. Thus, this study provides support for the application of therapeutic CD226 blocking in inflammatory autoimmune diseases in which Treg suppression is maintained via the upregulation of CTLA-4 and TIGIT expression.

Materials and methods

Mice

Wild-type C57BL/6 mice were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). The CD226 knockout (*Cd226*$^{-/-}$) mice with a C57BL/6 background were gifted by Professor Marco Colonna. To generate homozygous *Cd226*$^{-/-}$ mice, the *Cd226*$^{-/-}$ mice were back-crossed with C57BL/6 mice and then propagated by *Cd226*$^{+/-}$ × *Cd226*$^{+/-}$ mating. The WT (*Cd226*$^{+/+}$) mice that were used as controls were littermates of the *Cd226*$^{-/-}$ mice. All mice were bred in specific pathogen-free (SPF) conditions at the experimental animal centre of the Fourth Military Medical University and were treated according to the Guide for the Care and Use of Laboratory Animals and Welfare Institute (NIH, Bethesda, MD).

EAE model

To induce EAE, mice (6–8 weeks old, female) were subcutaneously immunized in their flanks with 200 μg myelin oligodendrocyte glycoprotein peptide 35–55 (MOG_{35-55}) (Bankpeptide Biotec, China) in complete Freund's adjuvant (Sigma, USA) containing 4 mg of a thermally inactivated tuberculosis strain (Difco, USA). On day 0 and day 2, the immunized mice were intraperitoneally injected with 200 ng *Bordetella pertussis* toxin (Sigma, USA) [14]. The immunized mice were monitored daily and assigned, in a double-blinded manner, a score using the following standard clinical scoring system: 0, no disease; 1, tail paralysis; 2, wobbly gait or partial hind limb paralysis; 3, complete hind limb paralysis; 4, forelimb and hind limb paralysis; and 5, moribund or dead [15].

Cell isolation and stimulation

Splenic Tregs were isolated at 15 to 18 days after immunization using a regulatory T cell isolation kit (MACS Miltenyi Biotec, Germany) following the manufacturer's instructions. Purified CD4$^+$ T cells (cell purity ≥ 95%) from erythrocyte-depleted splenocyte suspensions from *Cd226*$^{-/-}$ or WT mice were negatively selected by using the MojoSort Mouse CD4$^+$ T Cell Isolation Kit (Biolegend, USA) and cultured in RPMI complete medium with 10% foetal bovine serum (FBS) (Gibco, USA). The purified cells were then stimulated with plate-bound anti-CD3 (3 μg/ml, LEAF purified anti-mouse CD3ε, BioLegend, USA) and soluble anti-CD28 (5 μg/ml, LEAF purified anti-mouse CD28, BioLegend, USA) antibodies.

Immunohistochemical staining

Mouse brains were removed at the peak of EAE after cardiac perfusion and were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, USA). Fixed tissues were embedded in paraffin. Brain sections were blocked with H_2O_2 and then stained with anti-IL-17 antibody (Bioss, China) at 4 °C overnight. On the second day, a biotin-streptavidin HRP detection system (ZSGB-BIO, China) was used according to the manufacturer's instructions.

Isolation of mononuclear cells in the spinal cord and brain

The spinal cord and brain were removed and washed twice with precooled PBS after cardiac perfusion with PBS. The tissues were collected and homogenized, and the mononuclear cells were harvested by centrifugation with a 70–30% Percoll gradient (GE Healthcare, Sweden) according to the manufacturer's protocol. The viability of the cells was detected using trypan blue staining [16].

Flow cytometry analysis

Single cells from the spleen, inguinal lymph nodes, and CNS and induced Tregs were harvested and washed with PBS supplemented with 2% foetal calf serum and then stained with mAbs specific for CD4 (PerCP anti-mouse CD4, BioLegend, USA), CD25 (PE anti-mouse CD25, BioLegend, USA), TIGIT (APC anti-mouse CD155, BioLegend, USA), CTLA-4 (APC anti-mouse CTLA-4, BioLegend, USA) and PD-1 (APC anti-mouse PD-1, BioLegend, USA).

To examine the intracellular expression of the cytokines IL-10 and IL-17A, the cells were stimulated with a cell activation cocktail (with brefeldin A) (BioLegend, USA) for 6 h according to the manufacturer's protocols. The mAbs used were anti-IL-10 (PE anti-mouse IL-10, BioLegend, USA) and anti-IL-

17A (PE anti-mouse IL-17A, BioLegend, USA). To determine the number of Foxp3$^+$ cells in the population, the cells were sequentially fixed, permeabilized (Fixation/Permeabilization Diluent, eBioscience, USA) and stained for Foxp3 (Alexa Fluor 488 anti-mouse FOXP3, BioLegend, USA). Each antibody was diluted according to the manufacturer's instructions. All stained cells were analysed by using a BD flow cytometer (BD Biosciences, San Jose, CA, USA).

ELISA analysis

CD4$^+$ T cells were sorted from splenocytes (MojoSort Mouse CD4$^+$ T Cell Isolation Kit, Biolegend, USA) from WT or *Cd226*$^{-/-}$ mice at the peak of EAE and cultured in RPMI 1640 complete medium with 10% FBS in the presence of irradiated (3000 rad) syngeneic splenocytes as antigen-presenting cells (APCs). The cell supernatant was collected at the appropriate time points after stimulation with $MOG_{35–55}$ peptide [17, 18]. The concentrations of IL-10 (eBioscience, USA) and transforming growth factor (TGF)-β (eBioscience, USA) in the cell culture supernatant were determined using ELISA kits according to the manufacturer's instructions.

Treg suppression assay

To determine the suppressive function of Tregs, negatively selected CD4$^+$ T cells labelled with carboxyfluorescein succinimidyl ester (CFSE, Biolegend, USA) were used as CD4$^+$ effector T (T_{eff}) cells. The magnetically sorted Tregs were cultured together with different ratios of T_{eff} cells in RPMI 1640 complete medium containing 10% FBS with plate-bound anti-CD3 (3 μg/ml, LEAF purified anti-mouse CD3ε, BioLegend, USA) and soluble anti-CD28 (5 μg/ml, LEAF purified anti-mouse CD28, BioLegend, USA) antibodies for 5 days.

In vitro induced regulatory T cell induction

Purified naïve CD4$^+$ T cells (cell purity ≥ 95%) that were negatively selected from splenocyte suspensions by using the MojoSort Mouse CD4$^+$ Naïve T Cell Isolation Kit (BioLegend, USA) were added into 96-well plates coated with 3 μg/ml anti-CD3 antibody at 4 °C overnight. Afterwards, soluble anti-CD28 (5 μg/ml) antibody, IL-2 (2 ng/ml, PeproTech, USA) and recombinant TGF-β1 (5 ng/ml, PeproTech, USA) were added, and the cells were cultured in RPMI 1640 complete medium with 10% FBS for 3 days to polarize the induced regulatory T cells (iTregs) [15].

