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Role for MyD88, TLR2 and TLR9 but Not TLR1, TLR4 or TLR6 in Experimental Autoimmune Encephalomyelitis

Socorro Miranda-Hernandez,* Nicole Gerlach,*1 Julie M. Fletcher,*1 Erik Biros,* Matthias Mack,† Heinrich Körner,* and Alan G. Baxter*

The potential roles of TLRs in the cause and pathogenesis of autoimmune CNS inflammation remain contentious. In this study, we examined the effects of targeted deletions of TLR1, TLR2, TLR4, TLR6, TLR9, and MyD88 on the induction of myelin oligodendrocyte glycoprotein 35–55 (MOG35–55) peptide/CFA/pertussis toxin-induced autoimmune encephalomyelitis. Although C57BL/6. Tlr1−/−, C57BL/6.Tlr4−/−, and C57BL/6.Tlr6−/− mice showed normal susceptibility to disease, signs were alleviated in female C57BL/6.Tlr2−/− and C57BL/6.Tlr9−/− mice and C57BL/6.Tlr2/9−/− mice of both sexes. C57BL/6.Myd88−/− mice were completely protected. Lower clinical scores were associated with reduced leukocyte infiltrates. These results were confirmed by passive adoptive transfer of disease into female C57BL/6.Tlr2−/− and C57BL/6.Tlr9−/− mice, where protection in the absence of TLR2 was associated with fewer infiltrating CD4+ cells in the CNS, reduced prevalence of detectable circulating IL-6, and increased proportions of central (CD62L+) CD4+CD25+Foxp3+ regulatory T cells. These results provide a potential molecular mechanism for the observed effects of TLR signaling on the severity of autoimmune CNS inflammation. The Journal of Immunology, 2011, 187: 000–000.

Multiple sclerosis (MS) is a complex genetic trait resulting from the contributions of multiple genetic and environmental factors. It is a chronic immune-mediated inflammatory/demyelinating disease of the CNS that occurs in young adulthood and is more common in women. To date, there is no cure or preventative therapy for MS, and the mechanisms underlying the initiation and progression of MS are still unclear. Genetic and epidemiological studies have identified some reproducible associations, such as HLA alleles, UV light exposure, cigarette smoking, and microbial exposure. The hygiene hypothesis, originally proposed by David Strachan of the London School of Hygiene and Tropical Medicine, postulated that the inverse association between the prevalence of hay fever and family size was causally related to infection in early childhood transmitted by unhygienic contact with older siblings. Evidence of causality was provided when a cohort of schoolchildren transmitted by unhygienic contact with older siblings.

Ascaris and Trichuris infections were subsequently twice as likely to have positive skin-prick tests for house dust mite. Similarly, in a population in Guinea-Bissau, measles infection was associated with an 80% reduction in the risk for skin-prick test positivity to house dust mite. Almost 10 y after Strachan first published his idea, Rook and Stanford extraplated it from allergy to autoimmunity, including MS, arguing that vaccination replaced an infectious stimulus that generated a predominately Th1 response with a much weaker stimulus that generated a predominately Th2 response. The original proposed association with cytokine deviation did not survive testing; regression analysis of the Third National Health and Nutrition Examination Survey demonstrated a strong positive association between a reported history of “Th2-mediated allergic disorder” (hay fever, asthma, or both) and “Th1-mediated autoimmune disorders” (thyroid disorder, rheumatoid arthritis, or type 1 diabetes treated with insulin and not tablets). Our new understanding of the IL-17/IFN-γ/IL-4 counterregulation paradigm, and the central role of IL-17 in experimental autoimmune encephalomyelitis (EAE; an animal model of MS), may give this aspect of the hypothesis new life.

Despite this, key issues remain. Without knowing what microbial species are protective, what expressed constituents are responsible for this activity, or how their presence is recognized by the immune system, attempts to establish immunotherapies based on manipulating microbial exposure are fraught with danger. For example, the proposed use of bacillus Calmette-Guérin to treat MS (8) and diabetes (9) was associated with the precipitation of a lupus-like disease in mice.

One potential mechanism by which microbial flora could regulate autoimmune responses is via the TLR system and associated pathways. The family of TLRs plays an essential role in innate and adaptive immune recognition in mammals. TLRs are type 1 transmembrane receptors and recognize distinct pathogen-associated molecular patterns through their leucine-rich repeats in the extracytoplasmic domain. Because many of these molecular patterns are shared with nonpathogens, they are now commonly referred to as microbial-associated molecular patterns.
(15). The cytoplasmic portions of the receptors include a conserved motif, termed the Toll/IL-1R (TIR) domain. The TIR domains of TLRs are homologous with the respective domain of the IL-1R and the cytoplasmic adaptor protein family. The TIR domains of the adaptor proteins interact with the TIR domains of the TLRs or IL-1R and trigger the activation of downstream protein kinases and multiple transcription factors, including the NF-κB family. All TLRs except TLR3 signal through the adaptor protein MyD88; TLR3 signals through a MyD88-independent, TRIF-dependent pathway, and TLR4 uses both MyD88-dependent and -independent pathways (16).

To date, 10 human and 12 mouse TLRs have been identified, and each TLR recognizes a characteristic collection of microbial constituents. For example, TLR2 detects microbe-derived peptidoglycan and lipopeptides (17–19), TLR4 binds LPS (20), and TLR5 binds bacterial flagellin (21). The range of microbial products recognized by TLR is extended by the ability of some TLRs to heterodimerize. For example, the TLR1/TLR2 heterodimer binds triacylated lipopeptides, whereas the TLR2/TLR6 heterodimer binds diacylated lipopeptides (22–24). TLR3, TLR7, and TLR9 are expressed in endosomes and are ligated by microbial nucleic acids (25). TLR9, for example, recognizes bacterial CpG DNA sequences (26).

The role of TLR signaling in the pathogenesis and regulation of CNS inflammation is far from clear. Eighteen patients with chronic progressive MS were treated in an open phase I trial with the TLR3 agonist 2′-O-Methyl-β-cyclodextrin (22–24). TLR3, TLR7, and TLR9 are expressed in endosomes and are ligated by microbial nucleic acids (25). TLR9, for example, recognizes bacterial CpG DNA sequences (26).

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In summary, the role of TLR1 is unknown, and those of TLR2, TLR4, and TLR9 are still controversial. In this article, we revisit this issue in a specific pathogen-free facility specifically constructed to minimize environmental variation.

Materials and Methods

Mice

C57BL/6.Tlr1−/− and C57BL/6.Tlr6−/− mice, backcrossed to C57BL/6 mice six generations, and C57BL/6.Tlr2−/−, C57BL/6.Tlr4−/−, C57BL/6.Tlr9−/−, and C57BL/6.Myd88−/− mice, backcrossed to C57BL/6 mice for six generations, were kindly provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan) (19, 26, 36–39). C57BL/6.Tlr1−/− and C57BL/6.Tlr6−/− mice were further backcrossed to C57BL/6 mice for a total of 10 generations before intercrossing to generate homozygous mutants. C57BL/6.Tlr2−/− and C57BL/6.Tlr9−/− mice were intercrossed twice and homozygous breeders selected to obtain double-congenic TLR2/TLR9−/− deficient mice. All mice were bred and maintained in the Immunogenetics Research Facility at James Cook University under specific pathogen-free conditions. The facility has strict environment-controlled mouse holding rooms that are HEPA-filter ventilated and maintained at 19 ± 1.5°C and 40–50% humidity. Food is sourced from a specialty composition-defined manufacturer (Specialty Feeds, Glen Forrest, Western Australia) that purchases their grain from a restricted number of farms and subject it to gamma irradiation sterilization before delivery. A four-pass air filtration system, advanced drinking water sterilization and purification, as well as a unidirectional operation flow design with shower-in access limits microbial infections of the mice. These studies have been reviewed and approved by James Cook University Animal Care and Ethics Committee.

Induction and scoring of active EAE

Age-matched 7– to 13-week-old mice were immunized s.c. at days 0 and 7 in inguinal regions and flanks with 200 μg MOG35–55 peptide (Auspep, Parkville, VIC, Australia) in 100 μl PBS emulsified in an equal volume (1:1) of IFA (Sigma-Aldrich, Castle Hill, NSW, Australia) containing 0.5 mg heat-killed M. tuberculosis H37RA (Difco; BD Diagnostic Systems, Sparks, MD). Each animal received an i.p. injection of 250 ng PTX (List Biological Laboratories, Campbell, CA) at day of immunization and 2 d later [modified from Prinz et al. (32)].

