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ORIGINAL ARTICLE

Toll-Like Receptor 4 Promotes Th17 Lymphocyte Infiltration Via CCL25/CCR9 in Pathogenesis of Experimental Autoimmune Encephalomyelitis

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Abstract

Toll-like receptor 4 (TLR4) is a key component in innate immunity and has been linked to central nervous system (CNS) inflammation diseases, such as multiple sclerosis (MS), an inflammatory disorder induced by autoreactive Th17 cells. In our study, we found that TLR4 deficient ($TLR4^{-/-}$) mice were inadequate to induce experimental autoimmune encephalomyelitis (EAE), characterized by low clinic score and weight loss, alleviative demyelinating, as well as decreased inflammatory cell infiltration in the spinal cord. In the lesion area of EAE mice, loss of TLR4 down-regulated the secretion of inflammatory cytokines and chemokine CCL25. Furthermore, the expression of CCR9 was decreased and chemotactic migration was attenuated in $TLR4^{-/-}$ Th17 cells. Our results demonstrate that TLR4 may mediate Th17 infiltration through CCL25/CCR9 signal during pathogenesis of EAE.

Keywords Toll-like receptor 4 · Th17 cells · CCL25/CCR9 · Experimental autoimmune encephalomyelitis · Multiple sclerosis

Introduction

Multiple sclerosis (MS), a stubborn autoimmune disorder of central nervous system (CNS), is characterized by self-reactive $CD4^+$ T cells driven inflammation, demyelination in white matter, neuronal damage, and subsequent disability (Dileepan et al. 2016; Mycko et al. 2012; Rostami and Ciric 2013). Naïve $CD4^+$ T cells can differentiate into diverse cell lineages, such as Th17, up to the complicated conditions under which they are primarily activated (Toscano et al. 2007). Th17 lymphocytes, determined by the expression of transcription factor RORγt and secretion of inflammatory cytokine IL-17, play a causative role in MS, although activated microglia, invasion of macrophages and B cells also can promote the damage of myelin and axon (Kong et al. 2016; Zhang et al. 2015). The activated Th17 cells can migrate to the target site and secrete cytokines in situ, assisting with chemotaxis adhesion cytokine by endothelial cells and otherwise mediating tissue destruction (Grifka-Walk et al. 2013; Kong et al. 2016; McWilliams et al. 2015). Experimental autoimmune encephalomyelitis (EAE), which is used as a classical mice model of human MS, can be induced by injection of special Th17 cells that finally migrate into the brain and spinal cord (Braitch and Constantinescu 2010; Ummenthum et al. 2016). However, the mechanism of Th17 infiltration during MS pathogenesis remains incompletely understood.

Toll-like receptors (TLRs), reputed to be sensors for recognition of invading pathogenic microorganisms, are concerned with diverse autoimmune pathology (Priya et al. 2013; Severa et al. 2013; Sharma et al. 2016). TLR4, a well characterized TLR member, is widely distributed in Th17 cells with an increased expression in response to lipopolysaccharide

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(LPS) (Chang et al. 2011; Tukhvatulin et al. 2016). TLR4 activates NF-κB, an important nuclear transcription factor, resulting in the secretion of various cytokines like TNF-α, IL-1β and IL-6, which are indispensable in the process of Th17 differentiation (Nunez et al. 2012). In EAE mice, intracerebral injection of LPS promotes the pathology by activating of IL-17 producing Th17 cells in the CNS. Meaningfully, TLR4 deficient $CD4^+$ T cells almost completely abrogate EAE disease symptoms mainly through blunting Th17 cells (Reynolds et al. 2012). Interestingly, in Reynolds's paper, it has been shown that LPS has no effects on promoting Th17 polarization in vitro (Reynolds et al. 2012). One reason for this discrepancy may be that $TLR4^{-/-}$ $CD4^+$ T cells are not being fully activated and therefore fail to migrate and be reactivated in the CNS during EAE.

Although TLR4 has been linked with promoting CNS inflammation especially during MS and EAE (Mukherjee et al. 2016), the role of TLR4 on regulating Th17 infiltration in MS remains unknown. In this study, we find that TLR4 plays a significant role in pathology of EAE. In mechanism, that TLR4 up-regulates expression of C-C chemokine receptor type 9 (CCR9) and its special ligand C-C chemokine ligand 25 (CCL25) contributes to Th17 infiltration during EAE.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 mice and $TLR4^{-/-}$ mice were purchased from Peng Yue Laboratory Animal Breeding Co.Ltd. (Jinan, China) and the Model Animal Research Center of Nanjing University (Nanjing, China), separately. Mice were bred under semi-barrier system in the animal center of Xuzhou Medical University (Xuzhou, China). The use of mice in this study was approved by Xuzhou Medical University Experimental Animal Ethics Committee.

Induction of EAE

Using an appropriate immunization protocol, 6–8 weeks old female C57BL/6 WT and $TLR4^{-/-}$ mice were injected subcutaneously at the back with 200 μg $MOG_{35–55}$ peptide (GL Biochem, China) emulsified with the same volume of complete Freund's adjuvant (Sigma-Aldrich, USA) containing 4 mg/ml *Mycobacterium tuberculosis* H37RA (Becton, Dickinson and Company, USA). 200 ng of pertussis toxin (List Labs, USA) was applied intraperitoneally (i.p.) on day 0 and 2 after immunization. Control mice were treated without MOG. All of the mice were weighted and scored daily as follows (Mohamed et al. 2002): 0, no symptoms; 1, loss tone of tail; 2, hind limb weakness; 3, hind limb complete paralysis; 4, paralysis of forelimbs; 5, moribund or death.