Quantitative reverse transcription-polymerase chain reaction

RNA was isolated with RNAiso Plus (Takara, Japan) according to the manufacturer's protocol. cDNA was synthesized with PrimeScript^{TM} RT Master Mix (Takara, Japan), and PCR was performed using SYBR PremixEx Taq II (Takara, Japan). The primer sequences were as follows: 5′-GCTT CCTAGATTACCCCTTCTGC-3′ and 5′-CGGG CATGGTTCTGGATCA-3′ for *Ctla-4*; 5′-AGAA AGCTCAGTGGCTCAGT-3′ and 5′- GAGACTCC TCAGGTTCCATTCC-3-′ for *Tigit*; 5′-GCCT GGCTCACAGTGTCAG-3′ and 5′-TCCAGGGCTCTCCT CGATT-3′ for *Pd-1*; and 5′-AGGTCGGTGTGAAC GGATTTG-3′ and 5′ TGTAGACCATGTAGTTGAGGTCA 3′ for *Gapdh*. The primers were purchased from Applied Biosystems (Augct, China). The samples were amplified for 40 cycles according to the following protocol: 15 s at 95 °C and 1 min at 60 °C. The gene *Gapdh* was used as an endogenous reference. The samples were normalized to *Gapdh* using a ΔΔ cycle threshold-based algorithm [15].

Statistical analysis

All statistical analyses were performed using Prism 7.0 software (GraphPad software, La Jolla, CA). Student's *t* test and one-way analysis of variance (ANOVA) were used depending on the type of the experiment. Differences with $P < 0.05$ were considered statistically significant. $P > 0.05$ indicated non-significant (ns) differences. The histology assays were analysed using Image-Pro software. Flow cytometry (FCM) data were analysed by FlowJo V10 software (Tree Star, Ashland, USA).

Results

Tregs failed to upregulate CD226 during EAE

CD226 is expressed on NK cells, T cells and monocytes, as well as on a small subset of B cells, and plays a critical role in the adaptive immune response [4, 19], but its role in CD4$^+$ T cells and whether it is involved in the function of CD4$^+$ T cell subsets during pathological conditions is still not fully understood. To address this issue, we evaluated the expression level of CD226 in the CD4$^+$ T cell subsets in peripheral immune organs during healthy and EAE conditions. CD226 expression was assessed in total CD4$^+$ T cells, CD4$^+$ Foxp3$^-$ traditional T (T_{conv}) cells and CD4$^+$CD25$^+$Foxp3$^+$ Tregs from the spleens (Fig. 1a) and inguinal lymph nodes (Fig. 1b) of WT mice. Under healthy conditions, the expression levels of CD226 in both total CD4$^+$ T cells and their subset cells from the spleens and inguinal lymph nodes were comparable. Notably, the expression of CD226 was markedly upregulated in total CD4$^+$ T

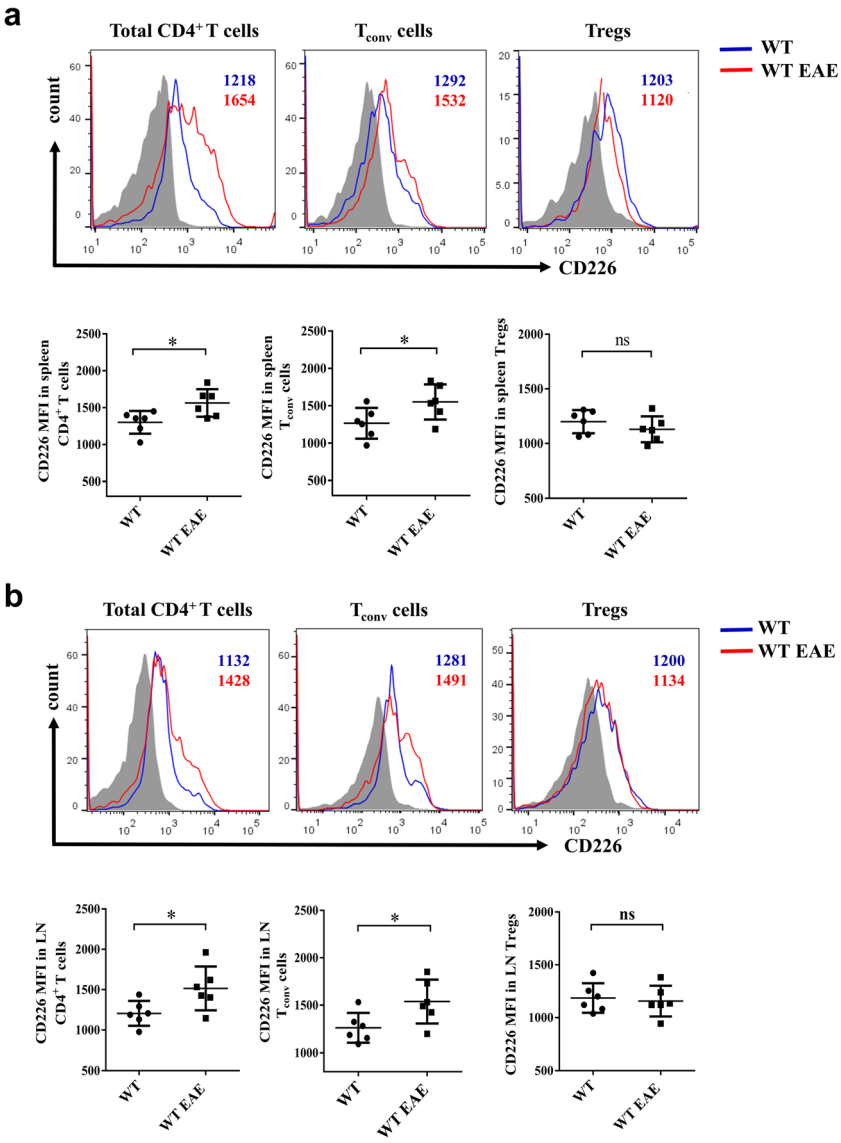

Fig. 1 The expression level of CD226 was increased in total $CD4^+$ T cells and T_{conv} cells but not Tregs during EAE. Mean fluorescence intensity (MFI) of CD226 in total $CD4^+$ T cells, $CD4^+$ $Foxp3^-$ T_{conv} cells and $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs from splenocytes (**a**) or inguinal lymph node cells (**b**) of 6~8-week-old WT mice under healthy conditions or during EAE was detected by FCM analysis (gated on total $CD4^+$ T cells, as shown in **a** and **b**). MFI was quantified below each histogram. All data are representative of six independent experiments. $^*P < 0.05$, $^{ns}P > 0.05$

cells and in T_{conv} cells under EAE conditions compared with that in healthy conditions, which was consistent with previous reports that CD226 is an active receptor on T cells [4] and plays an important role in promoting $CD4^+$ T cell activation in response to inflammatory autoimmune diseases [3, 20]. However, compared with the expression levels in the healthy condition, CD226 expression in Tregs from spleens or inguinal lymph nodes failed to increase and was slightly downregulated under EAE conditions. Based on these observations, we hypothesized that CD226 has differential effects in regulating the functions of $CD4^+$ T cell subsets during healthy and EAE conditions.