Mice were scored daily for clinical signs of EAE using the following scale: 0 = no detectable signs of EAE; 0.5 = hunching; 1 = slight tail weakness; 1.5 = distal limb tail or hind-limb weakness; 2 = total tail paraly- sis; 2.5 = hind leg totally paralyzed; 3 = both hind legs totally paralyzed; 3.5 = bilateral hind-limb paralysis in combination with unilateral fore-limb paralysis or weakness of forelimbs; 4 = bilateral hind-limb and forelimb paralysis; 5 = moribund state or death.

Histopathology

Brain and spinal cord (CNS) were collected on day 40 after EAE induction. Mice were euthanized by CO2 asphyxiation. The CNS was perfused with 24 ml ice-cold PBS to avoid leukocyte contamination from blood before being removed and fixed in 10% buffered formalin (Sigma-Aldrich). Tissues were embedded in paraffin, and 4-μm sections were stained with H&E or Luxol fast blue (LFB) to assess the degree of infiltration and demyelination. Protocols were adapted from Humason (40) and IHC World Science Information Network (http://www.ihcworld.com/_protocols/special_stains/fast_blue.htm).

Cell infiltration and defects in myelination of spinal cords were assessed using the following semiquantitative scoring system (modified from Ref. 41): 0 = no lesions, no cell infiltration, and no reduction in LFB staining; 1 = solitary lesions with cell infiltration of low cellular density with or without mildly reduced LFB staining; 2 = two to three lesions per level with moderate cell infiltration associated with reduced LFB staining; 3 = many lesions in almost all fields with extensive cell infiltration associated with severe reduction in LFB staining.
Isolation of CNS leukocytes and microglia

Mice were euthanized by CO2 asphyxiation. The CNS was perfused with 24 ml ice-cold PBS to avoid leukocyte contamination from blood, before being removed and minced through a 180-μm wire stainless steel mesh. The dissociated material was centrifuged at 400 × g for 15 min at 4°C. Enzymatic digestion was performed with Collagenase Type II (0.5 ml/mg) and DNase I (500 U/ml) at 37°C for 60 min. The resulting digest was pelleted and resuspended in 30% isotonic Percoll (Amersham Biosciences, Rydalmerne, NSW, Australia) underlayed with 70% isotonic Percoll and subsequently overlayed with PBS. The tube was centrifuged at 600 × g for 25 min at 25°C. Cells were collected between the 70 and 30% density layers [modified from Ford et al. (42)].

Cell suspensions

Splenocyte and lymph node cell suspensions were prepared by gently grinding organs between two frosted microscope slides in FACS buffer (PBS containing 1 mM EDTA (Amresco, Solon, OH) and 10% (v/v) bovine serum (Invitrogen Life Technologies, Mulgrave, VIC, Australia) and 0.02% sodium azide (BDH, Poole, U.K.). Splenocytes were treated with RBC lysis buffer (Sigma-Aldrich).

DC culture

DCs were derived from bone marrow. In brief, bone marrow cells were isolated from the tibias and femurs of mice by flushing with RPMI 1640 containing 5% (v/v) FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (Invitrogen Life Technologies), and 15% GM-CSF-containing supernatant of hybridoma cell line X63-Ag8. After one wash, cells were seeded in a 24-well plate at a concentration of 1 × 10⁶ cells in 500 μl and cultured at 37°C for 7 d.

In vitro stimulation of splenocytes

Isolated splenocytes were resuspended and plated at 5 × 10⁶/ml in RPMI 1640 supplemented with 10% (v/v) FCS (PAA Laboratories, Morningside, QLD, Australia), 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (Invitrogen Life Technologies).

Cells were treated for 24 h with TLR1/2 agonist synthetic tripalmitylated lipopeptide (Pam3CSK4), TLR4 agonist LPS, TLR9 agonist ODN1826, and/or TLR6/2 agonist FSL-1 (Invitrogen Life Technologies), dissolved at a concentration of 100 μg/ml in endotoxin-free water. Splenocytes were stimulated with a range of agonist concentrations and an optimal concentration for each agonist was chosen: 500 ng/ml for Pam3CSK4, FLS-1, and LPS, and 5 μg/ml for CpG1826. The cells were collected and analyzed for cell surface markers by flow cytometry.

Flow cytometry

Directly labeled fluorescent Abs anti–CD11b-FITC, –PE-Cy7, or –allophycocyanin-Cy7 (clone M1/70), anti–TCRβ-allelophycocyanin or -FITC (clone H57-597), anti–CD4-Pacific blue or -V450 (clone RM4-5), anti–CD3 allelophycocyanin (clone 145-2c11), anti–CD8-allelophycocyanin-Cy7 (clone 53-6.7), anti–CD11c PE-Cy7 (clone HL3), anti–CD19-PE (clone 1D3), anti–CD45/B220-PerCP (clone RA3-B2), or anti–CD69-PE-Cy7 (clone H1.2F3) and anti–Ly6C-PerCP-Cy5.5 (clone AL-21) were obtained from BD Biosciences (San Diego, CA). Anti–CD86-allelophycocyanin (clone GL1), anti–CD62L-PE-Cy7 (clone MEL-14), anti–TLR1–Alexa fluor (clone TR23), anti–TLR2–FITC (clone 6C2), and anti–TLR4–PE (clone UT41) were obtained from eBioscience (San Diego, CA), and anti–CD11c–PE (clone N418) was obtained from BioLegend (San Diego, CA). CCR2 staining with mAbs MC-21 (43) was detected by donkey anti–rabbit Dylight 649 (Jackson Immunoresearch, West Grove, PA). CD45 staining with biotin-conjugated anti-mouse CD45 (clone 30-F11; BD Biosciences) was detected by donkey anti-rat Dylight 649 (Jackson Immunoresearch, West Grove, PA). MyD88 expression was detected by intracellular markers, then fixed, permeabilized, and stained with anti-Foxp3 FITC (clone 236G9), anti-MHC II-PE (clone H57-597), anti–CD11c-PE-Cy7 (clone HL3), anti–CD19-PE (clone M1/70), and anti–CD25 allophycocyanin (clone IA4) was detected by goat anti–rabbit-FITC human and mouse absorbed (Southern Biotech, Birmingham, AL). TLR9 staining with biotin-conjugated anti–TLR9 (clone m9.D6; eBioscience) was detected with streptavidin-PE (BioLegend). CD45 staining with biotin-conjugated anti–CD45/B220-PerCP (clone RA3-B2), or anti–CD69-PE-Cy7 (clone H1.2F3) and anti–Ly6C-PerCP-Cy5.5 (clone AL-21) were obtained from BD Biosciences (San Diego, CA). CCR2 staining with mAbs MC-21 (43) was detected by donkey anti–rabbit Dylight 649 (Jackson Immunoresearch, West Grove, PA). CD45 staining with biotin-conjugated anti-mouse CD45 (clone 30-F11; BD Biosciences) was detected by streptavidin-Pacific orange (Invitrogen Life Technologies). TLR6 staining with rabbit polyclonal anti–TLR6 Ab (Abnova, Taipei, Taiwan) was detected by goat anti–rabbit-FITC human and mouse absorbed (Southern Biotech, Birmingham, AL). TLR9 staining with biotin-conjugated anti–TLR9 (clone m9.D6; eBioscience) was detected with streptavidin-PE (BD Biosciences).

For intracellular staining of Foxp3, the cells were stained with surface markers, then fixed, permeabilized, and stained with anti–Foxp3 FITC (clone FJK-16S; eBioscience). MyD88 expression was detected by intracellular staining using the affinity-purified goat anti-mouse-anti-mouse CD45 (clone 30-Fl1; BD Biosciences) and CD25 staining with biotin-conjugated anti-mouse CD25 (clone IHC; BD Biosciences) was detected by streptavidin-Pacific orange (Invitrogen Life Technologies). TLR6 staining with rabbit polyclonal anti–TLR6 Ab (Abnova, Taipei, Taiwan) was detected by goat anti–rabbit-FITC human and mouse absorbed (Southern Biotech, Birmingham, AL). TLR9 staining with biotin-conjugated anti–TLR9 (clone m9.D6; eBioscience) was detected with streptavidin-PE (BD Biosciences). FcR block (rat anti-mouse CD16/26 [FcRIII receptor, clone 93]; BioLegend) and, in some cases, 50% mouse serum and 10% rat serum were used to prevent nonspecific binding of Abs. If possible, a total of 1 million cells was analyzed per sample; a forward scatter area against forward scatter height gate was used to exclude doublets, and propidium iodide was used to gate out the dead cells from analyses. Samples were acquired using a CyAn ADP Flow Cytometer and analyzed with Summit software (Beckman Coulter, Gladesville, NSW, Australia).