Isolation and Differentiation of naïve T Cells

Splenocytes (SPs), obtained from 6 to 7 weeks old C57BL/6 mice and $TLR4^{-/-}$ mice, were dispersed for unicellular suspensions after the spleen digested. Naïve T cells were purified by $CD4^+CD62L^+$ T Cell Isolation Kit following manual methods (Miltenyi Biotec, Germany): First, indirect magnetic labeling non-$CD4^+$ T cells with biotin-antibody cocktail and anti-biotin microbeads were depleted. Then, $CD4^+CD62L^+$ T cells were directly labeled with CD62L-microbeads and enriched.

For Th17 polarization, naïve T cells were cultured for 3 days under Th17 cell differentiation condition (Qu et al. 2012): RPMI-1640 medium supplied with 10% fetal bovine serum (FBS), 1% nonessential amino acids (Sigma–Aldrich, USA), anti-CD3 and anti-CD28-coated beads (eBioscience, USA), 5 ng/mL IL-2 (R&D Systems, USA), 20 ng/mL IL-6, 5 ng/ mL TGF-β, 10 ng/mL IL-23, 2 μg/mL anti-IL-4 and 2 μg/mL anti-INF-γ (Miltenyi Biotec, Germany).

To enrich the induced $IL\text{-}17^+$ Th17, the cultured cells were incubated with Cell Stimulation Cocktail (eBioscience, USA) for 5 h, and then purified by magnetic cell sorting according to manufacturer's instructions (Miltenyi Biotec, Germany).

Th17 Cells Migration Assay

Migration assay was performed in 24 well transwell chambers with 8 μm pore polycarbonate filters (Corning, USA). Th17 cells (5×10^4) were added to the inner chamber in serum-free RPMI-1640 medium. Medium including 5% FBS and 0.1 or 1 μg/mL CCL25 (R&D Systems, USA) was placed in the bottom chamber. Transwell chambers were incubated at 37 °C for 2 h, and the number of cells in the lower chamber was counted. The chemotaxis index was shown as the ratio of the number of cells migrated in CCL25 group to that in medium containing FBS only group.

qRT-PCR

RNA was isolated from lymphocytes or spinal cords obtained from mice using Trizol reagent (Sigma, USA) according to the manufacturer's protocol, and then reverse-transcribed using a HiScript QRT SuperMix kit (Vazyme, China). Gene expression was quantified using a SYBR Green Master kit by a light Cycler® 480 System (Roche, Switzerland) and normalized to β-actin. The primers were listed in Table 1.

Histology

Mice were euthanized with pentobarbital, and then transcardially perfused with normal saline to rinse the blood and then with 4% paraformaldehyde (PFA). The lumbar spinal cords were embedded in paraffin after post-fixed for 24 h in 4% PFA, then cut into 5 μm slices for hematoxylin and eosin

Table 1 List of primers used for qRT-PCR

Name	Forward sequence(5′ to 3′)	Reverse sequence(5′ to 3′)
MAG	CTGCCGCTGTTTTGGATAATGA	CATCGGGGAAGTCGAAACGG
IL-17A	TTTAACTCCCTTGGCGCAAAA	CTTTCCCTCCGCATTGACAC
RORγt	AGTGTAATGTGGCCTACTCCT	GCTGCTGTTGCAGTTGTTTCT
IL-1β	CGACAGTGAGGAGAATGACC	ACCACTTGTTGGCTTATGTT
IL-6	ATGTAGCCGCCCCACACAGA	CATCCATCTTTTTCAGCCAT
TNF-α	AGAACTCCAGGCGGTG TCTGTG	GACCGATCACCCCGAAGTTCA
CCR9	CTTCAGCTATGACTCCACTGC	CAAGGTGCCCACAATGAACA
CCL25	TTACCAGCACAGGATCAAATGG	CGGAAGTAGAATCTCACAGC AC
β-actin	GAGACCTTCAACACCCCAGCC	AATGTCACGCACGATTTCCC

(H&E) and luxol fast blue (LFB) staining. Demyelinating in LFB stained slices were quantified by analysis of the ratio of demyelinating area relative to the whole area. Infiltration was evaluated by count of infiltrates per mm^2 in H&E stained slices.

For immunofluorescence staining, dehydrated spinal cords were cut into 15 μm sections after embedded in O.C.T Compound (Leica, Germany). Sections were prewarmed and blocked in 0.01% PBS contained 5% bovine serum albumin (BSA) and 0.3% Triton X-100 for 1 h at 37 °C. Then the slices were incubated overnight at 4 °C with primary antibodies: anti-myelin basic protein (MBP, Abcam, USA), anti-CCR9 (Santa Cruz, USA), anti-RORγt (Santa Cruz, USA), anti-CCL25 (Santa Cruz, USA). Then the specimens were incubated with related fluorescence labeling antibody (Santa Cruz, USA) for 2 h at room temperature, followed by cell nuclei counterstaining with 4′,6-diamidino-2-phenylindole (DAPI,