CD226 knockout attenuated Th17-mediated EAE pathogenesis

To further investigate the function of CD226, we generated homozygous *Cd226* $^{-/-}$ mice on a C57BL/6 genetic background. We detected that CD226 was greatly downregulated in both $CD4^+$ T cells and Tregs from *Cd226*$^{-/-}$ mice, indicating that CD226 was efficiently knocked out in the *Cd226*$^{-/-}$ mice (Supplementary Fig. 1). To explore the role of CD226 in the development and pathogenesis of $CD4^+$ T cell-mediated inflammatory disease, *Cd226*$^{-/-}$ and WT control mice were immunized with $MOG_{35–55}$ peptide to induce EAE and monitored closely. We compared EAE progression between *Cd226*$^{-/-}$ and WT mice and observed that the CD226 knockout mice were markedly protected from EAE pathogenesis, as shown by delayed onset and reduced clinical scores (Fig. 2a). Furthermore, the mice were assessed at 16–18 days after $MOG_{35–55}$ immunization (at the peak of EAE) to examine the effects on the immune system. Prior studies have shown that IL-17A is a key cytokine that mediates EAE pathogenesis because it attracts various immune cells into the CNS and initiates the inflammatory cascade [21]. In our study, immunohistochemical analysis revealed that the

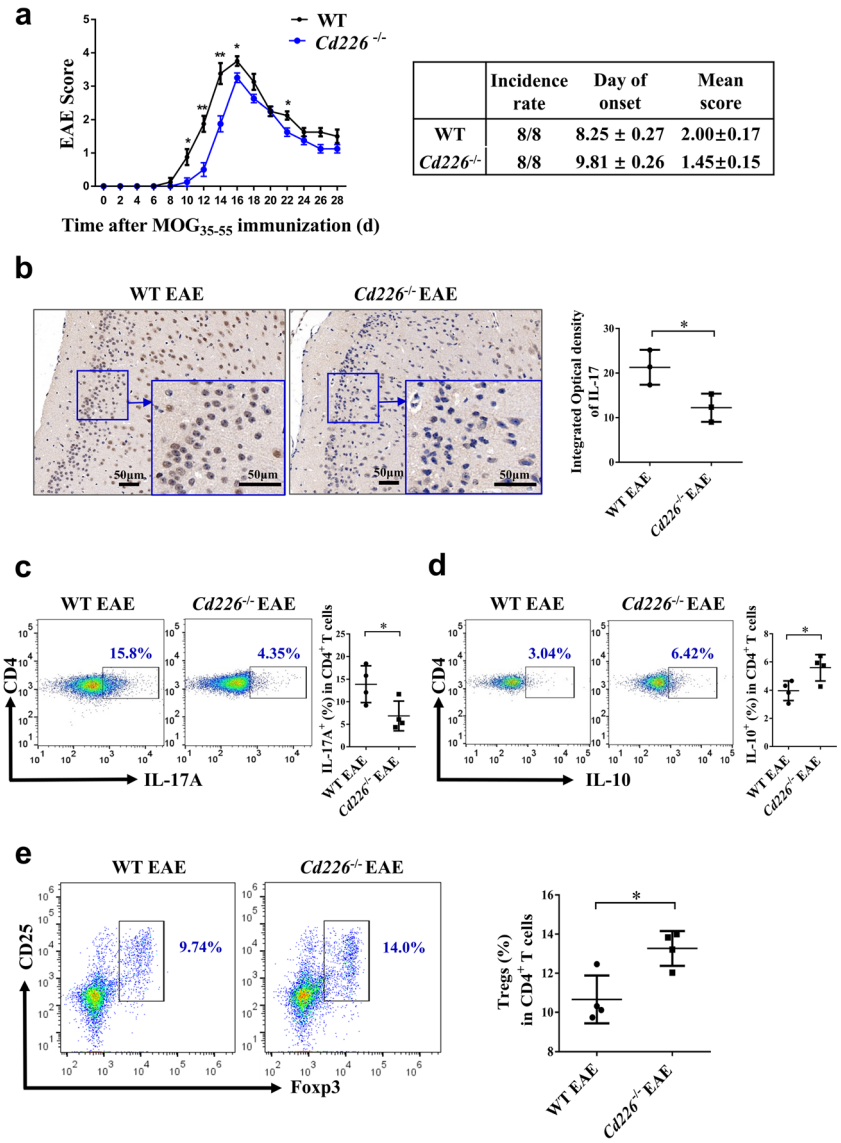

Fig. 2 Deletion of CD226 in mice attenuated the pathogenesis of EAE. **a** *Cd226*$^{-/-}$ and WT mice were immunized to induce EAE. The clinical score (mean ± SD) was monitored on the indicated days after immunization with $MOG_{35–55}$ in CFA. **b** IL-17$^+$ cell infiltration in brain tissues was analysed by immunohistochemistry at the peak of EAE. The percentage of IL-17A$^+$ cells (**c**), IL-10$^+$ cells (**d**) and Tregs (**e**) in CD4$^+$ T cells isolated from the CNS of WT and *Cd226*$^{-/-}$ mice was assessed by FCM at the peak of EAE (gated on total CD4$^+$ T cells, as shown in **c**, **d** and **e**). Representative flow cytometry analysis of IL-17A$^+$ cells, IL-10$^+$ cells and Tregs is plotted in **c**, **d** and **e**, respectively. The frequencies are shown on the right. All data are representative of at least three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$

infiltration of IL-17$^+$ cells in the brain was decreased in *Cd226*$^{-/-}$ mice compared with that in WT mice during EAE (Fig. 2b). We also observed a significant reduction in IL-17A-producing CD4$^+$ T cells in the CNS in *Cd226*$^{-/-}$ mice compared with that of WT EAE mice (Fig. 2c). These results suggest that the knockout of CD226 controls pathogenic Th17 cell responses and alleviates the severity of EAE.

Next, we investigated whether Treg infiltration in the CNS was affected in *Cd226*$^{-/-}$ mice during EAE. We found an increase in the percentage of IL-10$^+$ CD4$^+$ T cells in the CNS of *Cd226*$^{-/-}$ mice compared with that in WT mice during EAE (Fig. 2d). Moreover, the percentage of Tregs in the CNS of *Cd226*$^{-/-}$ EAE mice was increased compared with that in WT EAE mice (Fig. 2e). These results indicated that the CD226 knockout mice were more resistant to EAE, possibly due to downregulation of Th17 cells as well as enhanced infiltration of Tregs.