FIGURE 1. Clinical course of active MOG35-55/CFA + PTX -induced EAE in TLR-deficient C57BL/6 mice and C57BL/6 WT control mice. Each data point represents the mean ± SEM of at least five animals per group. Statistical differences in the clinical score between the controls and the TLR-deficient C57BL/6 mice are indicated (*p < 0.05, uncorrected Mann–Whitney U test; n = 5–10). Representative experiments are illustrated; data showing significant differences in clinical scores have been repeated at least once for female C57BL/6.Tlr2−/−, C57BL/6.Tlr9−/−, C57BL/6.Tlr2/9−/− and C57BL/6.MyD88−/− mice (see Table I).
Cytokine assays

Blood was collected by retro-orbital venipuncture, and plasma separated and stored at −80°C until assayed. Quantitative detection of plasma GM-CSF, IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, IL-23, TNF, CXCL1/KC, and MIP-1β were performed by cytometric bead array using Bender Medsystems (Vienna, Austria) mouse Th1/Th2 10plex. Mouse IL-23, and mouse CXCL1/KC, mouse IL-18, and mouse MIP-1 kits. Samples were analyzed following the manufacturer’s protocol. The detection limits were 10.9 pg/ml for GM-CSF, 6.5 pg/ml for IFN-γ, 15.7 pg/ml for IL-1α, 8.8 pg/ml for IL-2, 0.7 pg/ml for IL-4, 4.0 pg/ml for IL-5, 22 pg/ml for IL-6, 5.5 pg/ml for IL-10, 2.4 pg/ml for IL-17, 2.1 pg/ml for TNF, 10.0 pg/ml for IL-18, 14.5 pg/ml for IL-23, 30 pg/ml for CXCL1/KC, and 14.9 pg/ml for MIP-1β.

Data were acquired on either a CyAn ADP (Beckman Coulter) or an LSR Fortessa (BD Biosciences) Flow Cytometer and analyzed with a multiparameter logistic nonlinear regression model using Flow Cytomix Pro 2.2.1 (Bender Medsystems) or GraphPad Prism version 5.00 (GraphPad for Mac Software; GraphPad, San Diego, CA; http://www.graphpad.com).

Plasma samples were assayed in duplicates using an ELISA specific for mouse IFN-β (PBL Biomedical Laboratories, Piscataway, NJ), to quantitate the amounts of IFN protein. The ELISA was performed in accordance with the manufacturer’s protocol and analyzed at an absorbance of 450 nm. The limit of detection of IFN-β was 15.6 pg/ml.

Passive EAE

Donor mice were immunized with MOG35–55/CFA + PTX, as described earlier. The inguinal and axillary lymph nodes and spleens were collected 11 d after immunization. Cells were cultured for 3 d in RPMI 1640 medium (Invitrogen Life Technologies) containing 10% (v/v) PCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 1 mM sodium pyruvate, and 2 μM 2-ME (Invitrogen Life Technologies), supplemented with 50 μg/ml MOG35–55 Peptide (Auspep), 10 ng/ml IL-23 (eBioscience), 10 ng/ml IL-6 (Sigma-Aldrich or eBioscience), and 5 ng/ml recombinant human TGF-β1 (Jomar Bioscience, Kensington, South Australia). After 3 d, cells were harvested, washed, and dead cells were removed using Histopaque 1083 (Sigma-Aldrich). Recipient mice were pre-conditioned with 3 Gy gamma irradiation, and 4 h later, 5 × 10⁶ cells were injected i.p. to induce passive EAE. Each animal received an i.p. injection of 350 ng PTX (List Biological Laboratories) on the day of cell transfer and 2 d later.

Statistical analyses

Statistical differences in quantitative traits were compared by uncorrected Mann–Whitney U test or Kruskal–Wallis test with Dunn’s multiple-comparison posttest. Qualitative traits were compared by Fisher’s exact test. Statistical calculations were performed using InStat 3.0b (1992–2003) and GraphPad Prism 4.0c (San Diego, CA) software. Differences were considered statistically significant when the p values were <0.05.

Table I. Active EAE in TLR-deficient C57BL/6 mice and C57BL/6 WT control mice

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Strain</th>
<th>n</th>
<th>Incidence (%)</th>
<th>Onset (d)</th>
<th>Mean Maximum Score</th>
<th>CDI</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>C57BL/6</td>
<td>5</td>
<td>100</td>
<td>16.0 ± 0.0</td>
<td>3.1 ± 0.1</td>
<td>61.1 ± 3.8</td>
</tr>
<tr>
<td>C57BL/6.Tlr1−/−</td>
<td>6</td>
<td>100</td>
<td>15.2 ± 0.2**</td>
<td>2.7 ± 0.3</td>
<td>53.6 ± 9.7</td>
<td></td>
</tr>
<tr>
<td>C57BL/6.Tlr2−/−</td>
<td>6</td>
<td>100</td>
<td>15.7 ± 0.8</td>
<td>1.6 ± 0.3**</td>
<td>14.2 ± 8.8*</td>
<td></td>
</tr>
<tr>
<td>C57BL/6.Tlr4−/−</td>
<td>6</td>
<td>100</td>
<td>15.7 ± 1.6</td>
<td>3.2 ± 0.4</td>
<td>64.0 ± 19.2</td>
<td></td>
</tr>
<tr>
<td>C57BL/6.Tlr6−/−</td>
<td>6</td>
<td>100</td>
<td>15.5 ± 1.6</td>
<td>3.1 ± 0.5</td>
<td>65.1 ± 19.8</td>
<td></td>
</tr>
<tr>
<td>C57BL/6.Tlr9−/−</td>
<td>6</td>
<td>100</td>
<td>17.0 ± 1.2*</td>
<td>0.8 ± 0.5**</td>
<td>8.5 ± 7.8**</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>C57BL/6</td>
<td>6</td>
<td>100</td>
<td>13.7 ± 0.3</td>
<td>2.9 ± 0.3</td>
<td>57.7 ± 10.3</td>
</tr>
<tr>
<td>C57BL/6.Myd88−/−</td>
<td>6</td>
<td>100</td>
<td>17.0 ± 0.0***</td>
<td>2.2 ± 0.2*</td>
<td>32.0 ± 5.5*</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>C57BL/6</td>
<td>5</td>
<td>100</td>
<td>15.8 ± 0.6</td>
<td>2.6 ± 0.2</td>
<td>48.4 ± 2.8</td>
</tr>
<tr>
<td>4</td>
<td>C57BL/6</td>
<td>5</td>
<td>100</td>
<td>16.2 ± 0.3</td>
<td>2.4 ± 0.2</td>
<td>46.9 ± 4.3</td>
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<tr>
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<td>C57BL/6</td>
<td>8</td>
<td>100</td>
<td>15.9 ± 0.5</td>
<td>2.9 ± 0.1</td>
<td>53.3 ± 3.7</td>
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<td>C57BL/6</td>
<td>5</td>
<td>100</td>
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<td>2.6 ± 0.2</td>
<td>44.9 ± 3.5</td>
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<td>100</td>
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<td>45.5 ± 5.5</td>
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<td>47.7 ± 7.6</td>
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<td>54.8 ± 4.2</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
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<td>100</td>
<td>15.4 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>44.8 ± 7.3</td>
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<tr>
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<td>3.1 ± 0.1</td>
<td>71.8 ± 2.9</td>
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<td>14.7 ± 0.2</td>
<td>3.7 ± 0.3</td>
<td>77.3 ± 8.7</td>
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<td>100</td>
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<td>3.8 ± 0.4</td>
<td>80.2 ± 12.1</td>
</tr>
<tr>
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<td>C57BL/6</td>
<td>5</td>
<td>100</td>
<td>13.2 ± 0.2</td>
<td>3.2 ± 0.2</td>
<td>60.2 ± 4.3</td>
</tr>
<tr>
<td>6</td>
<td>C57BL/6</td>
<td>6</td>
<td>100</td>
<td>13.2 ± 0.2</td>
<td>3.5 ± 0.0*</td>
<td>76.7 ± 2.0</td>
</tr>
<tr>
<td>7</td>
<td>C57BL/6</td>
<td>5</td>
<td>100</td>
<td>13.0 ± 0.0</td>
<td>3.3 ± 0.2</td>
<td>67.3 ± 4.3</td>
</tr>
<tr>
<td>8</td>
<td>C57BL/6</td>
<td>6</td>
<td>100</td>
<td>15.2 ± 0.5</td>
<td>3.0 ± 0.0</td>
<td>64.9 ± 5.2</td>
</tr>
<tr>
<td>9</td>
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<td>5</td>
<td>100</td>
<td>15.3 ± 0.2</td>
<td>2.8 ± 0.2</td>
<td>60.5 ± 3.7</td>
</tr>
<tr>
<td>10</td>
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<td>6</td>
<td>100</td>
<td>13.4 ± 0.2</td>
<td>3.4 ± 0.1</td>
<td>80.9 ± 7.0</td>
</tr>
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<td>100</td>
<td>13.8 ± 0.3</td>
<td>3.8 ± 0.2</td>
<td>74.5 ± 8.3</td>
</tr>
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<td>12</td>
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<td>10</td>
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<td>2.8 ± 0.1**</td>
<td>49.4 ± 5.4***</td>
</tr>
<tr>
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<td>3.0 ± 0.0</td>
<td>70.3 ± 2.9***</td>
</tr>
<tr>
<td>14</td>
<td>C57BL/6</td>
<td>8</td>
<td>0</td>
<td>na</td>
<td>0.0 ± 0.03***</td>
<td>0.0 ± 0.0***</td>
</tr>
</tbody>
</table>