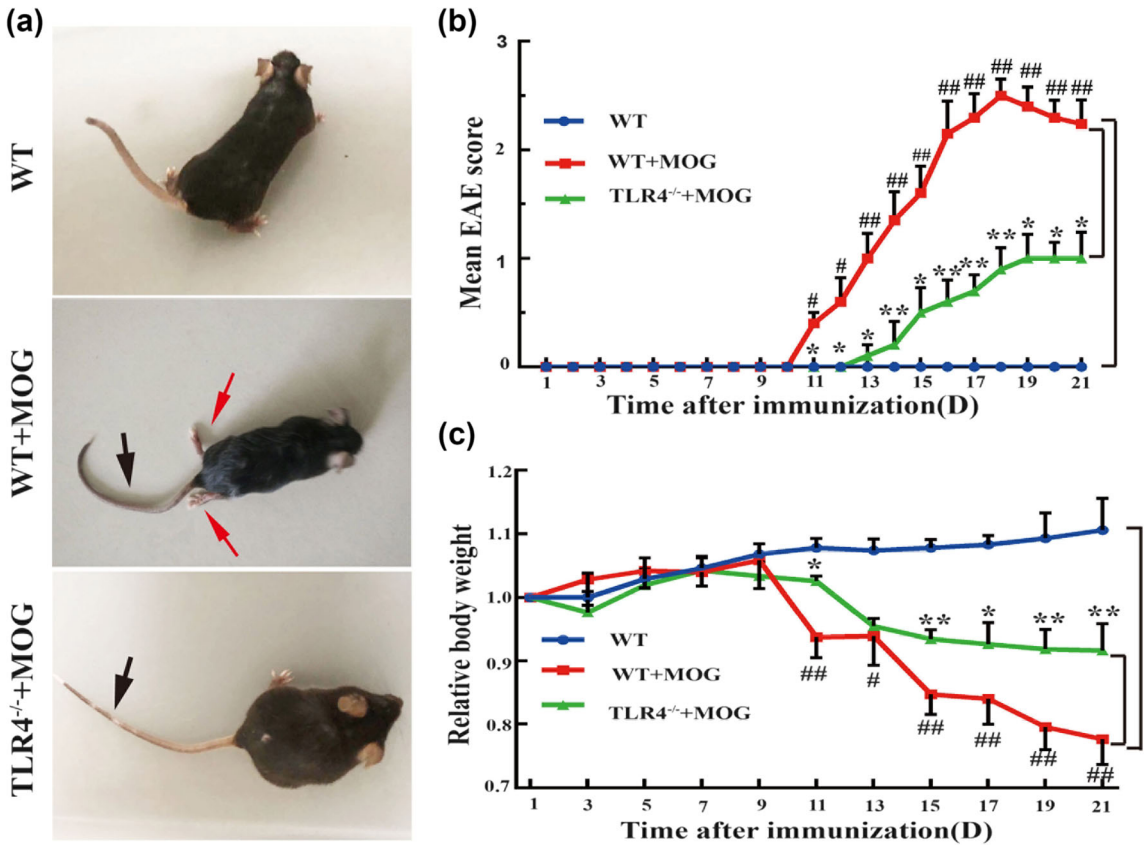

Fig. 1 TLR4 involves in EAE pathogenesis. (**a**) Behavioral symptoms of EAE mice. Red arrow shows hind limb paralysis, and black arrow shows limp tail. (**b**) Mean scores of EAE mice are recorded daily ($n = 20$ mice per group). (**c**) The ratio of body weight normalized to initial weight of each mouse (n = 20 mice per group). Data are presented as means ± standard deviation (SD). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs WT group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs WT + MOG group. WT, wild type mice without MOG immunization. WT + MOG, wild type mice with MOG immunization. $TLR4^{-/-}$ + MOG, $TLR4^{-/-}$ mice with MOG immunization

Table 2 EAE disease parameters

Group	Incidence	Max score	Mean score (mean ± SEM)	Day of onset (mean ± SEM)
WT	0(0/20)	0	0	0
WT + MOG	90%(18/20)##	4	2.1 ± 0.25##	13.2 ± 0.43
TLR4$^{-/-}$ + MOG	60%(12/20)*	2	0.9 ± 0.29 *	15.4 ± 0.36

($^{\#}$ P<0.05,$^{\#\#}$ P<0.01,vs WT group;* P<0.05,**P<0.01,vs WT + MOG group)

Vicmed, China) for 5 mins. Images were acquired using a fluorescence microscope system (Olympus, Japan) and mean fluorescence intensity (MFI) was analyzed using Image J software.

Elisa

Cerebrospinal fluid was collected from mice according to a previously described protocol (Liu and Duff 2008). The spinal cord was homogenized with cold RIPA buffer (Beyotime, China), then supernatant was collected for test. Quantitative ELISA kits for IL-17A, CCR9 and CCL25 were obtained from Jianglai Industrial Limited By Share Ltd. (Shanghai, China) and used according to the manufacturer's protocol. The measure of each sample was repeated three times.

Western Blot

The isolated spinal cord was homogenized with cold RIPA buffer (Beyotime, China). Proteins size-fractionated on SDS/PAGE were transferred to polyvinylidene fluoride membranes (Vicmed, China), followed by block for 1 h. Then membranes were incubated with monoclonal anti–MBP (Abcam, USA) and mouse monoclonal anti–β-actin (CST, USA) at 4 °C overnight. After washing 3 times, the membranes were incubated with proper secondary antibodies (LI-COR, USA) and

