Poly(ADP-ribose) polymerase-1 (PARP-1) has been implicated in the pathogenesis of several central nervous system (CNS) disorders. However, the role of PARP-1 in autoimmune CNS injury remains poorly understood. Therefore, we studied experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis in mice with a targeted deletion of PARP-1. We identified inherent physiological abnormalities in the circulating and splenic immune composition between PARP-1−/− and wild type (WT) mice. Upon EAE induction, PARP-1−/− mice had an earlier onset and developed a more severe EAE compared with WT cohorts. Splenic response was significantly higher in PARP-1−/− mice largely because of B cell expansion. Although formation of Th1 and Th17 effector T lymphocytes was unaffected, PARP-1−/− mice had significantly earlier CD4+ T lymphocyte and macrophage infiltration into the CNS during EAE. However, we did not detect significant differences in cytokine profiles between PARP-1−/− and WT spinal cords at the peak of EAE. Expression analysis of different PARP isozymes in EAE spinal cords showed that PARP-1 was down-regulated in WT mice and that PARP-3 but not PARP-2 was dramatically up-regulated in both PARP-1−/− and WT mice, suggesting that these PARP isozymes could have distinct roles in different CNS pathologies. Together, our results indicate that PARP-1 plays an important role in regulating the physiological immune composition and in immune modulation during EAE; our finding identifies a new aspect of immune regulation by PARPs in autoimmune CNS pathology.

Poly(ADP-ribose) polymerase-1 (PARP-1) belongs to a family of enzymes that regulate several cellular processes by adding poly(ADP-ribose) polymers to specific proteins (1, 2). Based on sequence homology, 18 PARP family members have been identified in the human genome (3). Of these, PARP-1 is the most abundantly expressed PARP in most cell types (2), accounting for up to 85% of maximally activated cellular PARP activity (4). Initially, PARP-1 function was associated only with DNA single-stranded break repair and maintenance of genomic integrity (5–7). However, recent evidence has shown that PARP-1 plays important roles in multiple cellular and disease processes (reviewed in Ref. 8). In several cell types, PARP-1 has been shown to have a direct role in gene expression by modulating specific transcription factors including those regulating inflammation (9–11). Recent studies have also reported their involvement in DNA replication (12) and epigenetics (13–15). In addition, excessive activation of PARP-1 has been shown to induce a form of caspase-independent cell death, which involves the nuclear translocation of the flavoprotein apoptosis-inducing factor from the mitochondria (16–18). This type of cell death by PARP-1 activation, termed “parthanatos,” (19) has been implicated as an important mediator of various central nervous system (CNS) disorders (20). Therefore, several studies have examined PARP-1 as a target for therapeutic intervention (21–24), and PARP-1 inhibitors are currently the focus of several clinical trials (25).

Mice lacking PARP-1 (PARP-1−/−) have also shown significant protection against various forms of CNS injury (26). These mice are resistant to neuronal cell death after ischemia-reperfusion injury and neurodegeneration (27, 28). Furthermore, PARP-1 deficiency is suggested to alter immune responses, as PARP-1−/− mice showed resistance to lipopolysaccharide-induced endotoxin shock (29). This immunomodulation has been suggested to be a result of PARP-1 regulation of transcriptional networks involved in immune cell activation and inflammatory cytokine production (30). Modulation of the activity of transcription factors, such as nuclear factor-κB (NFκB) and nuclear factor of activated T cells (NFAT) by PARP-1 has been reported in T lymphocytes (29, 31, 32). Moreover, T cell-dependent antibody responses are also found to be markedly reduced in PARP-1−/− mice (33). In addition to immune activation, there is also evidence indicating that PARP-1 could be required in earlier stages of immune system maturation. For example, in vitro experiments have suggested that PARP-1 is critical for the function...
tional maturation of dendritic cells (34). Furthermore, activation and proinflammatory gene expression in resident immune cells in the brain (microglia and astrocytes) have also been found altered in vitro by the deficiency of PARP-1 (11, 35) or in the presence of PARP inhibitors (35, 36).

In animal models, administration of putative PARP-1 inhibitors, such as 6(5H)-phenanthridinone, benzamide, Pj-34, and 5-aminoisoquinolinolone, has been shown to ameliorate clinical signs of experimental autoimmune encephalomyelitis (EAE) (37, 38), experimental autoimmune arthritis (39) and experimental autoimmune diabetes (40). These effects have been suggested to be caused mainly by attenuation of inflammatory responses involving multiple immune cell types (41). Although PARP-1 is believed to be the primary target in these studies, the specificity of these inhibitors on the different PARP isoforms has not been completely examined (42). Moreover, recent evidence suggests that there may be distinct but cooperative roles for PARP-1 and other PARP family members in their immunomodulatory effects (36).

Despite all of these previous studies, the specific role of PARP-1 in CNS immune injury seen in multiple sclerosis remains largely unclear. In this study, we examined the autoimmune response and progression of EAE in PARP-1−/− mice. Our results unexpectedly demonstrated that the deficiency of PARP-1 markedly alters the immune phenotype, leading to an exacerbated EAE in PARP-1−/− mice.