TLR-deficient C57BL/6 and C57BL/6 WT control mice were immunized with MOG35–55 emulsified in IFA and M. tuberculosis. Statistical differences between the TLR-deficient C57BL/6 and C57BL/6 WT control mice are indicated.

*p < 0.05, **p < 0.01, ***p < 0.001, uncorrected Mann–Whitney U test, na, not appropriate.
FIGURE 2. Histopathology of the spinal cords of male and female TLR-deficient C57BL/6 mice and C57BL/6 WT control mice after induction of active MOG35–55/CFA + PTX-induced EAE. Mice were culled 40 d after immunization; the spinal cords were harvested, fixed, sectioned, and stained with H&E or LFB. Representative spinal cord sections are shown. A. Whole spinal cord sections representative of the four gradings of inflammation applied in the histopathological assessment using a modification of the scoring system of Hempel et al. (41). Rectangular boxes indicate the general area from which the high-power sections in B were sampled. B. Representative sections from male (left two columns) or female (right two columns) mice from groups (in rows listed from top to bottom) of PBS-treated C57BL/6 mice; C57BL/6 mice treated with PBS/CFA + PTX; and then MOG35–55/CFA + PTX-treated mice from the following strains: C57BL/6 (positive control), C57BL/6.Tlr1−/−, C57BL/6.Tlr2−/−, C57BL/6.Tlr4−/−, C57BL/6.Tlr6−/−, C57BL/6.Tlr9−/−, C57BL/6.Tlr29−/−, and C57BL/6.MyD88−/−. Original magnification ×100.
Role of TLRs in active EAE

To study the potential role of TLR ligation by bacterial products in the regulation of autoimmune CNS inflammation, we rederived C57BL/6 mice deficient for TLR1, TLR2, TLR4, TLR6, TLR9 and MyD88 into an environmentally controlled “better than specific pathogen-free” facility. These lines were originally generated on the 129 background by Takeda and Akira and subsequently backcrossed to C57BL/6 mice (19, 26, 36–39). Mice deficient for TLR1 and TLR6 were supplied at the BC6 generation and were backcrossed to BC10 before being intercrossed to produce C57BL/6.Tlr1–/– and C57BL/6.Tlr6–/– breeders. Those bearing mutations of Tlr2, Tlr4, Tlr9 and Myd88 were supplied at BC10. C57BL/6.Tlr2–/– and C57BL/6.Tlr9–/– mice were intercrossed twice and the F2 progeny selected to produce a C57BL/6. Tlr2–/–.Tlr9–/– double-mutant line.

To confirm the lack of the corresponding TLR in the TLR-deficient C57BL/6 mice, we compared their constitutive expression of TLR1, TLR2, TLR4, TLR6, TLR9 and MyD88 with that in WT C57BL/6 mice by flow cytometry. As expected, C57BL/6. Tlr1+/+, C57BL/6.Tlr2+/-, C57BL/6.Tlr4+/-, C57BL/6.Tlr6+/-, C57BL/6.Tlr9+/+ and C57BL/6.Myd88+/+ mice did not express the corresponding TLR in comparison with the C57BL/6 WT mice (Supplemental Fig. 1).

In addition, we compared the responses to TLR ligands of B cells from TLR-deficient C57BL/6 mice with those of C57BL/6 WT mice (44). WT C57BL/6 splenic B cells showed an increased expression of the costimulatory molecules CD69 and CD86 after stimulation with TLR2/1 ligand Pam3CSK4, TLR2/6 ligand FSL-1, TLR4 ligand LPS, TLR9 ligand CpG1826, a combination of FSL-1 and CpG1826, or a combination of all four TLR ligands. In contrast, TLR2/1 ligand Pam3CSK4 failed to induce expression of either CD69 or CD86 in TLR1- or TLR2/9-deficient B cells; TLR2/6 ligand FSL-1 failed to induce expression in TLR2, TLR6, or TLR2/9-deficient B cells; TLR4 ligand LPS failed to induce expression in TLR4-deficient B cells; and CpG1826 failed to induce expression in TLR9- or TLR2/9-deficient splenic B cells (Supplemental Fig. 2). MyD88-deficient splenic B cells failed to respond to Pam3CSK4, FSL1, LPS, or CpG1826 (Supplemental Fig. 2). Taken together, these data confirmed that the TLR-deficient mice used in these studies do not express the corresponding TLR and do not respond to their corresponding ligands.

Role of TLRs in active EAE

To determine whether signaling through TLRs plays a role in the induction and/or effector phase of EAE, we immunized female C57BL/6 WT, C57BL/6.Tlr1–/–, C57BL/6.Tlr2–/–, C57BL/6. Tlr4–/–, C57BL/6.Tlr6–/–, C57BL/6.Tlr9–/–, C57BL/6.Tlr2/9–/– and C57BL/6.Myd88–/– mice (5–8 mice/group/experiment) with MOG35–55 peptide in IFA supplemented with EAE in one experiment (mean maximum score: Tlr1–/–,[23] Role of TLRs in active EAE deficient mice used in these studies do not express the corresponding TLR in comparison with the C57BL/6 WT mice (Supplemental Fig. 1).

In contrast, MyD88-deficient mice were completely resistant to EAE in one experiment (mean maximum score: p < 0.001; CDI: p < 0.001, Mann–Whitney U test); in the second experiment, two of five mice developed some weakness, but with delayed onset (p < 0.01, Mann–Whitney U test) and a very much reduced severity of disease (mean maximum disease score: p < 0.01; CDI: p < 0.01, Mann–Whitney U test) (Table I). These results confirm those previously published by Marta et al. (33) and Prinz et al. (32).

Less severe EAE was also observed for C57BL/6.Tlr2–/– mice with a mean maximum score of 1.6 ± 0.3 and a CDI of 14.2 ± 8.8 compared with WT C57BL/6 mice with a mean maximum score of 3.1 ± 0.1 (p < 0.01) and CDI of 61.1 ± 3.8 (p < 0.05, Mann–Whitney U test). Similar results were obtained in two of three additional independent experiments, with a comparable trend seen in the third (exact p value for CDI was 0.051) (Table I, experiment 4). A pooled analysis of the four cohorts of C57BL/6.Tlr2–/– mice and their respective control C57BL/6 mice showed highly significant differences in mean maximum score (p < 0.0005) and CDI (p < 0.0001, Mann–Whitney U test). Similarly, C57BL/6. Tlr9–/– mice exhibited a milder form of EAE compared with WT C57BL/6 mice, with a mean maximal score of 0.8 ± 0.5 and a CDI of 8.5 ± 7.8 (p < 0.01 for both comparisons, Mann–Whitney U test), again with similar results obtained in two additional experiments (Fig. 1, Table I).