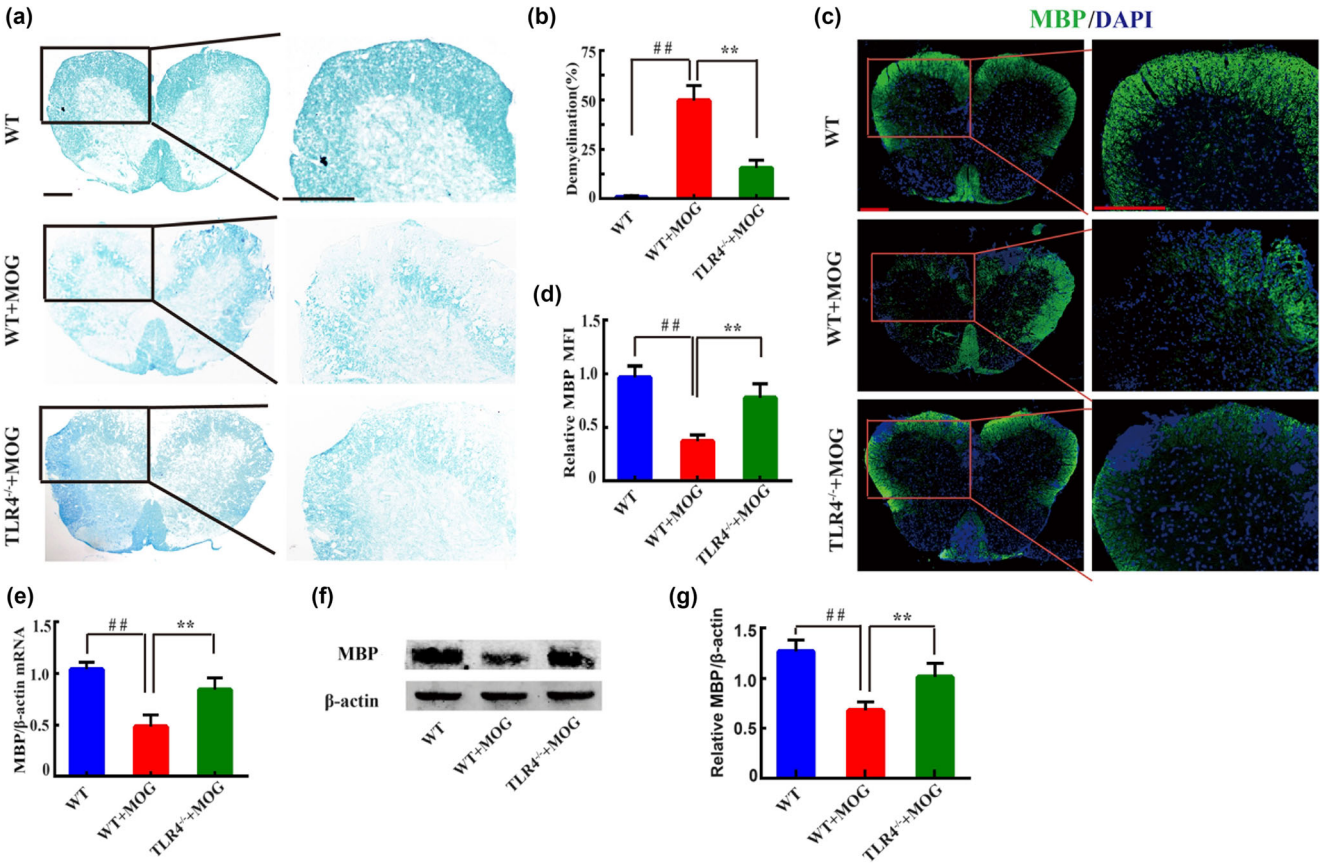

Fig. 2 TLR4 deficiency alleviates demyelination in spinal cord during EAE development. (**a**) Representative slices show LFB staining of spinal cords. Boxed areas are magnified on the right. Scale bars, 50 μm. (**b**) Analysis of demyelination in a. (**c**) MBP immunofluorescent staining in spinal cords. Scale bars, 50 μm. (**d**) MFI analysis of MBP in c (n = 5 per group). (**e**) Quantitative PCR analysis of MBP expression in spinal cords (n = 3 mice per group). (**f**, **g**) Western blot measure and analysis of MBP protein in spinal cords (n = 3 mice per group). Data are presented as means ± SD. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ vs WT group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs WT + MOG group. Results are representative of three separate experiments

scanned by Odyssey Infrared Imaging System Scanner (LI-COR, USA). Protein bands were quantified using ImageJ software.

Statistical Analysis

Two-tailed Student's t tests and Mann-Whitney tests (clinical score) were used to analyze differences. Data are presented as means ± standard deviation (SD). Differences were considered significant if $p < 0.05$.

Results

TLR4 Regulates EAE Pathogenesis

To evaluate the effect of TLR4 in EAE development, TLR4$^{-/-}$ mice were immunized by MOG for EAE induction. Twenty-one days later, WT EAE mice showed severe symptoms such as tail paralysis and complete paralysis of two hind limbs, while TLR4$^{-/-}$ mice suffering from EAE only had tail paralysis (Fig. 1a). EAE score data showed that TLR4$^{-/-}$ EAE mice had later onset (day 12), lower incidence (60%), less maximal score (2.0) and mean score (0.9 ± 0.29) compared to WT EAE mice, which began to suffer from EAE on day 10 with an incidence of 90%, maximal score 4.0 and mean score 2.1 ± 0.25 (Fig. 1b, Table 2). Additionally, TLR4$^{-/-}$ mice had less weight loss than WT mice after EAE induction (Fig. 1c).