**EXPERIMENTAL PROCEDURES**

**Reagents and Animals**—All chemicals were purchased from Sigma-Aldrich unless otherwise specified. New England Peptide (Gardner, MA) synthesized the peptide containing amino acids 35–55 of myelin oligodendrocyte glycoprotein (MOG) used in this study. Antibodies against CD4, CD8, B220, CD25, CD11c, CD11b, CD45, MHC Class II, IFNγ, and IL-17 were purchased from BD Biosciences, CD68 was from AbD Serotec (Raleigh, NC), myelin basic protein (MBP) was from Novus Biologicals (Littleton, CO), Iba1 was from Wako Pure Chemical Industries (Osaka, Japan), and AlexaFluor 488- and 555-conjugated secondary antibodies were from Invitrogen. Liberase RI and DNase I were purchased from eBioscience (San Diego), CD68 was from AbD Serotec (Raleigh, NC), myelin basic protein (MBP) was from Novus Biologicals (Littleton, CO), Iba1 was from Wako Pure Chemical Industries (Osaka, Japan), and AlexaFluor 488- and 555-conjugated secondary antibodies were from Invitrogen. Liberase RI and DNase I were purchased from eBioscience (San Diego), CD68 was from AbD Serotec (Raleigh, NC), myelin basic protein (MBP) was from Novus Biologicals (Littleton, CO), Iba1 was from Wako Pure Chemical Industries (Osaka, Japan), and AlexaFluor 488- and 555-conjugated secondary antibodies were from Invitrogen. Liberase RI and DNase I were purchased from eBioscience (San Diego), CD68 was from AbD Serotec (Raleigh, NC), myelin basic protein (MBP) was from Novus Biologicals (Littleton, CO), Iba1 was from Wako Pure Chemical Industries (Osaka, Japan), and AlexaFluor 488- and 555-conjugated secondary antibodies were from Invitrogen.
and positive controls using phorbol myristyl acetate (50 ng/ml) and ionomycin (750 ng/ml) during the last 2 h of incubation, to evaluate the viability and positive response of the CD4\(^+\) T lymphocytes in culture, were performed. At the end of incubation, cells were collected and processed for flow cytometry.

**Purification of Dendritic Cells**—Mouse spleens (three pooled for each sample) were collected in RPMI medium containing 0.5 Wünsch units/ml Liberase RI (Roche Applied Science) and 14 μg/ml DNase I (Roche Applied Science). Spleens were then sliced into small pieces using surgical blades and incubated with continuous shaking for 30 min at room temperature. During incubation, spleen pieces were triturated every 10 min by using a tube of equilibrated sheep anti-rat Biomag beads (Qiagen, Valencia, CA), and incubated for 30 min at 4 °C with 5% CO\(_2\) for 1 h. Negative controls were incubated at 37 °C with 5% CO\(_2\) for 1 h. Nega-

For the macropinocytosis assay, FITC-conjugated dextran was added to a tube of equilibrated sheep anti-rat Biomag beads (Qiagen, Valencia, CA), and incubated with FcR block (eBioscience) for 10 min at 4 °C. Cells were then incubated with CD11c-specific MACS separation, and the non-bound cells were processed for magnetic sorting (MACS, Miltenyi Biotec) using CD11c-specific MACS beads for 15 min in a rotary mixer at 4 °C. The incubation suspension was then loaded on an equilibrated MACS LS column; bound cells were washed three times and eluted. Cells were then counted, and the purity of each preparation was tested using flow cytometry for CD11c-positive cells as described below; only samples of >90% purity were used for further experiments.

**Dendritic Cell Phagocytosis and Macropinocytosis Assays**—To examine the antigen uptake potential of PARP-1\(^{-/-}\) dendritic cells, we performed specific assays for phagocytosis and macropinocytosis. In this experiment, purified dendritic cells were plated at a density of 3 \(\times\) 10\(^4\) cells on Costar\(^\) ultra-low-binding 96-well plates. For the phagocytosis assay, FITC-conjugated albumin was added at a final concentration of 20 μg/ml. For the macropinocytosis assay, FITC-conjugated dextran was added at a final concentration of 1 mg/ml. Cells under both conditions were incubated at 37 °C with 5% CO\(_2\) for 1 h. Negative controls without albumin or dextran and a more stringent temperature-based control in which cells exposed to either FITC-albumin or FITC-dextran were incubated at 4 °C for 1 h were also set up concurrently. At the end of incubation, the cells were processed for flow cytometry to estimate uptake by quantifying FITC fluorescence along with the detection of CD11c\(^+\) cells as described below.
FIGURE 2. Lymphocyte response during EAE in PARP-1−/− mice. A, changes in spleen weights during EAE in PARP-1−/− and WT mice. After MOG-CFA immune challenge, spleen weights of PARP-1−/− mice increased significantly compared with WT cohorts at Day 10 and Day 14 (p < 0.05, n = 8). B, changes in splenocyte numbers during EAE in PARP-1−/− and WT mice. Corresponding to spleen weight increase, spleen cellularity also increased significantly in PARP-1−/− compared with WT mice at Day 10 and Day 14 (p < 0.05, n = 8). C, CD4+ T lymphocytes in the spleen. Non-immunized PARP-1−/− mice (Day 0) had a significantly higher number of CD4+ cells in the spleen compared with WT (p < 0.05, n = 8). However, after MOG-CFA immune challenge, CD4+ cells in the spleen were not significantly different between PARP-1−/− and WT mice. D, CD8+ T lymphocytes in the spleen. Similar to CD4+ T lymphocytes, non-immunized PARP-1−/− mice had a significantly higher number of CD8+ cells in the spleen compared with WT (p < 0.05, n = 8). However, after MOG-CFA immune challenge, CD8+ cells in the spleen were not significantly different between PARP-1−/− and WT mice. E, representative scatter plots showing splenic CD4+ and CD8+ T lymphocyte proportions in non-immunized (Day 0) PARP-1−/− and WT mice. F, representative scatter plots showing splenic CD4+ and CD8+ T lymphocyte percentages at Day 10 of EAE in PARP-1−/− and WT mice. Although the percentages appear drastically different between PARP-1−/− and WT mice, absolute CD4+ and CD8+ cell numbers were not significantly different because of the increase in overall spleen cellularity. G, B220+ B lymphocytes in the spleen. Unlike CD4+ or CD8+ T lymphocytes, the B cell numbers in non-immunized PARP-1−/− spleens were not different compared with WT. However, after MOG-CFA immune challenge, the expansion of B220+ cells in the PARP-1−/− spleen was higher compared with WT mice and was statistically significant at Day 10 (p < 0.05, n = 8). H, representative scatter plots showing splenic B220+ B lymphocyte proportions in non-immunized PARP-1−/− and WT mice. There were no differences in base-line B cell numbers between PARP-1−/− and WT mice. I, representative scatter plots showing splenic B220+ B lymphocyte proportions at Day 10 of EAE in PARP-1−/− and WT mice. The percentages and associated absolute B cell numbers in the PARP-1−/− spleen were significantly higher compared with WT cohorts.
**PARP-1 and Multiple Sclerosis**