CD226 knockout upregulated Treg suppressive capacity

The inhibitory cytokines IL-10 and TGF-β are believed to mediate Treg function [22]. To investigate whether the attenuated EAE pathogenesis observed in *Cd226*$^{-/-}$ mice was dependent on Tregs, we measured the production of IL-10 and TGF-β in CD4$^+$ T cells. The results showed an increase in IL-10 expression in the CD4$^+$ T cell culture supernatants from *Cd226*$^{-/-}$ EAE mice after stimulation with 10 μg/ml $MOG_{35–55}$ for 2, 3 or 4 days (Fig. 3a) or stimulation with 5, 10 or 20 μg/ml $MOG_{35–55}$ for 3 days (Fig. 3b). Next, we evaluated the expression of TGF-β in the culture supernatants. There was no significant change in TGF-β secretion by CD4$^+$ T cells treated with $MOG_{35–55}$ at different times (2, 3 and 4 days) (Supplementary Fig. 2a) or then treated for 3 days with 5, 10

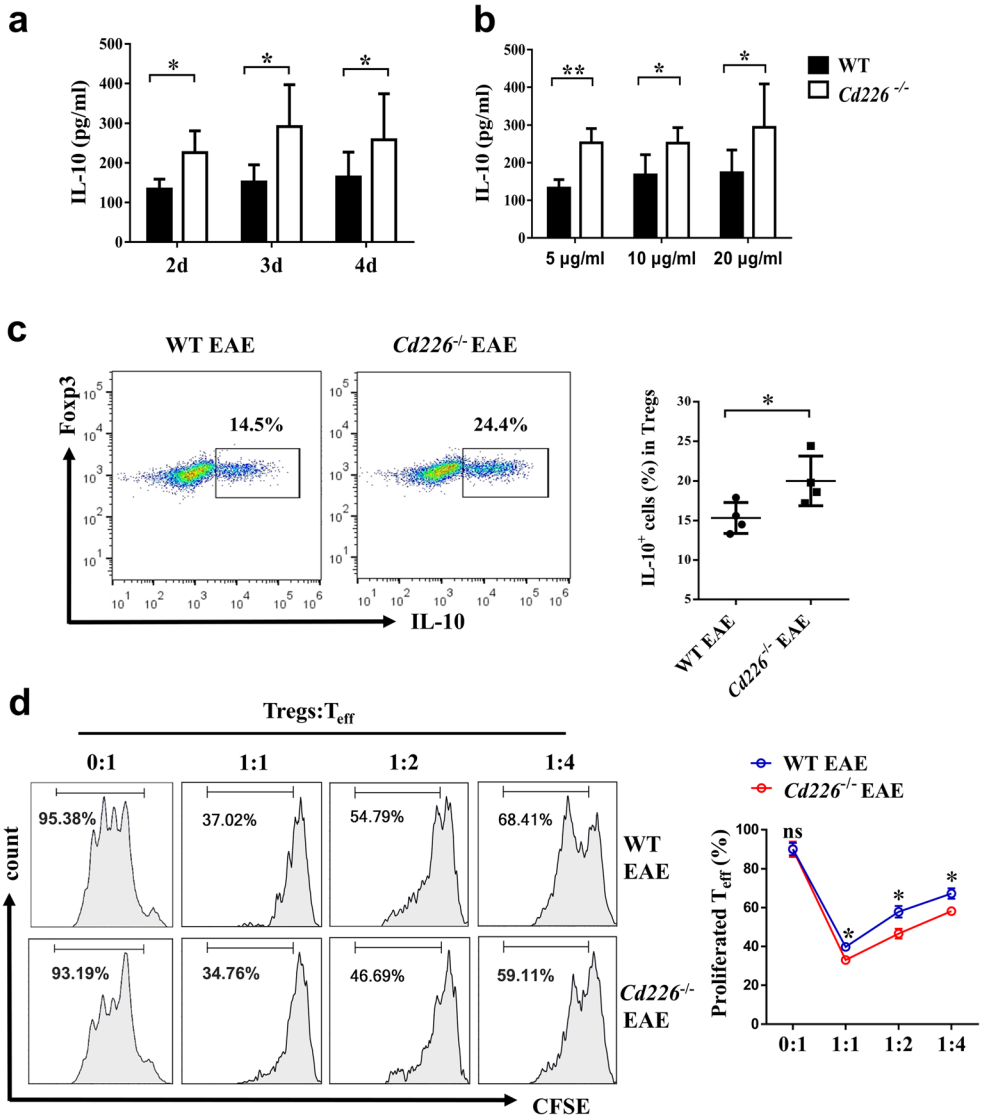

Fig. 3 Deletion of CD226 upregulated the suppressive capability of Tregs. IL-10 levels in the culture supernatant of $CD4^+$ T cells (isolated from the spleens of WT EAE or *Cd226*$^{-/-}$ EAE mice) stimulated for 2, 3 and 4 days with $MOG_{35–55}$ peptide (10 μg/ml) (**a**) or stimulated with increasing doses (5, 10, or 20 μg/ml) of $MOG_{35–55}$ peptide in the presence of irradiated syngeneic splenocytes as APCs for 3 days (**b**) were detected by ELISA. **c** Expression of IL-10 in splenic $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs from *Cd226*$^{-/-}$ and WT mice during EAE pathogenesis was detected by FCM. Representative flow cytometry of $IL\text{-}10^+$ Tregs was plotted (gated on $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs), and the frequencies are shown on the right. **d** Suppression of proliferation of CFSE-labelled T_{eff} cells by different ratios of Tregs from WT and *Cd226*$^{-/-}$ mice. The divisions of T_{eff} cells in vitro were detected by measuring CFSE dilution at the indicated ratios of cell numbers between Tregs and T_{eff} cells after 5 days. The percentage of the proliferated T_{eff} cells was assessed by FlowJo software V10. Student's *t* test and ANOVA were used for statistical analysis. The experiment was repeated three times. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$

or 20 μg/ml $MOG_{35–55}$ (Supplementary Fig. 2b) compared with those of WT EAE or *Cd226*$^{-/-}$ EAE mice, suggesting that the attenuated EAE pathogenesis in *Cd226*$^{-/-}$ mice was not dependent on TGF-β. Based on these results, we next analysed the production of IL-10 in splenic Tregs from *Cd226*$^{-/-}$ and WT mice during EAE. The results showed that Tregs from *Cd226*$^{-/-}$ mice generated increased amounts of IL-10 at the peak of EAE (Fig. 3c). To directly assess the capacity of CD226 in the inhibition of Tregs, we sorted Tregs from spleens at the peak of EAE and cultured them with different ratios of CFSE-labelled T_{eff} cells from healthy WT mice for 5 days according to the standard protocol [23]. CFSE dilution assays that measured the percentage of proliferated T_{eff} cells showed that T_{eff} cells cultured with *Cd226*$^{-/-}$ Tregs exhibited decreased proliferation compared to that of T_{eff} cells cultured with control cells (Fig. 3d).These findings are consistent with the high level of IL-10 secreted by CD226-deficient Tregs and suggest that knockout of CD226 enhances the suppressive function of Tregs.