To further identify the role of TLR4 in EAE pathogenesis, demyelination in spinal cord was measured. The results showed that spinal cord of WT EAE mice had a large area of demyelination while TLR4$^{-/-}$ EAE mice had a less demyelinated lesion (Fig. 2a, b). Consistently, the expression of MBP, a major constituent of myelin, was increased in spinal cord of TLR4$^{-/-}$ EAE mice compared with WT EAE mice (Fig. 2c-g). In conclusion, these data indicate that TLR4

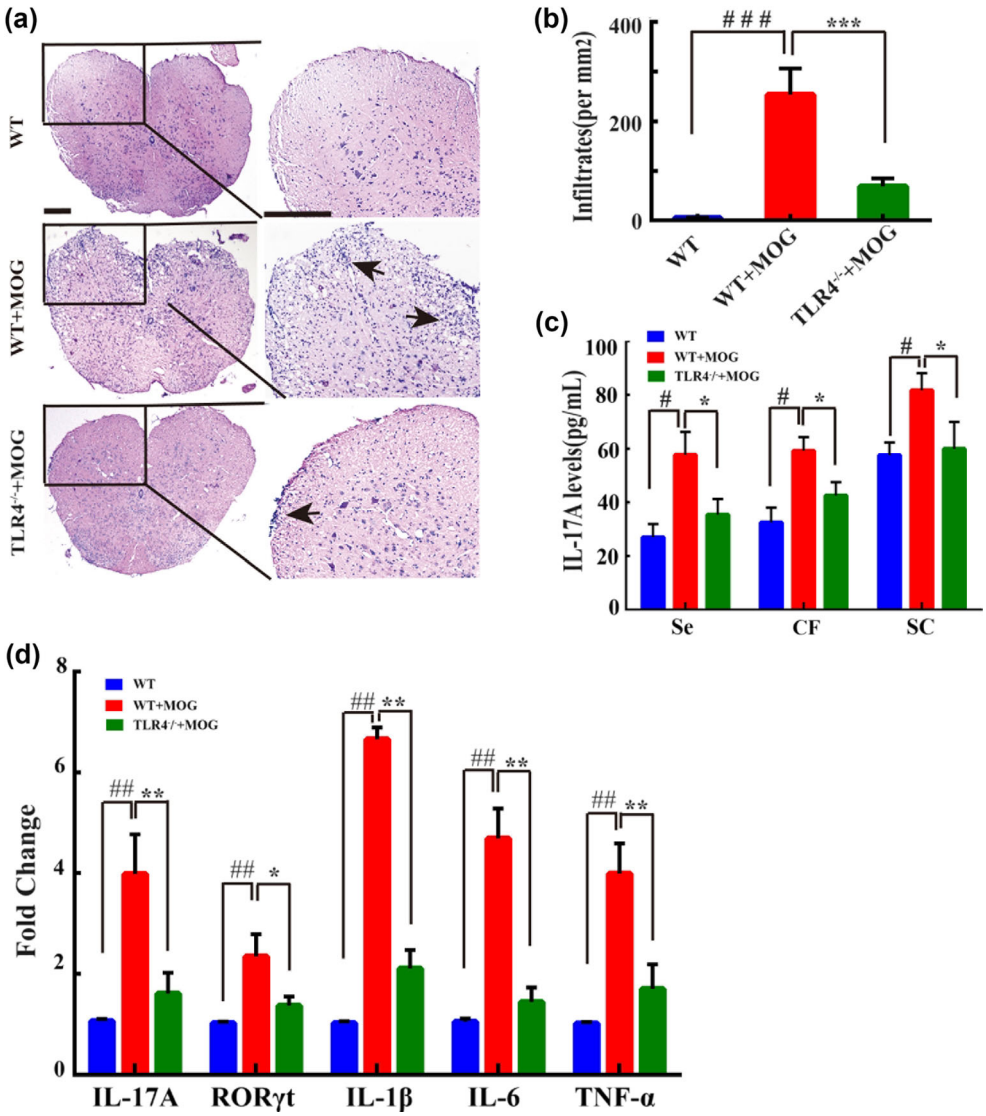

Fig. 3 TLR4 deficiency reduces lymphocyte infiltration in spinal cord during EAE development. (**a**) Representative H&E staining slices of spinal cord. Boxes show magnified areas. Arrows present lymphocyte infiltration. Scale bars, 50 μm. (**b**) Analysis of spinal cord infiltrates in a. (**c**) Concentration of IL-17A in serum (Se), cerebrospinal fluid (CF) and spinal cord homogenate (SC) is measured by ELISA (n = 5 per group). (**d**) IL-17A, RORγt, IL-1β, IL-6 and TNF-α mRNA levels in spinal cords are tested by qRT-PCR (n = 3 mice per group). Data are presented as means ± SD. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs* WT group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs WT + MOG group. Results are representative of three separate experiments

deficiency alleviates myelin damage in spinal cord during EAE development.

TLR4 Deficiency Reduces Inflammation in Spinal Cord during EAE

As EAE is a kind of autoimmune disease characterized by severe CNS inflammation for demyelination, we test lymphocyte infiltration and inflammatory factor levels after EAE induction. In our study, WT EAE mice developed extensive inflammatory cell infiltration in spinal cord, while TLR4$^{-/-}$ EAE mice had less (Fig. 3a, b). The level of IL-17A, an important participant in the development of EAE, was decreased in serum, cerebrospinal fluid and spinal cord when TLR4 was deficient (Fig. 3c). Moreover, the expression of RORγt, a Th17 cell lineage marker gene, as well as inflammatory cytokines such as IL-17A, IL-1β, IL-6 and TNF-α were down-regulated in spinal cord of TLR4$^{-/-}$ EAE mice (Fig. 3d). Therefore, TLR4 deficiency appears to lessen lymphocyte infiltration during EAE pathogenesis.

Th17 Cell Infiltration Is Regulated by TLR4 Via CCL25/CCR9

Since TLR4 plays a critical role on lymphocyte infiltration during EAE development, we next seek to grope for the potential mechanism involved in. As shown in Fig. 4, the level of CCL25 was increased in spinal cord when mice were suffered with EAE. Compared to WT EAE mice, CCL25 expression was decreased dramatically in spinal cord of TLR4$^{-/-}$ EAE mice (Fig. 4a, b), which was further supported by the result of reduced CCL25 mRNA (Fig. 4c). Consistently, the level of CCL25 was also decreased in serum and cerebrospinal fluid from TLR4$^{-/-}$ EAE mice (Fig. 4d).