Isolation of CNS Mononuclear Cells—Transcardial perfusion was carried out under anesthesia with 20 ml of sterile PBS to clear all vascular blood cells in the CNS. Immediately after perfusion, brain and spinal cord were removed by dissection in a laminar flow unit and placed in HBSS (with 2 mM EDTA) on ice. Collected tissues were chopped into small pieces (~1 mm³) in a Petri dish with digestion buffer (HBSS with 0.5 Wünsch Units/ml Liberase RI and 14 μg/ml DNase I) using a sterile scalpel blade. The tissue pieces were then incubated in a 37 °C incubator with 5% CO₂ and shaking at 100 rpm/min for 25 min. After incubation, the resulting isolated cells were passed through a 100-μm cell strainer and rinsed twice with HBSS. Cells were then pelleted by centrifugation at 1500 rpm for 10 min at 4 °C and subsequently separated in a step Percoll gradient as described for dendritic cells. Cells at the interface were collected with a Pasteur pipette into a separate tube and washed twice by centrifugation and resuspension in HBSS. These cells were counted and prepared for either flow cytometry or cell culture.

Flow Cytometry—Before antibody labeling, cells were spun down and resuspended in FACs buffer (PBS containing 1% bovine serum albumin with 0.1% azide). To reduce nonspecific antibody binding, surface Fc receptors were blocked using FcR block for 30 min. Surface labeling using fluorophore-conjugated antibodies (CD4, CD8, B220, CD14, F4/80, MHC Class II, CD11c, CD25, CD11b, CD45, and isotype controls) was carried out directly in FACs buffer with a 2-h incubation at 4 °C. After incubation, cells were washed by centrifugation and resuspension in FACs buffer to remove unbound antibodies. Cells were subsequently either fixed with 1% formaldehyde solution for analysis or permeabilized for intracellular labeling (IFNγ, IL-17, CD68, and isotype controls). For intracellular labeling for IFNγ and IL-17, cells were fixed and permeabilized using the BD Cytofix and Cytoperm buffers (BD Biosciences). Fluorophore-conjugated IFNγ and IL-17 were then added into BD Cytoperm buffer and allowed to label overnight at 4 °C. The next day, cells were washed with BD Cytoperm buffer, fixed using 1% formaldehyde solution, and analyzed. For intracellular labeling of CD68, cells were fixed and permeabilized using Leucopem (AbD Serotec) and processed as described above for IFNγ and IL-17. Data acquisition from labeled cells was performed in a CyAN flow cytometer (Dako Cytomation, Carpinteria, CA). Isotype and autofluorescence controls were examined for each assay. Acquired data were subsequently analyzed by calculating proportions/percentages and average intensities using FlowJo software (Treestar, Inc., San Carlos, CA). Absolute numbers of cellular subtypes were derived from percentages using the total cell numbers counted after isolation.

Immunohistochemistry—Spinal cords were dissected from transcardial perfusion-fixed animals and placed in 4% neutral buffered formalin for 24 h. Samples were then embedded in OCT compound (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen-cooled isopentane. Sections (10 μm thick) were prepared in a cryotome and stored at −20 °C until use. For routine histological examination, sections were stained with hematoxylin and eosin. For localization of MBP and Iba1, slides were post-fixed in 4% neutral buffered formalin containing 0.3% Triton X-100 for 30 min. Nonspecific binding of antibodies was blocked using 10% normal goat serum, and then samples were incubated with primary antibodies against either MBP or Iba1 overnight at 4 °C. Slides were then washed in PBS and incubated with fluorophore-conjugated secondary antibodies (1:500). Fluorescent nuclear counterstaining was carried out using 4,6-diamidino-2-phenylindole (1 μM final concentration; Invitrogen) for 10 min. Slides were then washed in PBS and mounted using ProLong Gold mounting medium (Invitrogen). Images were acquired on an Olympus BX61-DSU microscope (Olympus Corp., Melville, NY) using a Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Shizuoka, Japan).

RNA Isolation and qPCR—To examine the relative expression of immune cytokines, adhesion molecules, PARP isozymes and epigenetic regulators during EAE in PARP-1−/− mice, we used a real-time quantitative reverse transcriptase-polymerase chain reaction (qPCR) approach. Total RNA was extracted from lumbar spinal cords isolated (after clearing vascular blood cells as described earlier) from non-immunized mice (Day 0) and from mice at their peak of clinical EAE scores (Day 14) using a Qiagen RNeasy lipid mini kit following the standard protocol. For quality control, RNA purity was verified using the OD_{260/280} ratio to be between 1.8 and 2.0. Total RNA