The expression levels of CTLA-4 and TIGIT in splenic Tregs were increased in *Cd226*$^{-/-}$ EAE mice

To understand the mechanisms of the enhanced suppressive capacity of *Cd226*$^{-/-}$ Tregs, the expression levels of CTLA-4, TIGIT and PD-1 were detected since they are essential for the suppressive ability of Tregs. Therefore, we assessed the expression of these molecules at the protein and gene levels to understand whether the enhanced suppressive capabilities of CD226-deficient Tregs were attributed to the altered expression of these molecules. In healthy conditions, the expression level of CTLA-4 in CD226-deficient Tregs was comparable to that of CD226-expressing Tregs (Supplementary Fig. 3). Strikingly, Tregs isolated from the spleens of *Cd226*$^{-/-}$ EAE mice exhibited higher expression levels of CTLA-4 compared to those from WT EAE control mice (Fig. 4a). Furthermore, the expression of TIGIT in Tregs was monitored by flow cytometry, and we observed that the deletion of CD226 was also associated with elevated expression of TIGIT compared to that of control cells not only in healthy conditions but also during EAE (Fig. 4a and Supplementary Fig. 3). However, there were no differences in PD-1 expression among the WT and *Cd226*$^{-/-}$ mice under healthy conditions or at the peak of EAE (Fig. 4a and Supplementary Fig. 3). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses revealed that the expression levels of *Ctla-4* and *Tigit* mRNA in *Cd226*$^{-/-}$ Tregs were increased compared to those in Tregs from WT mice during EAE (Fig. 4b, c). However, there was no difference in the *Pd-1* mRNA expression level between these two groups (Fig. 4d). Taken together, these findings demonstrated that CD226 knockout increases the CTLA-4 and TIGIT expression levels in Tregs at the protein and mRNA levels during EAE.

Enhanced CTLA-4 and TIGIT expression in iTregs and Tregs sorted from the CNS of *Cd226*$^{-/-}$ EAE mice

Accumulating evidence shows that in vitro-derived iTregs play important roles in the control of inflammation and that

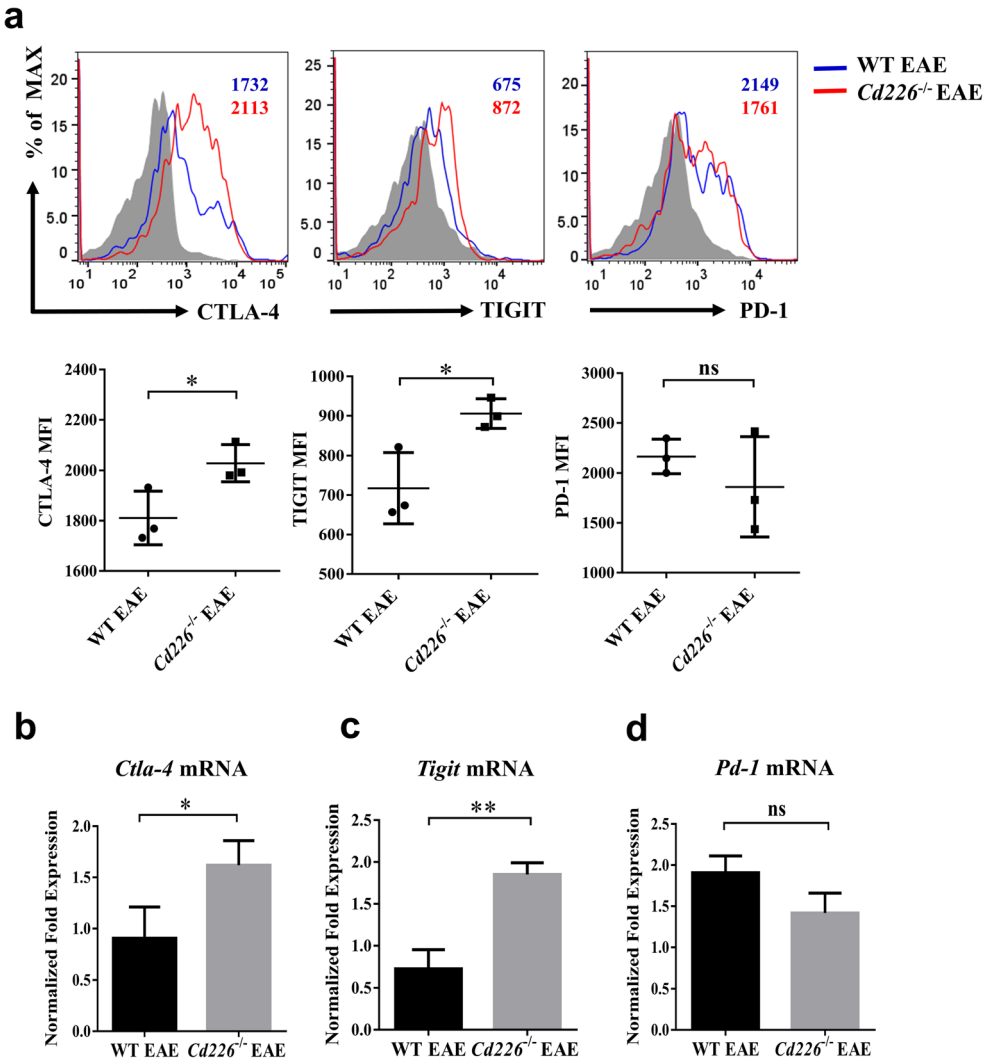

Fig. 4 The expression levels of CTLA-4 and TIGIT in splenic Tregs were increased in *Cd226*$^{-/-}$ EAE mice. **a** MFI of CTLA-4, TIGIT and PD-1 in splenic Tregs from *Cd226*$^{-/-}$ mice and WT mice were analysed by FCM at the peak of EAE (gated on CD4^{+} CD25^{+} Foxp3^{+} Tregs) and quantified below. The relative mRNA expression levels of *Ctla-4* (**b**), *Tigit* (**c**) and *Pd-1* (**d**) in splenic Tregs from WT or *Cd226*$^{-/-}$ mice during EAE were assessed by qRT-PCR. All data are representative of at least three

they contribute to tolerance in the autoimmune response [24, 25]. Although we demonstrated that CD226 deletion facilitated CTLA-4 and TIGIT expression in Tregs, how the function of CD226 is regulated in iTregs in vitro is poorly understood. We sorted naïve CD4$^+$ T cells from *Cd226*$^{-/-}$ or WT mice at the peak of EAE and differentiated them under iTreg-polarizing conditions for 3 days, followed by detection of TIGIT and CTLA-4 to assess expression levels in Tregs. Compared with the expression levels in the WT EAE group, iTregs from *Cd226*$^{-/-}$ EAE mice exhibited increased expression levels of CTLA-4 and TIGIT (Fig. 5a). Moreover, the qRT-PCR results also showed increased expression levels of *Ctla-4* mRNA (Fig. 5b) and *Tigit* mRNA(Fig. 5c) in *Cd226*$^{-/-}$ iTregs, which further confirmed that the deletion of CD226 maintained the suppressive phenotype of iTregs. EAE is characterized by widespread inflammatory processes in the CNS [26]. Thus, it will be necessary to determine the expression level of CTLA-4 and TIGIT in infiltrated Tregs in the CNS at the peak of EAE. The expression levels of CTLA-4 and TIGIT on EAE CNS Tregs were increased in *Cd226*$^{-/-}$ mice compared to those of Tregs from WT mice, but there were no differences in PD-1 expression among these two groups (Fig. 5d). Taken together, our findings demonstrated that the enhanced suppressive capacity of *Cd226*$^{-/-}$ Tregs is associated with increased expression of CTLA-4 and TIGIT.