Many evidences have indicated that Th17 cell is essential in the pathogenesis of MS and EAE (Quinn and Axtell 2018; Volpe et al. 2015). To study whether CCL25 is related with Th17 cell infiltration during EAE development, we tested the expression of CCR9, a receptor of CCL25, in Th17 cells. Our data showed that more CCR9^{+}RORγt^{+} inflammatory cells appeared in spinal cord of WT EAE mice, while TLR4 deficiency decreased the inflammatory infiltrates (Fig. 5a, b), as well as down-regulated the expression of CCR9 in the

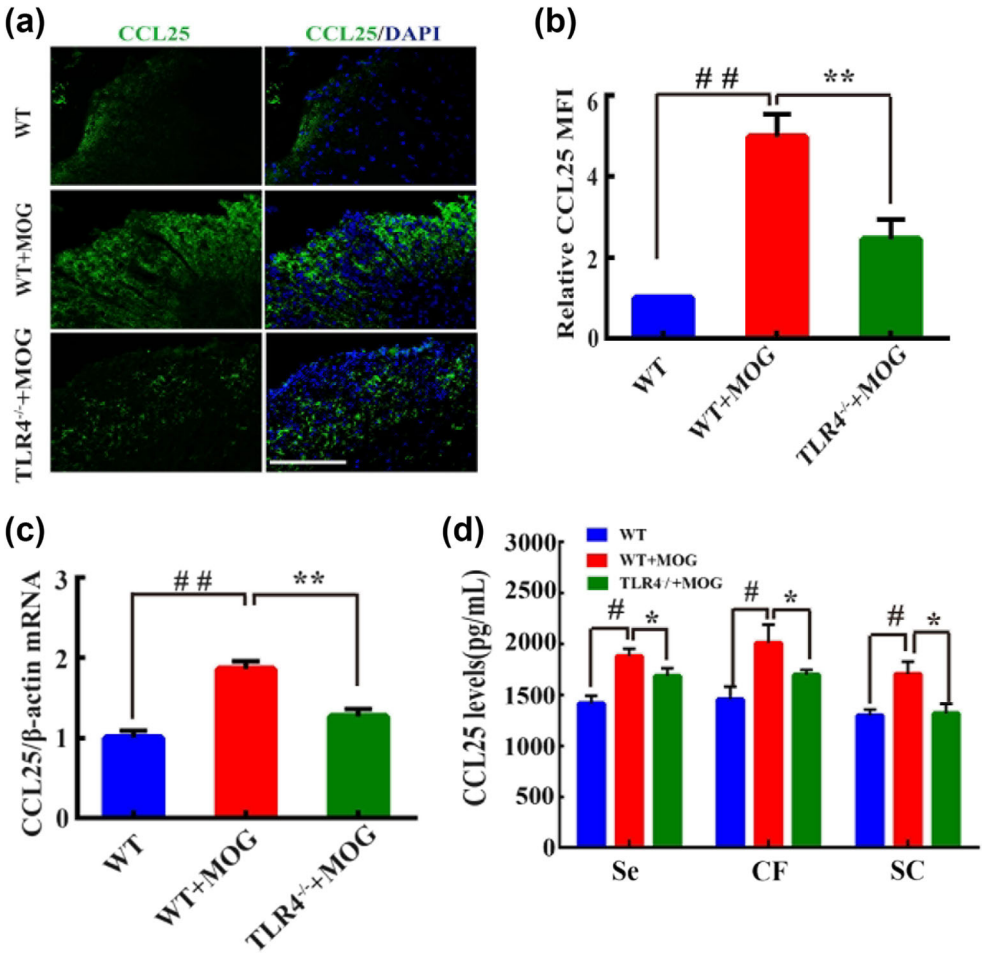

Fig. 4 CCL25 is decreased in TLR4$^{-/-}$ EAE mice. (**a**) CCL25 immunofluorescent staining (green) in spinal cords. Scale bars, 50 μm. (**b**) MFI analysis of CCL25 in a. (**c**) Quantitative PCR analysis of CCL25 expression in spinal cords (n = 3 mice per group). (**d**) Concentration of CCL25 in serum (Se), cerebrospinal fluid (CF) and spinal cord homogenate (SC) is measured by ELISA (n = 5 per group). Data are presented as means ± SD. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ vs WT group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs WT + MOG group. Results are representative of three separate experiments

infiltrated lymphocytes (Fig. 5c). Moreover, during Th17 differentiation in vitro, the stimulation of LPS, a ligand of TLR4, could up-regulate the expression of CCR9, which was dramatically decreased in the TLR4$^{-/-}$ Th17 cells (Fig. 6a, b).

To evaluate the effect of TLR4 on the chemotactic migration of Th17 cells in response to CCL25, transwell assay was used and the result showed that chemotactic migration of Th17 cells by CCL25 was in a dose dependent manner. In response to CCL25, LPS stimulation had a greater increase in Th17 cell chemotaxis, while TLR4 deficiency attenuated Th17 chemotactic migration (Fig. 6c). Above all, these findings demonstrate that TLR4 regulates the expression of CCR9 in Th17 cells, and CCL25/CCR9 signal may mediate the proinflammatory infiltration of Th17 cells into spinal cord during EAE pathogenesis.