Because active MOG-induced EAE appears to be at least partially dependent on signaling through both TLR2 and TLR9, the severity of disease was also examined in C57BL/6.Tlr2/9–/– doubly deficient mice. Compared with either C57BL/6.Tlr2–/– or C57BL/6. Tlr9–/– mice, the mean onset of disease (16.2 ± 0.8), the mean maximal score (1.6 ± 0.2), and the CDI (19.1 ± 4.9) were not statistically significantly different (Fig. 1, Table I). Given the strong suppression of EAE in C57BL/6.Myd88–/– mice, these results indicate that MyD88 plays a role in the pathogenesis of EAE in addition to its role as the adaptor molecule for TLR2 and TLR9.

Evidence of sexual dimorphism was sought by analyzing MOG35–55/CFP-induced EAE in male C57BL/6 WT, C57BL/6. Tlr1–/–, C57BL/6.Tlr2–/–, C57BL/6.Tlr4–/–, C57BL/6.Tlr6–/–,

FIGURE 3. Histopathological assessment of the severity of inflammation in the spinal cords of male and female TLR-deficient C57BL/6 mice and C57BL/6 WT control mice after induction of active MOG35–55/ CFA + PTX-induced EAE applying a modification of the scoring system of Hempel et al. (41). The mean scores of individual mice are represented by the data points and the mean value for each group by the horizontal bar. The first and second groups of each panel are negative control C57BL/6 mice treated with PBS or PBS/CFA + PTX, respectively, followed by MOG35–55/ CFA + PTX-treated C57BL/6 (positive control; closed circles), C57BL/6.Tlr1–/– (open circles), C57BL/6.Tlr2–/– (closed squares), C57BL/6.Tlr4–/– (open downward triangles), C57BL/6.Tlr6–/– (open upward triangles), C57BL/6.Tlr9–/– (closed downward triangles), C57BL/6. Tlr2/9–/– (closed upward triangles), and C57BL/6.Myd88–/– mice (closed diamonds). Statistical differences between the C57BL/6 control mice and the TLR-deficient mice are indicated (* p < 0.05, ** p < 0.01, *** p < 0.001, Mann–Whitney U test for C57BL/6 versus TLR1, TLR2, TLR4, TLR6, TLR9, TLR29, MyD88; n = 5–14).
C57BL/6.Tlr9<sup>−/−</sup>, C57BL/6.Tlr2/9<sup>−/−</sup>, and C57BL/6.Myd88<sup>−/−</sup> mice (5–10 mice/group/experiment). After immunization with MOG<sub>35–55</sub> in CFA accompanied by PTX injection, male C57BL/6 WT mice developed significantly more severe clinical signs of EAE than female C57BL/6 WT mice (mean maximum score: \( p < 0.01 \); CDI: \( p < 0.0001 \); \( n = 30–55 \), Mann–Whitney U test; Fig. 1). Male C57BL/6 WT, C57BL/6.Tlr1<sup>−/−</sup>, C57BL/6.Tlr4<sup>−/−</sup>, and C57BL/6.Tlr6<sup>−/−</sup> mice developed EAE with the same onset of disease (13–17 d), maximum score (3.1 ± 0.1, 3.7 ± 0.3, 3.5 ± 0.0, and 3.3 ± 0.2, respectively), and CDI (71.8 ± 2.9, 77.3 ± 8.7, 76.7 ± 2.0, and 67.3 ± 4.3, respectively) as each other, as observed for the female groups of mice. Similarly, like their female counterparts, male C57BL/6.Myd88<sup>−/−</sup> mice showed no clinical signs of EAE. In contrast with C57BL/6.Tlr2<sup>−/−</sup> and C57BL/6.Tlr9<sup>−/−</sup> female mice, which showed less severe EAE, disease

**FIGURE 4.** Representative flow cytometry showing the proportions of leukocytes from CNS of female C57BL/6.Tlr2<sup>−/−</sup>, C57BL/6.Tlr9<sup>−/−</sup>, C57BL/6.Tlr2/9<sup>−/−</sup>, C57BL/6.MyD88<sup>−/−</sup> and WT C57BL/6 control mice 21 d after MOG/CFA immunization. CD4<sup>hi</sup> and βTCR in combination with CD4 or CD8 were used to detect CD4 or CD8 T cells, respectively. Other cell populations were identified based on the differential expression of CD4<sup>int</sup>, CD11c, and CD11b. The values in plots indicate mean ± SD of four to six mice, with the exception of the PBS-treated control (\( n = 1 \)).

**FIGURE 5.** Absolute numbers of CNS leukocyte subsets from CNS of male (left column) and female (right column) C57BL/6.Tlr2<sup>−/−</sup> (closed squares), C57BL/6.Tlr9<sup>−/−</sup> (closed downward triangles), C57BL/6.Tlr2/9<sup>−/−</sup> (closed upward triangles), C57BL/6.MyD88<sup>−/−</sup> (open diamonds), and WT C57BL/6 control mice (closed circles) 21 d after MOG/CFA immunization. The cell numbers of individual mice are represented by the data points and the mean value for each group by the horizontal bar, with 4–8 mice/group, with the exception of the PBS-treated control (\( n = 1 \); closed diamonds). Statistical differences between the C57BL/6 control group and the TLR-deficient C57BL/6 mice are indicated (*\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), Kruskal–Wallis test with Dunn’s multiple-comparison posttest).
in male C57BL/6.Tlr2−/− and C57BL/6.Tlr9−/− mice was not alleviated and showed the same pattern as that in male C57BL/6 WT mice. Examination of the individual scores shows a very substantial overlap in clinical scores between the two sexes of WT mice. If a threshold in disease severity were responsible for the failure of Tlr2 or Tlr9 deletion to modulate EAE in male mice, then a reduction in the average scores of TLR-deficient mice should still be seen (reflecting reduced scores in a proportion of mice in the group). Such a situation may be occurring in TLR9-deficient mice, which trend toward lower average scores, but not in TLR2-deficient mice, which do not show this trend.

Although neither male C57BL/6.Tlr2−/− nor male C57BL/6. Tlr9−/− mice were protected, alleviation of EAE was observed in male C57BL/6.Tlr2−/− double-mutant mice, with a maximum EAE score of 2.8 ± 0.1 and a CDI of 49.4 ± 5.4 compared with WT male C57BL/6 mice (mean maximum score: 3.4 ± 0.1; CDI: 80.9 ± 7.0; p < 0.01 and p < 0.001, respectively, Mann–Whitney U test).

Histological analysis of the role of TLRs in active EAE

C57BL/6 WT, C57BL/6.Tlr1−/−, C57BL/6.Tlr2−/−, C57BL/6. Tlr4−/−, C57BL/6.Tlr6−/−, C57BL/6.Tlr9−/−, C57BL/6.Tlr2/9−/− and C57BL/6.Myd88−/− mice (5–14 mice/group) were treated with MOG/CFA + PTX and killed ~40 d postimmunization for histological analysis, semiquantitative scoring, and statistical analysis. Representative histological sections of the spinal cord are shown in Fig. 2. Histological evaluation of spinal cord sections showed multiple inflammatory foci in female C57BL/6 WT mice, and the histopathological scores observed in spinal cords averaged 2.39 ± 0.15. In contrast, female C57BL/6.Myd88−/− mice had no inflammation in the spinal cord (histopathological score: 0; p < 0.0005, Mann–Whitney U test) (Fig. 3). Female C57BL/6.Tlr2−/− (histopathological score: 0.93 ± 0.52), C57BL/6.Tlr9−/− (histopathological score: 0.53 ± 0.23), and C57BL/6.Tlr2/9−/− double-mutant mice (histopathological score: 1.35 ± 0.46) also had significantly reduced scores (p < 0.05, p < 0.001, and p < 0.05, respectively, uncorrected Mann–Whitney U test) (Fig. 3). These results confirmed the clinical assessments.

Similarly, like their female counterparts, male C57BL/6. Myd88−/− mice lacked inflammatory lesions in their spinal cords (p < 0.0001, Mann–Whitney U test) (Fig. 3). Significantly reduced infiltration was also found in male C57BL/6.Tlr2−/− mice (2.15 ± 0.23; p < 0.01, Mann–Whitney U test) compared with C57BL/6 WT mice (2.8 ± 0.05) (Fig. 3).