Discussion

EAE contributes to a breakdown of self-tolerance and eventually leads to auto-reactive CD4$^+$ T cells, especially Th17 cells, infiltrating the CNS to mediate inflammation and neuronal injury [21]. Published studies have demonstrated that the migration of Th17 cells into the CNS is the key pathogenic process in EAE mice [27]. Tregs are known to suppress the response of myelin-reactive CD4$^+$ T_{eff} cells and are pivotal in regulating CNS inflammation [28]. Therefore, imbalance in the Th17/Treg ratio is associated with the onset and progression of MS and EAE [29, 30]. Several lines of evidence support the role of CD226 in promoting T cell- and NK cell-mediated immune responses [4, 31, 32]. We previously reported that EAE susceptibility in mice treated with anti-CD226 pAb was markedly decreased via balancing the Th17/Treg ratio [9]. However, the molecular mechanisms by which CD226 regulates the Th17/Treg balance involved in decreased EAE susceptibility remain unclear. Here, we observed that

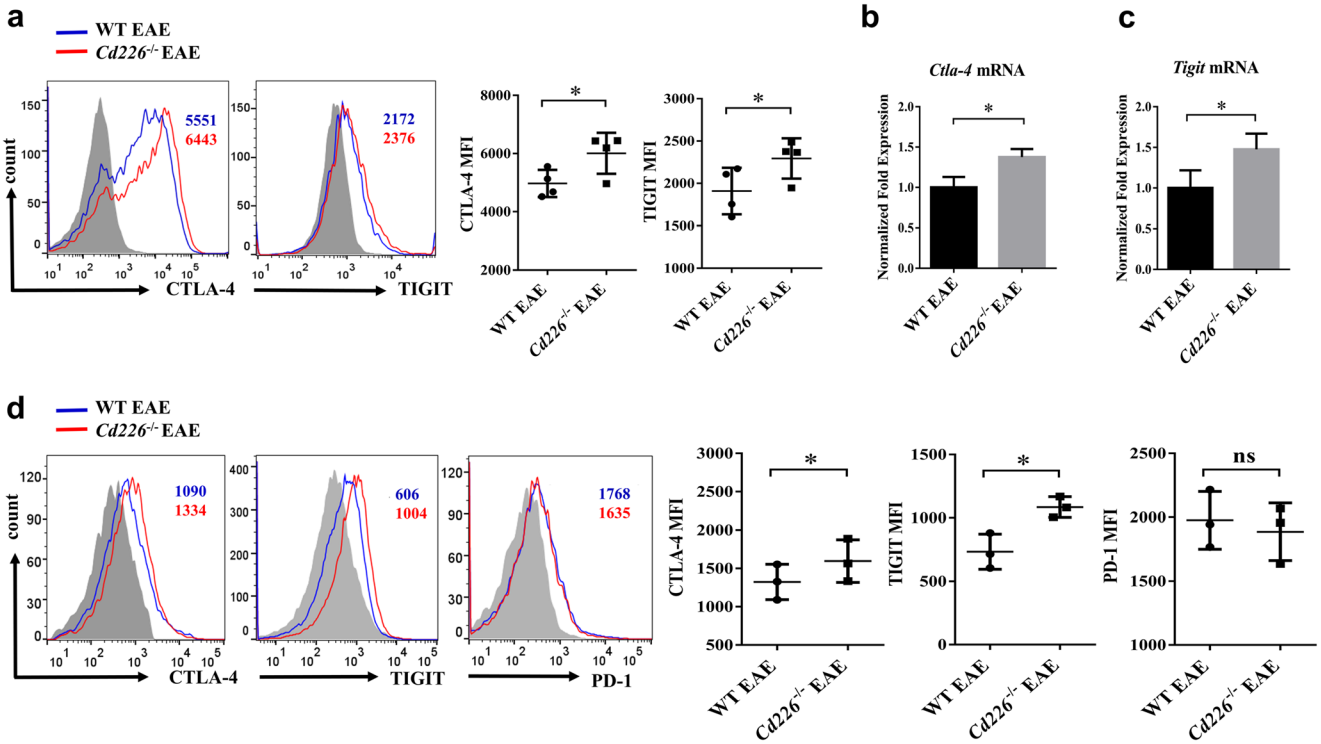

Fig. 5 CTLA-4 and TIGIT expression was increased in iTregs and Tregs sorted from the CNS of *Cd226*$^{-/-}$ EAE mice. **a** Naïve CD4$^+$ T cells were sorted from splenocyte suspensions from WT or *Cd226*$^{-/-}$ EAE mice and then cultured under iTreg-polarizing conditions. Cells were harvested 3 days later to detect CTLA-4 and TIGIT expression (gated on CD4$^+$ CD25$^+$ Foxp3$^+$ iTregs), and MFI was quantified and is shown to the right of the histogram. *Ctla-4* mRNA (**b**) or *Tigit* mRNA (**c**) in iTregs was assessed by qRT-PCR. **d** The expression levels of CTLA-4, TIGIT and PD-1 in Tregs isolated from the CNS of WT EAE or *Cd226*$^{-/-}$ EAE mice were assessed by FCM (gated on CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs). MFI was quantified and is shown to the right of the histogram. All data are representative of at least three independent experiments. $^{ns}P > 0.05$, $^{*}P < 0.05$

while both total $CD4^+$ T cells and T_{conv} cells upregulate CD226 during EAE, Tregs failed to upregulate CD226. These results are consistent with the report that upon T cell receptor (TCR) activation, Tregs exhibited lower CD226 expression than T_{eff} cells [33]. These observations suggest a potentially distinct role for CD226 in controlling $CD4^+$ T cell lineages, especially in Tregs.

To investigate the specific function of CD226 in the Th17/Treg balance during EAE, *Cd226*$^{-/-}$ mice were raised, and an EAE model was constructed. We observed that CD226 deletion delayed the onset and alleviated the development of EAE. The immunohistochemistry results showed that the infiltration of IL-17^+ cells in the *Cd226*$^{-/-}$ EAE mouse brain was reduced. Moreover, the expression level of IL-17A was decreased but IL-10 was increased in sorted *Cd226*$^{-/-}$ EAE CNS $CD4^+$ cells. It is clear that the stable expression of Foxp3 is necessary to maintain Treg function [34], and increasing MS severity is associated with impaired Tregs [35]. Consistent with this, the Treg percentage in $CD4^+$ T cells was measured, and the percentage of $Foxp3^+$ Tregs in the CNS of *Cd226*$^{-/-}$ EAE mice was increased compared with that in WT EAE mice. Accumulating evidence has demonstrated that Th17 cells play a pathogenic role in promoting and enhancing autoimmune tissue injuries [36], and these cells are enriched in lesions during the pathogenesis of EAE [37]. To the opposite, IL-10 produced by Tregs inhibits Th17 cell pathogenicity to negatively regulate autoimmune responses [38]. Therefore, our results confirmed that the absence of CD226 in mice attenuates EAE, which may be related to decreased Th17 and increased Treg infiltration in the CNS during EAE.