Discussion

TLR4 is expressed on almost all cell phenotypes of mammals, including cells in CNS. It has been shown that microglia has a high expression of TLR4, while other kinds of nerve cells express less (Naseemuddin et al. 2012; Suh et al. 2009). The function of TLR4 has been linked to many CNS inflammation diseases, especially autoimmune diseases (Grace et al. 2014; Panaro et al. 2008). TLR4 is able to promote EAE by increasing the secretion of IFN- and IL-17 (Imanishi et al. 2007). Recently, based on the fact that Th17 cells have higher TLR4 expression than other CD4$^+$ Th cell types (Brummelman et al. 2016; Reynolds et al. 2012), more and more attention are paid to the function of TLR4 on CD4$^+$ Th cells (Gonzalez-Navajas et al. 2010). Evidences show that TLR4 activated by LPS fails to induce naïve T cells differentiation, but CD4$^+$ T cells exert improved proliferation ability and tenacious survival when TLR4 pathway is stimulated. In EAE mice, TLR4$^{-/-}$ Th17 cells mainly exhibit reduction of proliferation and failure of crossing blood-brain barrier, leading to an alleviated inflammatory response in CNS (Reynolds et al. 2012). TLR4 can directly regulate lymphocyte migration by its binding to fibronectin (Zanin-Zhorov et al. 2007). In our research, we find TLR4 is necessary for EAE development. After $MOG_{35–55}$ immunization, TLR4$^{-/-}$ mice have lower clinic score and weight loss, as well as less demyelination compared to WT mice. One reason for this phenomenon is that fewer lymphocytes, especially Th17 cells, which are regarded as the major inflammatory subset involved in promoting EAE (t Hart et al. 2011; Tan et al. 2013), infiltrate into CNS when TLR4 is deficient. However, the mechanism of regulating lymphocytes infiltration needs further study.

Lymphocytes recruitment to the CNS is a vital step for the development of inflammatory infiltration in EAE. C-C chemokine receptor (CCR) is a micromolecular protein that

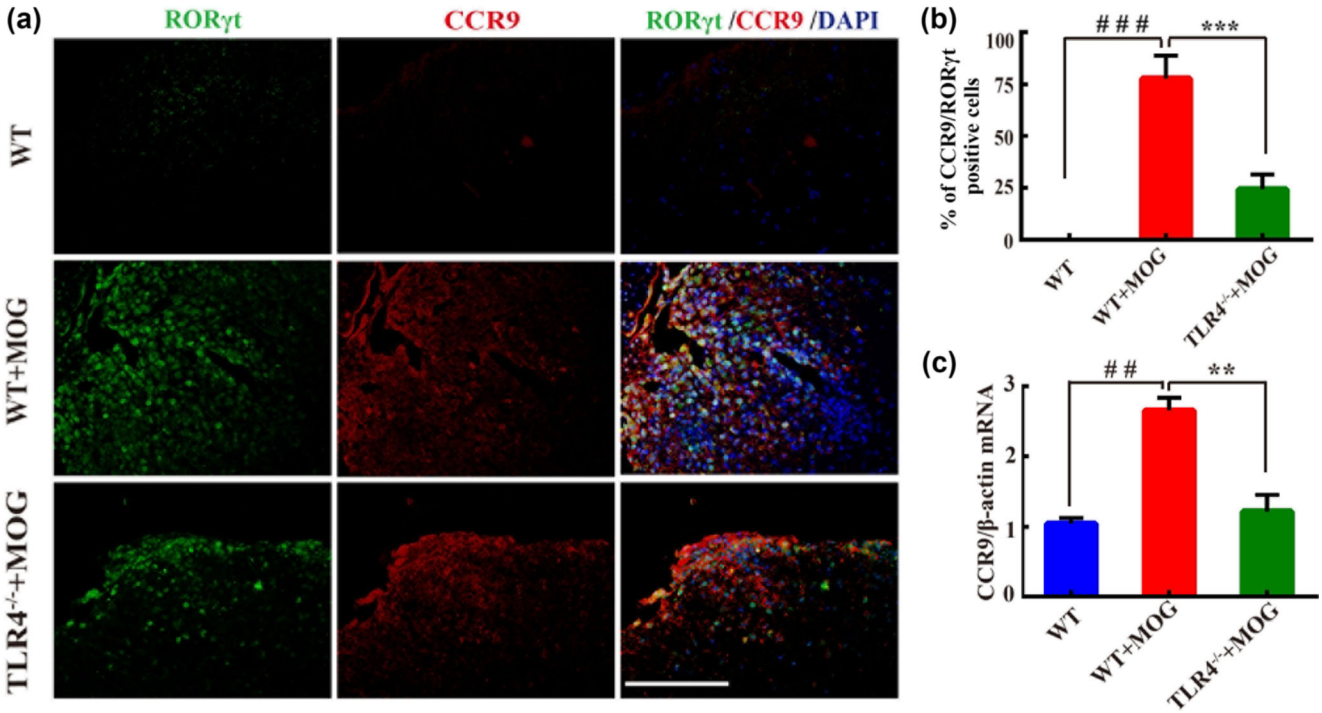

Fig. 5 TLR4 deficiency down-regulates CCR9 expression in infiltrating lymphocytes. (**a**) Immunofluorescent staining of RORγt (green) and CCR9 (red) in spinal cords. Scale bars, 50 μm. (**b**) Quantification of CCR9/RORγt positive cells in a. (**c**) Spinal cords are digested by type I collagenase, and then lymphocytes are collected by Percoll gradient centrifugation for qRT-PCR analysis (n = 3 mice per group). Data are presented as means ± SD. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs* WT group. $^{**}p < 0.01$, $^{***}p < 0.001$ vs WT + MOG group. Results are representative of three separate experiments