**FIGURE 3. MOG-specific Th17 and Th1 polarization during EAE in PARP-1−/− mice.** A, IL-17-producing CD4+ T cells at Day 7 after MOG-CFA immunization. Although the mean number of Th17 cells significantly increased after in vitro stimulation with MOG and phorbol myristate acetate (PMA), there were no differences between the numbers of Th17 cells generated by PARP-1−/− and WT CD4+ T cell populations collected from the spleen and lymph nodes (n = 7). B, IL-17-producing CD4+ T cells at Day 10 after MOG-CFA immunization. Similar to Day 7, there were no differences in Th17 cell numbers between PARP-1−/− and WT (n = 10). C, IFN-γ-producing CD4+ T cells at Day 7 after MOG-CFA immunization. Although the mean number of Th1 cells significantly increased after in vitro stimulation with MOG and phorbol myristate acetate, there were no differences between the mean numbers of Th1 cells generated by PARP-1−/− and WT CD4+ T cell populations collected from the spleen and lymph nodes (n = 7). D, IFN-γ-producing CD4+ T cells at Day 10 after MOG-CFA immunization. The number of CD4+ T cells polarizing to a Th17 subtype dramatically increased compared with Day 7. However, there were no differences in mean Th1 cell numbers generated by PARP-1−/− and WT CD4+ T cells (n = 10).
(1.0 µg) was reverse-transcribed to cDNA using Multiscribe™ reverse transcriptase (Applied Biosystems). Subsequent qPCR reactions for TNFα, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-10, iNOS, and TGFβ were performed in triplicate on a Roche Lightcycler 480 using SYBR Green Master Mix (Roche Applied Science) with specific primers (supplemental Table 1) either designed or selected from published literature (44, 45). The specificity of products from each primer set was validated by analyzing melting curves ($T_m$). To examine the induction of PARP-1 and relative changes in PARP-2, PARP-3, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), DNA methyltransferase 1 (DNMT1), and CTCF binding factor (CTCF) expression during EAE in WT and PARP-1−/− mice, qPCR reactions were performed in duplicate using validated TaqMan gene expression assays (Applied Biosystems), PARP-1 (Mm00500171_g1), PARP-2 (Mm01319555_m1), PARP-3 (Mm00467486_m1), ICAM-1 (Mm 01175876_g1), VCAM-1 (Mm01320970_m1), DNMT1 (Mm01151065_g1), CTCF (Mm004840 27_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Mm99999915_g1). For both SYBR Green and TaqMan primers, validation was performed by calculating qPCR efficiencies by amplification of a standardized dilution series and constructing a relative efficiency plot comparing target and reference $ΔC_t$ values to ensure that the absolute slope of fit line was less than 0.1 (46). Subsequently, all experimental samples were analyzed and normalized with expression level of the internal control gene, GAPDH. Relative quantification of fold-change was performed comparing $C_p$ values (calculated by second derivative maximum) from individual mice by applying the $2^{-ΔΔC_t}$ method (46).

**RESULTS**

**EAE in PARP-1−/− Mice—**We first studied EAE in PARP-1−/− versus WT mice. In our paradigm of EAE induction, WT mice showed a mild to moderate EAE, consistent with the anticipated outcome for the 129S/J strain of mice (47). Surprisingly, PARP-1−/− mice had an earlier onset and a more severe disease compared with WT cohorts (Fig. 1A). Clinical signs of EAE were evident by Day 11 in PARP-1−/− compared with Day 13 in WT mice. Mean clinical scores were significantly higher on Days 11–17 in PARP-1−/− compared with WT mice. At the peak of disease (Days 14–15) mean clinical scores for PARP-1−/− mice were 2-fold higher compared with WT (1.5 in WT versus 3.0 in PARP-1−/−). In addition, disease incidence was also higher in PARP-1−/− mice (90.0%) compared with WT cohorts (66.7%). During EAE, mean decrease in body weight was also significantly greater in PARP-1−/− mice compared with WT ($p < 0.05$, Fig. 1B). This significant decrease in body weight of PARP-1−/− mice corresponded to the time points of peak clinical scores despite the fact that initial body weights of PARP-1−/− mice corresponded to the time points of peak clinical scores despite the fact that initial body weights of PARP-1−/− mice were higher than their WT counterparts. Corresponding to their disease severity, PARP-1−/− mice also had an increased mortality rate during progression of EAE (Fig. 1C). Survival rates were 86.7% for PARP-1−/− compared with 93.3% for WT mice. Clinical scores, incidence, and survival rate were highly reproducible during subsequent experiments when MOG-CFA was injected to examine the immune response in these mice.

**Differences in Circulating Immune Cells between PARP-1−/− and WT Mice—**To evaluate the physiological peripheral immune profile, we performed hematologic on circulating blood from 10-week-old PARP-1−/− mice and compared values with WT cohorts from the same genetic background. Our results showed that PARP-1−/− mice had physiologically abnormal immune cell counts and mean corpuscular volume (Table 1). Compared with WT, PARP-1−/− mice had an overall...
A significant decrease in total leukocytes (0.79-fold, \( p < 0.05 \)), mainly because of lower lymphocyte numbers (0.65-fold, \( p < 0.05 \)). However, the numbers of circulating neutrophils (1.46-fold, \( p < 0.05 \)) and monocytes (2.97-fold, \( p < 0.05 \)) were significantly higher in PARP-1\(^{-/-}\) mice compared with the WT. It should also be noted that erythrocyte numbers were significantly decreased (\( p < 0.05 \)), and values of mean corpuscular volume were significantly increased (\( p < 0.05 \)) in PARP-1\(^{-/-}\) mice. These results suggest that there are global effects on the immune phenotype due to the deficiency of PARP-1. These changes in the immune system due to the deficiency of PARP-1 could result in altered immune responses in these mice.

**Immune Response during EAE in PARP-1\(^{-/-}\) Mice**—As an indicator of lymphocyte expansion after an immune challenge, we monitored spleen weights and cellularity in PARP-1\(^{-/-}\) and WT mice at four different time points (Days 0, 7, 10, and 14). Interestingly, PARP-1\(^{-/-}\) mice showed a greater increase in spleen weight compared with WT mice and were highly significant at Day 10 and Day 14 of EAE (\( p < 0.05 \), Fig. 2A). Corresponding to spleen weight increases, PARP-1\(^{-/-}\) mice also showed increased spleen cellularity that was also significant at Day 10 and Day 14 of EAE (\( p < 0.05 \), Fig. 2B). These findings suggested that PARP-1\(^{-/-}\) mice had a higher index of immune cell expansion compared with WT. On investigating different immune subsets in the spleen, we found that at Day 0, PARP-1\(^{-/-}\) mice had higher absolute numbers of CD4\(^{+}\) and CD8\(^{+}\) T lymphocytes compared with WT cohorts (\( p < 0.05 \), Fig. 2, C and D). However, this difference in absolute CD4\(^{+}\) and CD8\(^{+}\) T lymphocyte numbers was not significant at 7, 10, and 14 days after EAE induction. Although scatter plots visualizing the proportions of CD4\(^{+}\) and CD8\(^{+}\) cells in the spleen showed significantly different values between PARP-1\(^{-/-}\) and WT mice (Fig. 2, E and F), this difference was nullified by the significant increase in spleen cellularity in PARP-1\(^{-/-}\) compared with WT mice. Unlike T lymphocytes, B220\(^{+}\) B lymphocytes were not significantly different before EAE induction; however, there was an increase in absolute B cell numbers that was significant at Day 10 of EAE (\( p < 0.05 \), Fig. 2G). In agreement with B cell numbers, scatter plots visualizing proportions of B220\(^{+}\) cells in the spleen were not significantly different