Altogether, these results show that female C57BL/6.Tlr2−/− and C57BL/6.Tlr9−/− mice, as well as female and male C57BL/6. Tlr2/9−/− mice, show less severe EAE signs, whereas female and male C57BL/6.Myd88−/− mice are resistant to EAE.

Phenotypic analysis of cells infiltrating the CNS of TLR-deficient mice in active EAE

Potential explanations for the relative resistance of C57BL/6.Tlr2−/−, C57BL/6.Tlr9−/−, C57BL/6.Tlr2/9−/− and C57BL/6.Myd88−/− mice to active EAE induced with MOG35–55/CFA + PTX include effects on recruitment of inflammatory cells to the CNS and differences in subsets recruited. To determine the nature of cells infiltrating the CNS of TLR-deficient C57BL/6 mice, CNS infiltrating leukocytes from female and male C57BL/6, C57BL/6. Tlr2−/−, C57BL/6.Tlr9−/−, C57BL/6.Tlr2/9−/− and C57BL/6. Myd88−/− mice were compared by flow cytometric analysis 21 d after immunization with MOG35–55/CFA + PTX (Fig. 4). We observed a significant decrease in the total number of CD4+ and CD8+ T cells infiltrating the CNS of female C57BL/6.Myd88−/− mice compared with C57BL/6 mice (p < 0.001 and p < 0.01, respectively, Kruskal–Wallis test with Dunn’s multiple-comparison posttest; Fig. 5) and a significant reduction in CD8+ T cells in female C57BL/6.Tlr9−/− mice (p < 0.05). Because TLR2- and TLR9-deficient mice showed a trend to lower T cell numbers, an additional cohort of C57BL/6, C57BL/6. Tlr2−/− and C57BL/6.Tlr9−/− mice was assessed and the data combined. Numbers of CD4+ T cells infiltrating the CNS of female C57BL/6. Tlr2−/− and C57BL/6.Tlr9−/− mice of the combined experiment were significantly decreased (p < 0.05 and p < 0.01, respectively, Kruskal–Wallis test with Dunn’s multiple comparison posttest; n = 11–13), as were the numbers of CD8+ T cells infiltrating the CNS of C57BL/6.Tlr9−/− mice (p < 0.01).

Table II. Passive EAE in TLR-deficient C57BL/6 mice and C57BL/6 WT control mice

<table>
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<tr>
<th>Experiment No.</th>
<th>Strain</th>
<th>n</th>
<th>Incidence (%)</th>
<th>Onset (d)</th>
<th>Mean Maximum Score</th>
<th>CDI</th>
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</thead>
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<td></td>
<td></td>
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<td></td>
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<td>2.8 ± 0.2</td>
<td>34.4 ± 4.7</td>
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<tr>
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<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>C57BL/6.Tlr9−/−</td>
<td>5</td>
<td>13.8 ± 1.8</td>
<td>1.6 ± 0.4</td>
<td>25.1 ± 9.3</td>
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</tr>
<tr>
<td>2</td>
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<td>2.7 ± 0.2</td>
<td>21.8 ± 2.1</td>
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<td>0.0 ± 0.0**</td>
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<tr>
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<td>4</td>
<td>23.3 ± 1.3</td>
<td>1.5 ± 0.2**</td>
<td>4.4 ± 1.5**</td>
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<td>19.4 ± 2.8</td>
<td>2.1 ± 0.5</td>
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<td>29.1 ± 3.0</td>
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<tr>
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<td>C57BL/6.Tlr9−/−</td>
<td>14</td>
<td>18.8 ± 0.5</td>
<td>1.8 ± 0.2**</td>
<td>19.5 ± 5.5</td>
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<td>2.8 ± 0.1</td>
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<td>0.0 ± 0.0***</td>
<td>0.0 ± 0.0***</td>
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Passive EAE was induced by adoptive transfer of in vitro-activated lymph node and splenic T cells from MOG/CFA-immunized C57BL/6 mice into C57BL/6.Tlr2−/−, C57BL/6.Tlr9−/−, and C57BL/6 control WT mice. Statistical differences between recipients of cells transferred from TLR-deficient C57BL/6 and C57BL/6 WT control mice are indicated.

* p < 0.05, ** p < 0.01, *** p < 0.001, uncorrected Mann–Whitney U test.

na, not appropriate.
Whereas male C57BL/6/Mdy88^−/− mice showed a statistically significant decrease in only the total number of infiltrating CD4^+ T cells (p < 0.01, Kruskal–Wallis test with Dunn’s multiple-comparison posttest; n = 7–8/group; Fig. 5), neither the C57BL/6/MTr2^−/− nor C57BL/6/MTr9^−/− male mice showed any significant difference in CNS infiltration, consistent with the clinical data. As the C57BL/6/MTr2/9^−/− double-mutant and C57BL/6/Mdy88^−/− male mice showed trends from the control mice in terms of some infiltrating cell subsets, an additional cohort of C57BL/6, C57BL/6/MTr2^−/−, and C57BL/6/Mdy88^−/− male mice was assessed and the data combined. Numbers of CD4^+ and CD8^+ T cells infiltrating the CNS of male C57BL/6/Mdy88^−/− mice of the combined experiment were significantly decreased (p < 0.001 and p < 0.05, respectively, Kruskal–Wallis test with Dunn’s multiple comparison posttest; n = 13/group).

CD45 staining in combination with CD11b and/or CD11c was used to distinguish between DCs (CD45^hiSS^hiCD11c^+), macrophages/monocytes (CD45^hiSS^hiCD11b^+), and inflammatory monocytes (CD45^hiSS^hiCD11c^+CD11b^+)(Fig. 4). In contrast with female C57BL/6 mice, which developed severe EAE with high numbers of infiltrating cells, female C57BL/6/Mdy88^−/− mice had statistically significantly fewer infiltrating DCs, macrophages, and inflammatory monocytes (p < 0.05, p < 0.01, and p < 0.001, respectively, Kruskal–Wallis test with Dunn’s multiple comparison posttest; n = 4–6/group). Analysis of the combined experiment also found a significant reduction in CNS infiltrating macrophages in both C57BL/6/MTr2^−/− and C57BL/6/MTr9^−/− female mice (p < 0.05 in both cases) and a significant reduction in inflammatory monocytes in female C57BL/6/MTr2^−/− mice (p < 0.01).

As for the T cells, male mutant mice tended to a phenotype more similar to that of WT mice in terms of macrophage/monocyte infiltration. Only male C57BL/6/Mdy88^−/− mice showed statistically significant decreases in numbers of CNS-infiltrating DCs and macrophages (p < 0.05 and p < 0.01, respectively, Kruskal–Wallis test with Dunn’s multiple comparison posttest; n = 7–8/group) (Fig. 5). Analysis of the combined experiment confirmed reduction of DCs, macrophages, and inflammatory monocytes in only the male C57BL/6/Mdy88^−/− mice (p < 0.05, p < 0.01, and p < 0.05, respectively, Kruskal–Wallis test with Dunn’s multiple comparison posttest; n = 13/group).

Thus, the milder clinical disease and resistance to EAE seen in female C57BL/6/MTr2^−/−, C57BL/6/MTr9^−/−, C57BL/6/MTr2/9^−/−, and C57BL/6/Mdy88^−/− mice and male C57BL/6/Mdy88^−/− mice is associated with decreased numbers of CNS-infiltrating cells compared with C57BL/6 WT mice.