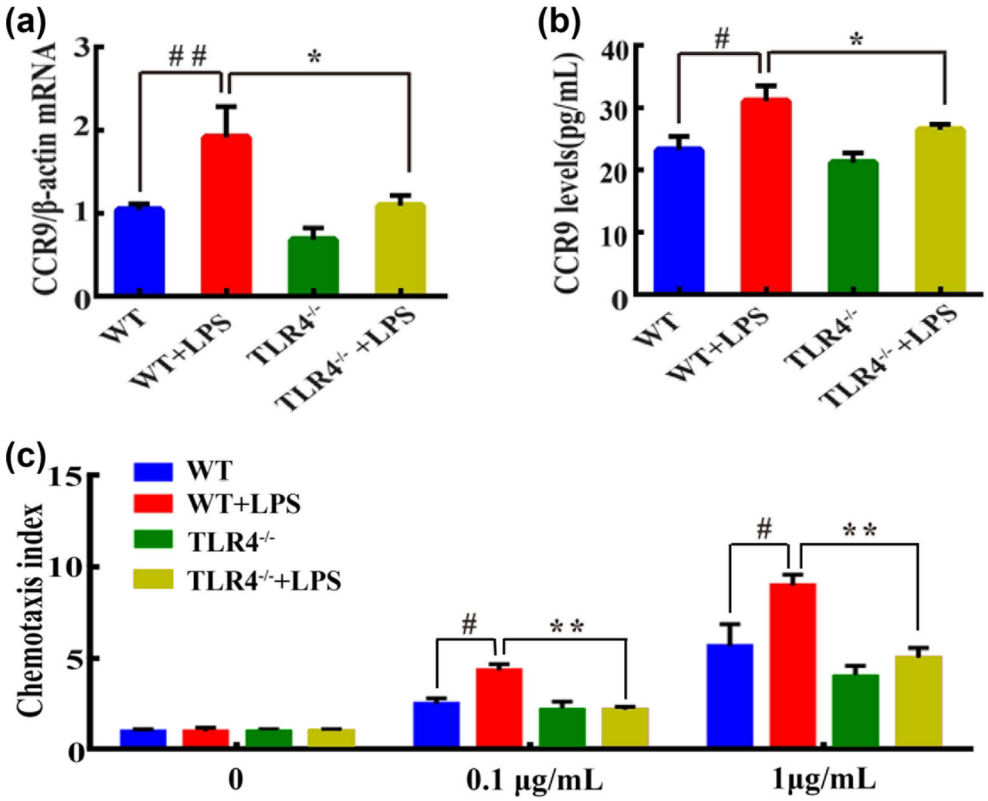

Fig. 6 CCR9 is decreased in TLR4$^{-/-}$ Th17 cells in vitro. Naïve T cells from WT and TLR4$^{-/-}$ mice are stimulated with or without LPS under Th17-polarizing conditions for 3 days. The induced Th17 cells are purified for testing. (**a**) Quantitative PCR analysis of CCR9 expression (n = 3 per group). (**b**) ELISA of CCR9 in 1×10^5 cells homogenate (n = 5 per group). (**c**) Transwell assay shows Th17 chemotaxis in response to different dose of CCL25 (0, 0.1, 1 μg/mL) normalized to migration in medium containing FBS only. Data are presented as means ± SD. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs* WT group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs WT + LPS group. Results are representative of three separate experiments

participates in T cell development and tissue specific homing by binding with its proper ligand (Chantakru et al. 2001; Huber et al. 2002; Labovsky et al. 2017). Th17 cells have been known to express CCR2, CCR5 and CCR6, together with related ligands to mediate effector T cells infiltration into CNS, where effector T cells release inflammatory cytokines contributing to the destruction of myelin and axons (Di Prisco et al. 2014; Gaupp et al. 2003). CCR9 is a pretty important chemokine receptor expressed on most CD4$^+$ thymocytes, and participates in regulating thymocytes proliferation and migration by binding with its homologous ligand CCL25. CCL25/CCR9 can adjust CD4$^+$ T lymphocyte chemotaxis in many physiological and pathological process, such as immune cell growth and differentiation, leukocyte transportation, regulation of immune system stability and allergic disease (Krishnamoorthy et al. 2015b; Tubo et al. 2012; Uehara et al. 2002). Previous studies have found that CCL25/CCR9 is involved in ulcerative colitis, neonatal necrotizing enteritis, and asthma with playing an important role in infiltration of T lymphocytes (Bratke et al. 2013; Egan et al. 2016; Holt and Upham 2004). Here, we explore that the expression of CCL25 in lesion area and CCR9 in activated Th17 cells are both increased during pathological process of EAE, resulting in more lymphocytic infiltration and severe demyelination.

In summary, we demonstrate that TLR4 plays a role on Th17 infiltration during pathogenesis of EAE through regulating CCL25/CCR9 expression. Recent data have shown that CCR9 blocking or inhibition leads to decreased lymphocyte infiltration and improved clinical symptoms in many clinical inflammatory diseases (Krishnamoorthy et al. 2015a; Tubo et al. 2012). The fact that CCR9 mediates infiltration of effector T cells into CNS suggests CCL25/CCR9 is a potential new biological target to inhibit pathologic lymphocyte recruitment in MS treating (Trivedi et al. 2016).

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Authors' Contributions XQ and RY designed the study. YZ performed experiments, analyzed the data and wrote the article. JH, MW, YW, WY, LX and FD performed experiments. FH, HF, XQ and RY reviewed the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflicts of interest.

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