![Image](image-url)
between PARP-1−/− and WT mice at Day 0 (Fig. 2H) but showed significantly different values at Day 10 (Fig. 2I) of EAE. This increased B cell expansion in PARP-1−/− mice could in part contribute to its significantly higher spleen weight and cellularity during EAE progression compared with WT mice.

Subsets of spleen and lymph node CD4+ T lymphocytes that polarize to IL-17-producing T helper type 17 (Th17) cells were not significantly different between PARP-1−/− and WT mice at both Day 7 (Fig. 3A) and Day 10 (Fig. 3B) of EAE. Likewise, CD4+ lymphocyte subsets that polarize to IFNγ-producing Th1 cells did not show any difference between PARP-1−/− and WT mice at either Day 7 (Fig. 3C) or Day 10 (Fig. 3D) of EAE. Thus, the deficiency of PARP-1 did not affect Th1 and Th17 polarization by spleen and lymph node CD4+ T cells.

Antigen presentation by macrophages plays an important role in lymphocyte expansion in the spleen. We therefore examined the number of CD14+ F4/80+ macrophages in the spleens of PARP-1−/− and WT mice during EAE. Despite the increased number of circulating monocytes, our results showed that CD14+ F4/80+ cell numbers were not significantly different between PARP-1−/− and WT spleens at either Day 0 or Day 14 (Fig. 4, A and B). Moreover, evaluation of MHC Class II expression by CD14+ F4/80+ cells revealed no differences between PARP-1−/− and WT spleen macrophages at either Day 0 or Day 14 of EAE (Fig. 4, C and D). Thus, spleen macrophage numbers and their antigen-presenting properties were not affected by the deficiency of PARP-1.

**Dendritic Cell Function in PARP-1−/− Mice**—It has been suggested that PARP-1 may be critical for dendritic cell maturation (34). However, we found that CD11c+ dendritic cell numbers were not different between PARP-1−/− and WT spleens before and during EAE (Fig. 5A). The expression of co-stimulatory markers indicative of maturation, CD80 and CD86, was also not different between PARP-1−/− and WT mice (Fig. 5, B–E). In addition, functional antigen uptake evaluated by phagocytosis of FITC-albumin was not significantly different between purified dendritic cells from PARP-1−/− and WT spleens (Fig. 5, F and G). However, we found that antigen uptake by macrophagocytosis of FITC-dextran was significantly decreased in PARP-1−/− dendritic cells compared with WT (Fig. 5, H and I).

**CNS Immune Infiltration and Microglial Activation during EAE in PARP-1−/− Mice**—We then compared CNS immune infiltrates between WT and PARP-1−/− mice. We found significantly higher numbers of CD4+ T lymphocytes at Day 10 of EAE in PARP-1−/− mice (p < 0.05; Fig. 6, A and B). However, the number of CD8+ T lymphocytes was not different at Day 10 and was comparatively low (Fig. 6A). At Day 14 of EAE, although there was an increase in the mean numbers of both CD4+ and CD8+ T lymphocytes in PARP-1−/− mice, these differences were not statistically significant compared with WT mice (Figs. 6, C and D). When specific polarized nature of CD4+ CNS infiltrates to CD4+CD25+ T regulatory cells was examined in the CNS, we found no differences in the numbers between PARP-1−/− and WT mice at either Day 10 or Day 14 of EAE (Fig. 6F). In addition, when either the Th1 or the Th17 cell type was examined, we also found no differences in numbers between PARP-1−/− and WT mice (Fig. 6, F and G). To distinguish any differences in histopathological lesions between the two groups, spinal cord pathology and MBP staining for myelin were compared between mice of equivalent clinical EAE scores at Day 14. The results showed no differences in the severity and extent of incident lesions between PARP-1−/− and WT spinal cords (Fig. 6H).

Similar to CD4+ T lymphocytes, the number of CD11b+CD45hi macrophages in the CNS was also significantly greater in PARP-1−/− mice compared with WT mice at Day 10 of EAE (Fig. 7, A and B). However, there were no differences between macrophage infiltrates in the CNS at Day 14 of the disease (Fig. 7A). This finding correlated with the earlier onset of clinical signs in PARP-1−/− mice. Corresponding to this macrophage infiltration, we also found that the number of CD11b+CD45lo microglia was significantly higher in PARP-1−/− mice at Day 10 of EAE but not at Day 0 or Day 14 (Fig. 7, B and C). However, the rate of activation of these CD11b+CD45lo microglia indicated by the expression level of CD68 was not significantly different between WT and PARP-1−/− mice at either Day 10 or Day 14 of the disease (Fig. 7, D and E). However, because of the significant difference in mean microglia numbers at Day 10, it should be noted that the overall numbers of activated microglia in PARP-1−/− mice was significantly higher in PARP-1−/− mice compared with WT mice (p < 0.05). Immunohistochemical localization of Iba1-expressing macrophages and microglia indicated similar enrichments at the sites of lymphocyte infiltration in both PARP-1−/− and WT mice at Day 14 (Fig. 7F). Although circulating neutrophil numbers were significantly elevated in PARP-1−/− mice, there were no differences in mean CNS neutrophil infiltration when compared with WT cohorts (Fig. 7, G and H).