Role of TLRs in passive EAE

Because the induction of EAE in this model involves the use of killed M. tuberculosis as a component of the adjuvant, and M. tuberculosis possesses agonists for TLR2 (45–48), TLR4 (47, 49), and TLR9 (50), one potential explanation for the inhibitory effect of targeted deletion of TLR2 and TLR9 is that suppression of TLR signaling via these receptors has inhibited the adjuvancing action of CFA. Although this finding would be of significant interest, its relevance to human health is unclear. The effects of targeted deletion of TLR2 and TLR9 are therefore examined in the passive model of EAE. Although we found no significant role for TLR4 in active EAE, it remained possible that it did contribute to the activity of PTX in a passive model. Similarly, it has been proposed that irradiation triggers the release of intrinsic TLR ligands (51), as well as the translocation of commensal gut microflora into mesenteric lymph nodes and elevation of circulating LPS (52). We therefore examined the dependence on irradiation, PTX, and TLR4 of passive EAE induced by adoptive transfer of in vitro-activated lymph node and splenic T cells from MOG35–55/CFA + PTX immunized C57BL/6 mice into C57BL/6/MTr2^−/−, C57BL/6/MTr9^−/−, and C57BL/6 control WT mice. A. Data pooled from three experiments using female donors and recipients, with three to six replicates/group/experiment, as indicated in Table II. Each data point represents the mean ± SEM of 6–10 animals/group. B. Adoptive transfer of in vitro-activated lymph node and splenic T cells from MOG35–55/CFA + PTX immunized male C57BL/6 mice into male C57BL/6/MTr2^−/− and C57BL/6 control WT mice (n = 6). Each data point represents the mean ± SEM of 6–10 animals/group.
of disease severity, similar in intensity to that seen in C57BL/6. Tlr4−/− mice (Supplemental Fig. 3).

Passive EAE was induced in 3 Gy irradiated, PTX-treated female C57BL/6.Tlr2−/−, C57BL/6.Tlr9−/− and C57BL/6 recipients by i.p. injection of in vitro-activated lymph node cells and splenocytes harvested from C57BL/6 CFA/MOG + PTX immunized mice 11 d postimmunization. Data were pooled from 3 independent experiments with 3–6 replicates/group/experiment (total n = 13–15/group) (Table II). In none of the experiments did lymphocytes from C57BL/6 mice adoptively transfer EAE into C57BL/6.

Tlr2−/− mice, resulting in a highly significant and highly reproducible decrease in mean maximum score and CDI compared with WT recipients (p < 0.001 for both, uncorrected Mann–Whitney U test) (Fig. 6A, Table II). Suppression of the mean maximum score of C57BL/6.Tlr9−/− recipients occurred in two of the three trials and was confirmed in the pooled analysis (p < 0.01, uncorrected Mann–Whitney U test) (Fig. 6A, Table II). Although the onset of disease in C57BL/6.Tlr9−/− mice consistently appeared earlier than that in C57BL/6 control mice in each trial, this difference did not reach significance in any trial or in the analysis of pooled data.

The greater effect of TLR2 deficiency on passive EAE than on active disease was unexpected. To gain further insight into the role of TLR2 in passive EAE, we performed adoptive transfers in which TLR2 was deleted from either the donor cells, the recipient, both, or neither. Again, adoptive transfer of WT lymphocytes into C57BL/6.Tlr2−/− mice resulted in no disease, in stark contrast with the transfer of aliquots of the same pool of cells into WT mice. The adoptive transfer of C57BL/6.Tlr2−/− cells into either WT or C57BL/6.Tlr2−/− recipients resulted in alleviated disease (Fig. 6B). These results are consistent with previously published comparisons of passive EAE induced by adoptive transfer of WT or TLR2-deficient T cells (35) and confirm that TLR2 plays a role in exacerbating the severity of EAE. In this context, the prevention of disease in TLR2-deficient recipients of WT cells suggests that the presence of the receptor at induction of disease creates a dependence on TLR2 signaling in the effector phase (Fig. 6C). Adoptive transfer of in vitro-activated cells from male C57BL/6 mice into male C57BL/6.Tlr2−/− recipients also failed to induce disease (Fig. 6C), consistent with this hypothesis.

Phenotypic analysis of cells infiltrating the CNS of TLR-deficient mice in passive EAE

Flow cytometric analysis of CNS-infiltrating leukocytes from female C57BL/6.Tlr2−/−, C57BL/6.Tlr9−/− and C57BL/6 WT control mice 34 d after adoptive transfer of C57BL/6 MOG-reactive leukocytes. A, CD45 and SS area were used to distinguish between lymphocytes (high CD45 expression and low SS area) and monocytes/macrophages/DCs (high to intermediate CD45 and high SS area). Lymphocytes were stained with βTCR, CD4, and CD8 to determine major T cell subsets, whereas the APC population was stained with Ly6C, CD11b, and CD11c. B, Total numbers of CD4, CD8 T cells and inflammatory macrophages and mean fluorescence intensity (MFI) of CD11c on myeloid DCs in the CNS of female C57BL/6. Tlr2−/− (closed squares), C57BL/6.Tlr9−/− (closed triangles), and C57BL/6 WT control mice (closed circles). The values in plots indicate mean ± SD of four to six mice, with the exception of the PBS control (n = 1; closed diamonds). Statistical differences between the C57BL/6 control group and C57BL/6.Tlr2−/− or C57BL/6.Tlr9−/− mice are indicated (*p < 0.05, **p < 0.01, Mann–Whitney U test; n = 4–6) (B).
control mice was performed 34 d after adoptive transfer of MOG-reactive leukocytes from female C57BL/6 donors. CD45 and side scatter (SS area) were used to distinguish between lymphocytes (high CD45 expression and low SS area) and monocytes/macrophages/DCs (high to intermediate CD45 and high SS area) (Fig. 7A). Coexpression of CD45a and βTCR in combination with CD4 or CD8 were used to detect CD4+ or CD8+ T cells (Fig. 7A). Consistent with the clinical data, C57BL/6.Tlr2−/− recipients had fewer CNS-infiltrating CD4+ T cells (p < 0.01, pairwise Mann–Whitney U test, n = 4–6/group) (Fig. 7B) and reduced activation (CD11c expression levels) of myeloid DCs (*p < 0.05). Numbers of inflammatory (CCR2+Ly6C+CD11b+) macrophages did not differ significantly (Fig. 7B).

At least two mechanisms could account for the reduced recruitment of CD4 T cells to the brains of C57BL/6.Tlr2−/− mice in this system. Recruitment of leukocytes to the CNS is at least partly dependent on the expression of CCR2 (53, 54). CCR2 levels were examined on the infiltrating CD4 T cells of female C57BL/6.Tlr2−/−, C57BL/6.Tlr9−/− and C57BL/6 recipients of MOG-reactive leukocytes, but virtually no expression was found (data not shown). This was not due to technical difficulties because CCR2 expression on inflammatory macrophages was easily detected (Fig. 7). Because CCR2 expression on T cells may be downregulated after recruitment to the CNS (55), its levels of expression were compared on splenic CD4 T cells of TLR-deficient mutants, but again no difference was found (data not shown).

Role of central regulatory T cells in modulating passive EAE in C57BL/6.Tlr2−/− mice

An alternative explanation for the reduced recruitment of CD4+ T cells to the brains of C57BL/6.Tlr2−/− mice is the suppression of activation and expansion of autoreactive T cells via the activity of regulatory T cells (Tregs). Because prior flow cytometric characterization of NKT cell numbers and subsets in TLR-deficient C57BL/6 mice had failed to identify any significant differences (data not shown), we examined the numbers of splenic CD4+CD25+Foxp3+ Tregs of female C57BL/6.Tlr2−/−, C57BL/6, Tlr9−/− and C57BL/6 recipients of MOG-reactive leukocytes, 34 d after adoptive transfer. Because expression of CD62L has been reported to be associated with suppressive activity of Tregs in vivo in three models of autoimmune/inflammatory disease, including MOG/CFA + PTX-induced EAE (56–59), we specifically examined the CD62L-expressing subset of splenic Tregs. Whereas CD4+CD25+Foxp3+ Tregs were inconsistently increased in spleens of C57BL/6.Tlr2−/− recipients of WT cells, absolute numbers of CD62L-expressing Tregs were significantly increased in C57BL/6.Tlr2−/−, but not C57BL/6.Tlr9−/− recipients of MOG-reactive leukocytes compared with WT recipients (p < 0.01, Mann–Whitney U test, n = 4–6/group) (Fig. 8). This pattern was also seen in the male transfers; numbers of CD62L-expressing Tregs were significantly increased in C57BL/6.Tlr2−/− mice (p < 0.05; Mann–Whitney U test, n = 6/group; data not shown).

In an attempt to identify a molecular mechanism for the increased numbers of central (CD62L+) Tregs in C57BL/6.Tlr2−/− mice, we examined plasma cytokine levels of IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, IFN-γ, TNF, and GM-CSF at 10 or 34 d after adoptive transfer in two independent experiments. As a generalization, most of the cytokines were undetectable in the plasma of most mice. A significant exception to this was IL-6, which was detected in 4 of 6 C57BL/6 recipients at 10 d and 3 of 5 at 34 d, but was undetectable in the plasma of any C57BL/6.Tlr2−/− mice (7/11 versus 0/9; p < 0.005, Fisher’s exact test).