To assess whether CD226 influences Treg suppressive functions, in vitro suppression assays were performed, it was found that the suppressive capacity of *Cd226*$^{-/-}$ Tregs was enhanced. Published studies have shown that the expression levels of Treg signature molecules such as CTLA-4, TIGIT and PD-1 are associated with the development and suppressive functions of Tregs [39]. We examined the expression levels of CTLA-4, TIGIT and PD-1 on splenic Tregs, iTregs and CNS Tregs. Although the CTLA-4 expression level was comparable between WT and *Cd226*$^{-/-}$ splenic Tregs under healthy conditions, the Tregs from *Cd226*$^{-/-}$ EAE mice exhibited increased CTLA-4 and TIGIT expression levels compared to those from WT EAE mice. It is well known that CTLA-4 is a target gene that is regulated by Foxp3 [40] and promotes the suppressive function of Tregs [41]. Moreover, genetic abnormalities in CTLA-4 have been found in patients with MS, and patients with rheumatoid arthritis exhibit unregulated CTLA-4 expression [42, 43]. On the other hand, $TIGIT^+$ Tregs are an activated Treg subset that specifically inhibits pro-inflammatory Th1 and Th17 cells [12]. In the absence of CD226, TIGIT interacts with CD155 or CD112 to enhance Treg proliferation and function [20]. There is evidence that $TIGIT^+$ Tregs are highly suppressive and more so [33]. Furthermore, the expression level of TIGIT on Tregs is enhanced in the absence of CD226 in graft-versus-host disease, which may promote donor Treg expansion and function [20]. We observed that CTLA-4 was expressed at higher levels in iTregs but not in splenic Tregs during healthy conditions. The reason may be that naïve T cells receive anti-CD3/CD28 stimulation during iTreg polarization in vitro. Based on these findings, we hypothesize that the enhanced suppressive capacity of *Cd226*$^{-/-}$ Tregs during EAE may be related to the increased expression levels of CTLA-4 and TIGIT. PD-1 is expressed on activated T cells and contributes to maintaining immune tolerance during inflammation. However, the role of PD-1 in Tregs remains controversial. Some studies showed the absence of PD-1 induced Treg instability and increased the number of ex-Tregs [44]. Another study reported that high PD-1 expression on circulating human Tregs identified a dysfunctional Treg population that secretes interferon (IFN)-γ, and PD-1 deficient Tregs are highly immunosuppressive in mice [39, 45]. Our results showed that the PD-1 expression on Tregs was not obviously different between WT and *Cd226*$^{-/-}$ mice under healthy or EAE conditions, suggesting that PD-1 may not be involved in the enhanced suppressive function of *Cd226*$^{-/-}$ Tregs. Taken together, our results indicated that the increased suppressive capacity of *Cd226*$^{-/-}$ Tregs may be partially contact-dependent, which is associated with high expression of CTLA-4 and TIGIT. Further work is needed to determine which downstream factors of CTLA-4 and TIGIT are involved in the regulation of *Cd226*$^{-/-}$ Treg during EAE and whether IL-10 signalling plays a role in this regulation.

In summary, our current study has demonstrated that CD226 plays a vital role in impeding the function of Tregs in the pathogenesis of EAE. Additionally, the increased expression levels of the signature Treg molecules CTLA-4 and TIGIT are associated with the enhanced suppressive capacity of *Cd226*$^{-/-}$ Tregs in EAE. In the future, it would also be interesting to determine the role of CD226 in Treg differentiation, proliferation and stability during EAE.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Xu Z, Jin B. A novel interface consisting of homologous immunoglobulin superfamily members with multiple functions. Cell Mol Immunol. 2010;7:11–9.
2. Lozano E, Joller N, Cao Y, Kuchroo VK, Hafler DA. The CD226/CD155 interaction regulates the proinflammatory (Th1/Th17)/anti-inflammatory (Th2) balance in humans. Journal of immunology (Baltimore, MD: 1950). 2013;191:3673–80.