**FIGURE 5.** Dendritic cell response and function in PARP-1−/− mice. A, dendritic cell numbers in the spleen. PARP-1−/− mice did not show differences in splenic CD11c+ cells compared with WT during EAE progression (n = 6–8/group). B, dendritic cell maturation indicated by a CD11c+CD80+ subpopulation was not affected in PARP-1−/− mice when examined at Day 0 and Day 14 (n = 6/group). C, representative scatter plots at Day 14 from mice with a clinical EAE score 4 showing the CD11c+ and CD11c CD80+ subpopulations in the spleen. Associated absolute CD11c+CD80+ cell numbers from these percentages in the PARP-1−/− spleen were not significantly different compared with WT cohorts. D, dendritic cell maturation indicated by a CD11c+CD86+ subpopulation was not affected in PARP-1−/− mice when examined at Day 0 and Day 14 (n = 6/group). E, representative scatter plot at Day 14 from mice with a clinical EAE score 4 showing the CD11c+ and CD11c CD86+ subpopulations in the spleen. Associated absolute CD11c+CD86+ cell numbers from these percentages in the PARP-1−/− spleen were not significantly different compared with WT cohorts. F, phagocytosis in PARP-1−/− dendritic cells. FITC-albumin uptake estimated as intracellular mean fluorescence intensity (MFI) showed no differences between PARP-1−/− and WT dendritic cells (n = 3/group). Control mean fluorescence intensity was estimated by incubating WT dendritic cells with FITC-albumin at 4 °C. G, representative histogram showing no differences in FITC-albumin uptake between PARP-1−/− and WT dendritic cells. Also shown are histograms for autofluorescence (Auto) and FITC-albumin uptake by a WT control at 4 °C (Control). H, macrophagocytosis in PARP-1−/− dendritic cells. FITC-dextran uptake estimated as intracellular mean fluorescence intensity showed significant decrease in PARP-1−/− compared with WT dendritic cells (p < 0.05, n = 3/group). Control MFI was estimated by incubating WT dendritic cells with FITC-dextran at 4 °C. I, representative histogram showing an increased uptake in WT dendritic cells compared with PARP-1−/−. Also shown are histograms for autofluorescence and FITC-dextran uptake by a WT control at 4 °C.
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Differences in Cytokine and Adhesion Molecule Expression during EAE in PARP-1−/− Mice—Cytokine expression in the CNS during EAE can highlight specific pathology of both immune state and tissue injury. Therefore we examined the relative expression of selected factors during Day 0 and Day 14 of EAE between PARP-1−/− and WT lumbar spinal cords (Fig. 8). We found significant up-regulation of TNFα, IFNγ, IL-1β, IL-5, TGFβ, and iNOS at Day 14 of EAE in both PARP-1−/− and WT mice. However, we did not detect any significant differences between PARP-1−/− and WT mice either before induction (Day 0) or at the peak of EAE (Day 14). As other homologs exist for PARP-1, they may functionally compensate in substituting for the function of disrupted gene in PARP-1−/− mice. Moreover, recent evidence suggests that there may be cooperative roles for PARP-1 and other PARP family members in immunomodulatory effects (36). We thus examined the expression of three PARP family members (PARP-1, PARP-2, and PARP-3, which have the highest sequence homologies) in PARP-1−/− and WT lumbar spinal cords during EAE. The comparison of PARP-1 expression during EAE in spinal cord tissues of WT mice showed that PARP-1 mRNA levels were significantly down-regulated (0.8-fold, p < 0.05) at the peak of EAE (Day 14) compared with non-immunized controls (Day 0; Fig. 9A). PARP-2 expression when compared in these same tissues showed that mRNA levels did not change significantly during the course of EAE in either PARP-1−/− or WT mice (Fig. 9B). PARP-3 expression was found significantly up-regulated (≥6-fold, p < 0.05) in both PARP-1−/− and WT mice at the peak of EAE compared...
with Day 0 controls (Fig. 9C), suggesting a previously unknown role for PARP-3 in EAE.

**DNMT1 and CTCF mRNA Expression during EAE**—Several recent studies have shown that PARP-1 can regulate genomic methylation patterns by either modulating the activity of CTCF or directly regulating DNMT1 gene transcription (48, 49). We therefore examined the expression of DNMT1 and CTCF in PARP-1^{−/−} and WT lumbar spinal cords during EAE. A comparison of DNMT1 expression during EAE in spinal cord tissues of WT mice showed that DNMT1 mRNA levels were significantly up-regulated (1.5-fold; \( p < 0.05 \)) at the peak of EAE (Day 14) compared with non-immunized controls (Day 0; Fig. 9D). However, DNMT1 expression levels remained unchanged during EAE in PARP-1^{−/−} mice (Fig. 9D). CTCF expression levels, on the other hand, showed a significant decrease at the peak of EAE compared with non-immunized controls (0.8-fold in WT versus 0.7-fold in PARP-1^{−/−} mice; Fig. 9E). In pairwise comparisons, CTCF levels in PARP-1^{−/−} mice at Day 0 were not different from WT mice at Day 14. However, there were no significant differences between PARP-1^{−/−} and WT mice either before induction (Day 0) or at the peak of EAE (Day 14).

**DISCUSSION**

Although several studies have suggested that PARP-1 is a potential therapeutic target for the treatment of autoimmune diseases including multiple sclerosis (37, 38), the specific role of PARP-1 in disease development and progression has not been studied. We investigated autoimmune responses during EAE in mice lacking PARP-1.