FIGURE 8. Representative flow cytometry of splenic Treg cells from female C57BL/6.Tlr2−/−, C57BL/6.Tlr9−/− and C57BL/6 WT control mice 34 d after adoptive transfer of MOG-reactive leukocytes. A, Lymphocytes were gated by forward scatter and SS and cell clusters excluded by forward scatter height versus forward scatter area gating. Dead cells were excluded by propidium iodide staining. Viable cells were gated on CD3+ and CD4+, and Tregs were identified by CD25 and Foxp3 expression. The histograms show the expression of CD62L on Treg cells. B, Total numbers of CD4 T cells, Treg cells and CD62L-expressing Tregs in the spleen of C57BL/6.Tlr2−/−, C57BL/6.Tlr9−/− and C57BL/6 WT control mice. At least four mice per group were analyzed and the mean value of each group is indicated as a bar. Statistical differences between the C57BL/6 control group and C57BL/6.Tlr2−/− or C57BL/6.Tlr9−/− mice are indicated (**p < 0.01, Mann–Whitney U test; n = 4–6) (B).
Discussion

Although encephalomyelitis can be experimentally induced in laboratory animals in the absence of adjuvant, the severity and incidence of disease is greatly enhanced by its addition (reviewed in Ref. 60). Although the conventional model applies CFA as an adjuvant, zymosan (a ligand for TLR2 and TLR6) (61), CpG-ODN (TLR9) (62), and/or LPS (TLR4) (35, 61, 63) have been successfully (but inconsistently) (61, 63, 64) substituted for killed M. tuberculosis in the induction of EAE, indicating the capacity of TLR signaling to promote the disease. Paradoxically, LPS can also inhibit the induction of conventional CFA-induced EAE (65, 66).

In this article, we have shown that, although targeted deletion of TLR1, TLR4, or TLR6 did not affect the incidence or severity of MOG35–55 peptide-induced EAE, signaling via MyD88 is necessary, and TLR2 and TLR9 contribute to the severity of the disease. The dependence of EAE induction on MyD88 signaling has been reported previously (32, 33, 67), as has the lack of an EAE phenotype in TLR6-deficient mice (33); the role of TLR1 in EAE has not been previously reported. TLR2 can signal as a heterodimer reported previously (32, 33, 67), as has the lack of an EAE phenotype in TLR6-deficient mice (33); the role of TLR1 in EAE has not been previously reported. TLR2 can signal as a heterodimer acting in the absence of either TLR1 or TLR6 has been reported (68) and may be responsible for the dependence of EAE on TLR2 expression in our hands. Alternately, functional redundancy between the effects of ligands for TLR2/1 and TLR2/6 are possible but difficult to test, because Tlr1 and Tlr6, the genetic loci encoding these two TLRs, are in strong linkage disequilibrium.

Although we found that TLR4 deficiency did not affect the severity of active EAE, Marta et al. (33) reported mild exacerbation and Kerfoot et al. (34) reported inconsistent patterns of disease (protection, amelioration, or normal severity) in TLR4-deficient mutants. This variability is in keeping with the inconsistent action of LPS when used as an adjuvant in the induction of disease, as well as its variable ability to prevent EAE (65), and may reflect the effects of natural LPS exposure early in life (69), possibly a consequence of the induction of tolerance to LPS induced by previous exposure (70). Similarly, although we found a partial dependence of both TLR2 and TLR9 signaling for the full expression of active EAE, Marta et al. (33) reported slight exacerbation of disease in TLR9-deficient mice, whereas Prinz et al. (32) reported mild alleviation and found no effect of TLR2 deficiency. Nevertheless, the results published in this study were highly reproducible and are consistent with the known roles of TLR2 and TLR9 in mediating many of the adjuvant-like effects of killed M. tuberculosis.

The observation that although MyD88-deficient mice were completely protected from the induction of active EAE, TLR2/9 doubly deficient mice showed no more resistance than TLR2 or TLR9 singly deficient mice, indicating that MyD88 plays a role in the pathogenesis of EAE in addition to its role as the adaptor molecule for TLR2 and TLR9. This result could either be explained by a degree of redundancy between TLR2/TLR9 and TLR4 signaling or by dependence of EAE induction on IL-18 and/or IL-1 signaling, which is also MyD88 dependent, as has been shown for the analogous animal model experimental autoimmune uveitis (71).

Sexual dimorphism was observed in this study in the active EAE experiments, particularly in TLR2-deficient mice, but also to a lesser extent in TLR9-deficient and TLR2/9 doubly deficient mice. Although sexual dimorphism of TLR effects on disease has not been previously reported, postpubertal mice show sex differences in cytokine responses to TLR ligands, with females producing less IL-1 in response to the TLR2 ligand lipoteichoic acid (72), and it is a typical feature of both EAE (73–76) and MS [reviewed in Orton et al. (77)]. Paradoxically, although the incidence and severity of MS is generally higher in women, it is also more readily affected by environmental change (77). Although these data do not contribute to understanding the greater susceptibility of women to autoimmunity, they do provide a potential mechanism by which environmental factors could play a greater role in the prevalence of disease in women than in men.

Although it could be postulated that in active EAE the role of TLR2 and TLR9 signaling was merely to mediate the adjuvanting activity of CFA (45–48, 50), the demonstration of inhibition of passive (adoptively transferred) EAE in targeted deletion mutants for TLR2 and TLR9 not only confirms the importance of these TLRs in the pathogenesis of EAE, but also implicates these pathways in ongoing immune control of the effector phase of the autoimmune response. Although Prinz et al. (32) also reported a partial dependency on TLR9 expression in the adoptive transfer recipient, our finding of complete dependency on TLR2 expression in recipients of WT cells is novel but consistent with Reynolds et al.’s report (35) that bone marrow chimeras reconstituted with TLR2-deficient marrow expressed ameliorated disease after the induction of active EAE.

The dependency of passive induction of EAE on TLR2 expression in the recipient indicates the presence of tonic signaling through the receptor. This could occur via endogenous or exogenous ligands. Although many putative endogenous ligands for TLR2 have been proposed, none of them meets the reasonable criteria of purity and validation required to demonstrate their ability to directly bind and signal through TLR2 (78). In contrast, microbial products from intestinal microflora have been detected in the blood of healthy humans (79) and mice (80). Specifically, the mucosal translocation and systemic circulation of enterically derived peptidoglycan was confirmed using an NF-κB reporter cell line transfected with Nod1 and by comparing the phagocytic priming of bone marrow neutrophils in WT and Nod1-deficient targeted mutant mice (80). Extracellular recognition of high m.w. polymeric peptidoglycan is mediated by CD14 and TLR2 (17–19, 81, 82).

To our knowledge, we show for the first time, in the context of CNS autoimmune inflammatory disease, that TLR2 signaling is associated with detectable levels of circulating IL-6, reduced numbers of central (CD62L-expressing) Tregs, and increased recruitment of activated CD4 T cells to the brain. EAE is suppressed by the activity of Tregs (83–86), including central Tregs (54, 87), via the action of IL-10 (84, 86, 88). Tregs are induced by CD4+ T cell activation in the presence of TGF-β (86), and their induction is inhibited by IL-6 via trans signaling of soluble IL-6R into T cells causing SMAD7 suppression of TGF-β signaling (89). IL-6 is a proinflammatory growth and survival factor whose expression is induced by TLR2 ligation (19) and is, in part, controlled by the IL-6–NF-κB transcription factor binding site 5’ to the gene’s transcription initiation site (90). IL-6–deficient targeted mutant mice are completely resistant to the induction of EAE by MOG/CFA + PTX (91).

These data therefore provide molecular insight into the differential environment-dependent susceptibility of females to CNS autoimmune inflammatory disease: female C57BL/6 mice were dependent on TLR2 signaling driving the production of IL-6, which inhibited the induction of central Tregs, thereby enhancing the proliferation and/or inhibiting the cell death of encephalitogenic CD4+ T cells.

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Disclosures

The authors have no financial conflicts of interest.

References

8. Tlr4
ROLE OF TLR IN EAE