3. Lozano E, Dominguez-Villar M, Kuchroo V, Hafler DA. The TIGIT/CD226 axis regulates human T cell function. J Immunol (Baltimore, MD: 1950). 2012;188:3869–75.
4. Shibuya K, Shirakawa J, Kameyama T, Honda S, Tahara-Hanaoka S, Miyamoto A, et al. CD226 (DNAM-1) is involved in lymphocyte function-associated antigen 1 costimulatory signal for naive T cell differentiation and proliferation. J Exp Med. 2003;198:1829–39.
5. Mastorodemos V, Ioannou M, Verginis P. Cell-based modulation of autoimmune responses in multiple sclerosis and experimental autoimmmune encephalomyelitis: therapeutic implications. Neuroimmunomodulation. 2015;22:181–95.
6. Joller N, Hafler JP, Brynedal B, Kassam N, Spoerl S, Levin SD, et al. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. J Immunol (Baltimore, MD: 1950). 2011;186:1338–42.
7. Luchtman DW, Ellwardt E, Larochelle C, Zipp F. IL-17 and related cytokines involved in the pathology and immunotherapy of multiple sclerosis: current and future developments. Cytokine Growth Factor Rev. 2014;25:403–13.
8. Danikowski KM, Jayaraman S, Prabhakar BS. Regulatory T cells in multiple sclerosis and myasthenia gravis. J Neuroinflammation. 2017;14:117.
9. Zhang R, Zeng H, Zhang Y, Chen K, Zhang C, Song C, et al. CD226 ligation protects against EAE by promoting IL-10 expression via regulation of CD4+ T cell differentiation. Oncotarget. 2016;7:19251–64.
10. Fuhrman CA, Yeh WI, Seay HR, Saikumar Lakshmi P, Chopra G, Zhang L, et al. Divergent phenotypes of human regulatory T cells expressing the receptors TIGIT and CD226. J Immunol (Baltimore, MD: 1950). 2015;195:145–55.
11. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science (New York, NY). 2008;322:271–5.
12. Joller N, Lozano E, Burkett PR, Patel B, Xiao S, Zhu C, et al. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. Immunity. 2014;40:569–81.
13. Asano T, Meguri Y, Yoshioka T, Kishi Y, Iwamoto M, Nakamura M, et al. PD-1 modulates regulatory T-cell homeostasis during low-dose interleukin-2 therapy. Blood. 2017;129:2186–97.
14. King IL, Dickendesher TL, Segal BM. Circulating Ly-6C+ myeloid precursors migrate to the CNS and play a pathogenic role during autoimmune demyelinating disease. Blood. 2009;113:3190–7.
15. Geng J, Yu S, Zhao H, Sun X, Li X, Wang P, et al. The transcriptional coactivator TAZ regulates reciprocal differentiation of TH17 cells and Treg cells. Nat Immunol. 2017;18:800–12.
16. Pino PA, Cardona AE. Isolation of brain and spinal cord mononuclear cells using percoll gradients. J Visualized Exp : JoVE 2011.
17. Klocke K, Sakaguchi S, Holmdahl R, Wing K. Induction of autoimmune disease by deletion of CTLA-4 in mice in adulthood. Proc Natl Acad Sci U S A. 2016;113:E2383–92.
18. Kim BS, Lu H, Ichiyama K, Chen X, Zhang YB, Mistry NA, et al. Generation of RORgammat(+) antigen-specific T regulatory 17 cells from Foxp3(+) precursors in autoimmunity. Cell Rep. 2017;21:195–207.
19. Zeng H, Zhang R, Jin B, Chen L. Type 1 regulatory T cells: a new mechanism of peripheral immune tolerance. Cell Mol Immunol. 2015;12:566–71.
20. Koyama M, Kuns RD, Olver SD, Lineburg KE, Lor M, Teal BE, et al. Promoting regulation via the inhibition of DNAM-1 after transplantation. Blood. 2013;121:3511–20.
21. Rostami A, Ciric B. Role of Th17 cells in the pathogenesis of CNS inflammatory demyelination. J Neurol Sci. 2013;333:76–87.
22. Miyara M, Gorochov G, Ehrenstein M, Musset L, Sakaguchi S, Amoura Z. Human FoxP3+ regulatory T cells in systemic autoimmune diseases. Autoimmun Rev. 2011;10:744–55.
23. Collison LW, Vignali DA. In vitro Treg suppression assays. Methods Mol Biol (Clifton, NJ). 2011;707:21–37.
24. Schmitt EG, Haribhai D, Williams JB, Aggarwal P, Jia S, Charbonnier LM, et al. IL-10 produced by induced regulatory T cells (iTregs) controls colitis and pathogenic ex-iTregs during immunotherapy. J Immunol (Baltimore, Md : 1950). 2012;189:5638–48.
25. Zhang P, Tey SK, Koyama M, Kuns RD, Olver SD, Lineburg KE, et al. Induced regulatory T cells promote tolerance when stabilized by rapamycin and IL-2 in vivo. J Immunol (Baltimore, Md : 1950). 2013;191:5291–303.
26. Bar-Or A, Hintzen RQ, Dale RC, Rostasy K, Bruck W, Chitnis T. Immunopathophysiology of pediatric CNS inflammatory demyelinating diseases. Neurology. 2016;87:S12–9.
27. Gao Q, Zhang Y, Han C, Hu X, Zhang H, Xu X, et al. Blockade of CD47 ameliorates autoimmune inflammation in CNS by suppressing IL-1-triggered infiltration of pathogenic Th17 cells. J Autoimmun. 2016;69:74–85.
28. Liu Y, Teige I, Birnir B, Issazadeh-Navikas S. Neuron-mediated generation of regulatory T cells from encephalitogenic T cells suppresses EAE. Nat Med. 2006;12:518–25.
29. Sun Y, Tian T, Gao J, Liu X, Hou H, Cao R, et al. Metformin ameliorates the development of experimental autoimmune encephalomyelitis by regulating T helper 17 and regulatory T cells in mice. J Neuroimmunol. 2016:292, 58–67.
30. Knochelmann HM, Dwyer CJ, Bailey SR, Amaya SM, Elston DM, Mazza-McCrann JM, et al. When worlds collide: Th17 and Treg cells in cancer and autoimmunity. Cell Mol Immunol. 2018;15:458–69.
31. Shibuya A, Campbell D, Hannum C, Yssel H, Franz-Bacon K, McClanahan T, et al. DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. Immunity. 1996;4:573–81.
32. Iguchi-Manaka A, Kai H, Yamashita Y, Shibata K, Tahara-Hanaoka S, Honda S, et al. Accelerated tumor growth in mice deficient in DNAM-1 receptor. J Exp Med. 2008;205:2959–64.
33. Fourcade J, Sun Z, Chauvin JM, Ka M, Davar D, Pagliano O, et al. CD226 opposes TIGIT to disrupt Tregs in melanoma. JCI insight. 2018;3:121157.
34. Qiu R, Zhou L, Ma Y, Zhou L, Liang T, Shi L et al. Regulatory T cell plasticity and stability and autoimmune diseases. Clin Rev Allergy Immunol 2018.
35. Carbone F, De Rosa V, Carrieri PB, Montella S, Bruzzese D, Porcellini A, et al. Regulatory T cell proliferative potential is impaired in human autoimmune disease. Nat Med. 2014;20:69–74.
36. Gu C, Wu L, Li X. IL-17 family: cytokines, receptors and signaling. Cytokine. 2013;64:477–85.
37. Yamada H. Current perspectives on the role of IL-17 in autoimmune disease. J Inflamm Res. 2010;3:33–44.
38. Hofmann SR, Rosen-Wolff A, Tsokos GC, Hedrich CM. Biological properties and regulation of IL-10 related cytokines and their contribution to autoimmune disease and tissue injury. Clin Immunol (Orlando, Fla). 2012;143:116–27.
39. Atretkhany KN, Mufazalov IA, Dunst J, Kuchmiy A, Gogoleva VS, Andruszewski D, et al. Intrinsic TNFR2 signaling in T regulatory cells provides protection in CNS autoimmunity. Proc Natl Acad Sci U S A. 2018;115:13051–6.
40. Chen C, Rowell EA, Thomas RM, Hancock WW, Wells AD. Transcriptional regulation by Foxp3 is associated with direct promoter occupancy and modulation of histone acetylation. J Biol Chem. 2006;281:36828–34.
41. Walker LS, Sansom DM. The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. Nat Rev Immunol. 2011;11:852–63.
42. Wagner M, Sobczynski M, Karabon L, Bilinska M, Pokryszko-Dragan A, Pawlak-Adamska E, et al. Polymorphisms in CD28,

CTLA-4, CD80 and CD86 genes may influence the risk of multiple sclerosis and its age of onset. J Neuroimmunol. 2015;288:79–86.
43. Cribbs AP, Kennedy A, Penn H, Read JE, Amjadi P, Green P, et al. Treg cell function in rheumatoid arthritis is compromised by ctla-4 promoter methylation resulting in a failure to activate the indoleamine 2,3-dioxygenase pathway. Arthritis Rheumatol (Hoboken, NJ). 2014;66:2344–54.
44. Zhang B, Chikuma S, Hori S, Fagarasan S, Honjo T. Nonoverlapping roles of PD-1 and FoxP3 in maintaining immune tolerance in a novel autoimmune pancreatitis mouse model. Proc Natl Acad Sci U S A. 2016;113:8490–5.
45. Lowther DE, Goods BA, Lucca LE, Lerner BA, Raddassi K, van Dijk D, et al. PD-1 marks dysfunctional regulatory T cells in malignant gliomas. JCI Insight. 2016;1:e85935.