Our results unexpectedly revealed that PARP-1^{−/−} mice had an earlier onset and developed a more severe EAE compared with wild type mice, indicating that PARP-1 likely has a rather complex role in regulating immune composition and function during EAE. Previously, it had been well accepted that PARP-1 activation in the presence of low levels of DNA damage promotes cell survival, but excessive activation in the presence of widespread DNA damage causes cell death (4, 50, 51). However, emerging evidence extends PARP-1 function beyond its role in DNA repair and cell death. Its role in epigenetic modulation of chromatin structure can dynamically control the functional state of DNA (14, 52). This is mediated by a direct interaction of PARP-1 within the DNMT1 promoter, protecting its unmethylated state (49). In addition, cross-talk between CTCF and poly (ADP-ribosyl)ation in the regulation of DNA
methylation has also been demonstrated (48). This specific effect on DNMT1 and global epigenetic regulation by PARP-1 could have different gene expression outcomes in different cell types, a conclusion that is supported by several studies (53, 54). This conclusion is also confirmed in this study, as significant differences were detected in the expression of DNMT1 and CTCF during EAE in PARP-1−/− mice. Thus, we believe that the autoimmune phenotype in the EAE model is significantly influenced by epigenetic changes involving multiple cell types in PARP-1−/− mice.

These epigenetic changes could occur in both immune and CNS cells. Changes observed in physiological peripheral circulating immune composition in PARP-1−/− mice could, for the most part, be contributed by cellular changes in gene regulation thresholds due to the complete absence of PARP-1. Immune cell type-specific regulation was also indicated in our studies by the fact that within the splenic lymphocyte population, physiological CD4+ and CD8+ T lymphocyte numbers were significantly elevated in PARP-1−/− mice, whereas B220+ B lymphocyte numbers were not significantly different from WT mice. In addition, the lack of PARP-1 could affect various functional endpoints of these cell types. For example, significant transcriptional differences during in vitro activation of PARP-1−/− T cells have been reported (30), and a critical requirement for PARP-1 in the terminal differentiation/maturity of dendritic cells has also been suggested (34). These changes could be due to alterations in epigenetic states based on cellular requirements during activation or in response to specific immune stimuli. Alternatively, specific functional interaction of PARP-1 either physically or by poly(ADP-ribosyl)ation of proteins involved in these activation pathways could also modulate immune responses (32).

Our observations clearly demonstrate that the lack of PARP-1 does not affect the mounting of an autoimmune response and that it in fact significantly enhances the severity of autoimmune injury. Populations of splenic CD4+ and CD8+ cells expanding after the MOG-CFA challenge were not different between PARP-1−/− and WT mice. Thus, their proliferation rates were similar, even after taking into account the fact that PARP-1−/− mice had a higher initial number of these cells in the spleen. This finding is in contrast to previous in vitro studies showing that proliferation rates of PARP-1−/− lymphocytes after anti-CD3 and anti-CD28 stimulation are significantly lower than WT controls (30). This discrepancy could be due to a more physiological antigen presentation milieu in vivo compared with the receptor cross-linking-based activation in vitro. Alternatively, it is possible that there is more efficient and abundant antigen presentation in PARP-1−/− mice than in WT mice. Supporting evidence for the latter is that in the same spleens, B lymphocyte expansion was significantly higher in PARP-1−/− mice. A recent study has shown that B lymphocytes from PARP-1−/− mice have normal proliferative responses in vitro (33). Therefore, the increases seen in our study could suggest an enhanced antigen presentation in PARP-1−/− mice in vivo. However, we did not find any differences in the number and MHC Class II expression of splenic macrophages in PARP-1−/− mice, thus arguing against this possibility. Although dendritic cell numbers were also not affected, there was a significant but modest decrease in the rate of macroinocytosis in PARP-1−/− dendritic cells. The relationship between the rate of macroinocytosis and dendritic cell function is not completely understood. In certain cases, macroinocytotic potential has been associated with dendritic cell maturation (55, 56). This could suggest that PARP-1−/− dendritic cells mature at a faster rate. However, we found that the expression of co-receptors CD80 and CD86 indicative of maturation was not different between PARP-1−/− and WT dendritic cells. Taken together, our PARP-1−/− dendritic cell data suggest that any deficits due to the deficiency of PARP-1 in dendritic cells are not detrimental to its function.

Downstream of antigen presentation, the establishment of EAE requires the activation of Th1 cells, which secrete the pro-inflammatory cytokines IFNγ, TNFα, and lymphotixin-α (57), and Th17 cells, which secrete IL-17 (58). We found no differences in the Th1 and Th17 responses between PARP-1−/− and WT mice. It has been previously shown that T cell-dependent antibody responses are markedly reduced in PARP-1−/− mice (33), suggesting a deficit in the Th2 response. Although Th2-mediated suppression can occur in chronic progressive EAE through immune deviation (59, 60), the CFA used for MOG immunization for EAE primarily directs a Th1 response. After disease induction, immunotolerant T regulatory cells contribute notably to the negative regulation of an autoimmune response (61). Our investigations into the potential for an altered EAE tolerance in PARP-1−/− mice also did not suggest a role for PARP-1 in this process. The above findings show that generation of effector Th1, Th17, and T regulatory cell types is not significantly affected by the deficiency of PARP-1.

Although we observed a significant B lymphocyte expansion in PARP-1−/− spleens, B cells are not necessary for the gener-

FIGURE 7. Macrophages, microglia, and neutrophils in the CNS during EAE in PARP-1−/− mice. A, number of CD11b+ CD45th macrophages in the CNS at Day 10 and Day 14 of EAE. PARP-1−/− mice had a significantly higher CD11b+ CD45th cell infiltration compared with WT at Day 10 of EAE (p < 0.05, n = 6). Although the means were different at Day 14, CD11b+ CD45th cell numbers were not different for this time point. B, representative contour plots showing proportions of CD11b+ CD45th and CD11b+ CD45sh cells in the CNS at Day 10 of EAE in a PARP-1−/− and WT cohort. Increased percentages of CD11b+ CD45th macrophages and CD11b+ CD45sh microglia were seen in the PARP-1−/− mice compared with WT. C, number of CD11b+ CD45th microglia in the CNS at Day 0, Day 10, and Day 14 of EAE. PARP-1−/− mice had a significantly higher CD11b+ CD45th microglia compared with WT at Day 10 of EAE (p < 0.05, n = 6). Microglia numbers were not significantly different on Day 0 or Day 14 of EAE. D, microglial activation indicated by the expression of CD68 in CD11b+ CD45th cells in the CNS expressing CD68 were not different between PARP-1−/− and WT mice at either Day 10 or Day 14 of EAE. E, representative histograms showing microglia (Mg) and macrophage (Mø) expression of CD68 in a PARP-1−/− and WT mouse, both with a clinical EAE score of 4 at Day 14. There were no differences in CD68 expression in either CD11b+ CD45th or CD11b+ CD45sh cells between PARP-1−/− and WT mice. F, immunohistochemistry for Iba1 in sections of lumbar spinal cord at Day 14 of EAE. Representative images from a PARP-1−/− and WT mouse, both with a clinical EAE score of 4 are shown. Region of infiltrating cells (4,6-diamidino-2-phenylindole) and Iba1 positive macrophages and microglia indicate activation-induced overexpression of Iba1 at a similar rate in both PARP-1−/− and WT mice (n = 6). Scale bars indicate 100 μm. G, CNS neutrophil infiltration during EAE in PARP-1−/− and WT mice. There were no differences in Ly6G+ neutrophils in the CNS at Day 10 or Day 14 of EAE between PARP-1−/− and WT cohorts (n = 6). H, representative histograms comparing CNS Ly6G+ cells in PARP-1−/− and WT mice, both with a clinical EAE score of 4. We detected no significant differences in Ly6G+ cell numbers in the absence of PARP-1.
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The extravasation of MOG-specific encephalitogenic T lymphocytes. In fact, the complete absence of B lymphocytes does not impair T effector functions (62, 63). However, several reports have shown data supporting the ability of activated B lymphocytes to stimulate naive T cells (64–66). Thus, the robust B lymphocyte expansion seen in PARP-1−/− mice could potentially enhance stimulation of naive T lymphocytes in vivo to generate MOG-specific T effector cells.

We found that the extravasation of activated myelin-specific T lymphocytes occurred significantly earlier in the CNS of PARP-1−/− mice, suggesting that an earlier breach in the tight endothelial junctions comprising the blood-brain barrier may account for the earlier onset of EAE clinical signs in PARP-1−/− mice. ICAM-1 and VCAM-1 have been implicated in CNS infiltration during EAE. ICAM-1 null mice show markedly reduced spinal cord T lymphocyte infiltration during EAE (67). PARP-1 is known to negatively regulate ICAM-1 expression in lymphocytes and their subsequent adhesion to endothelial cells (68, 69). In our knock-out model, we found that ICAM-1 and VCAM-1 levels were not different between PARP-1−/− and WT mice at the peak of EAE, suggesting that these adhesion molecules can also be regulated by pathways independent of PARP-1. Although infiltration was significantly higher at an earlier time point in PARP-1−/− mice, the specific subsets of Th1 and Th17 effector cells in the CNS were not different between PARP-1−/− and WT mice. However, there was a clear indication that the elaboration of chemokines and cytokines by these earlier invading myelin-specific T cells also led to the earlier influx of macrophages to the CNS in PARP-1−/− mice.

In addition to immune infiltrates, inflammatory effects on CNS-resident microglia were also evident in PARP-1−/− mice. Significant increases in PARP-1−/− microglial cell numbers leading to the pres-
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with an earlier disease onset and longer disease duration, resulted in the increased severity of EAE in PARP-1−/− mice.

Furthermore, the examination of different PARP isoforms allowed additional insight into the role of different PARPs in EAE. Our observation that PARP-1 in the CNS was significantly down-regulated to a modest degree at the peak of EAE suggested that cell death mediated by PARP-1 overactivation is not predominant during autoimmune injury. In accordance with this interpretation, activation of PARP-1 was observed only in astrocytes in a primate model of EAE (74). This led us to consider the question of how PARP inhibitors could bring about protection in EAE. One potential explanation is the differences that exist between PARP-1 inhibition versus genetic disruption as highlighted in a recent study on astrocytes suggesting a cooperative function for PARP-1, PARP-2, and PARP-3 in inflammatory responses (36).

Among all PARP family members, PARP-2 and PARP-3 carry the highest sequence homology to PARP-1. PARP-2 catalytic domain has the strongest resemblance to PARP-1 with 69% homology (3) and has partially redundant functions with PARP-1 (75–77). This functional overlap is key in the survival of PARP-1−/− mice because mice with double deletion of PARP-1 and PARP-2 are not viable (76, 78). Moreover, PARP-2 has a significant role in immune system development, and its deficiency significantly affects the survival of CD4+CD8+ double-positive thymic lymphocytes (79). However, we did not find any evidence for compensatory up-regulation of PARP-2 transcripts in PARP-1−/− mice. Compared with PARP-1 and PARP-2, PARP-3 is an understudied protein, which is not completely characterized. However, preliminary observations on PARP-3 have suggested that it is also involved in transcriptional silencing and cellular response to DNA damage (80) similar to PARP-1. The discovery that PARP-3 is significantly up-regulated at the peak of EAE in both PARP-1−/− and WT mice suggests a potentially important role for this molecule in CNS inflammatory injury. These observations suggest that there may be distinct roles for the different PARP isozymes in EAE development and pathology, forcing us to reevaluate current models explaining the mechanism of protective effects seen with PARP inhibitor therapy in CNS autoimmune diseases.

In conclusion, our results demonstrate that the deficiency of PARP-1 can bring about widespread changes in the physiological immune composition and the outcome of an autoimmune response. Our findings also identify previously unknown aspects of immune regulation by PARPs relevant to autoimmune CNS pathology. These results on the EAE in PARP-1−/− mice are unexpected, revealing a rather complex role for PARP-1 in regulating immune and CNS factors. Understanding these autoimmune responses and neurological outcomes are critical for the design of therapeutic strategies to target different PARPs for the treatment of multiple sclerosis.

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