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Exacerbation of Experimental Autoimmune Encephalomyelitis in P2X₇R⁻/⁻ Mice: Evidence for Loss of Apoptotic Activity in Lymphocytes

Lanfen Chen² and Celia F. Brosnan

The purinergic receptor P2X₇-R is a nucleotide-gated ion channel that has been proposed to function as a major regulator of inflammation. In this study we examined the role of this receptor in regulating inflammation in the CNS by determining the effects of the loss of this receptor (P2X₇-R⁻/⁻) on experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis. We show here that P2X₇-R⁻/⁻ mice developed more severe clinical and pathological expression of EAE than wild type (WT) controls and that spleen and lymph node cells from P2X₇-R⁻/⁻ mice proliferated more vigorously to Ag in vitro. Bone marrow (BM) radiation chimeras revealed that enhanced susceptibility to EAE was detected in chimeric mice of WT host engrafted with P2X₇-R⁻/⁻ BM cells, indicating that the genotype of the BM cells regulated disease susceptibility. Coculture of P2X₇-R⁻/⁻ macrophages with WT lymphocytes and vice versa showed that enhanced proliferative activity resided within the P2X₇-R⁻/⁻ lymphocyte population and correlated with reduced levels of IFN-γ and NO and apoptosis of lymphocytes. mRNA and protein for IFN-γ were also significantly reduced in the CNS of P2X₇-R⁻/⁻ mice with EAE. FACS analysis of cells isolated from the CNS showed significantly fewer annexin V/propidium iodide-positive lymphocytes in the CNS of P2X₇-R⁻/⁻ mice early in the disease, and TUNEL staining of infamed CNS tissues supported this result. From these data we conclude that enhanced susceptibility of P2X₇-R⁻/⁻ mice to EAE reflects a loss of apoptotic activity in lymphocytes, supporting an important role for this receptor in lymphocyte homeostasis. The Journal of Immunology, 2006, 176: 3115–3126.

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he P2X₇ receptor, P2X₇-R, is a member of the ionotropic family of purinergic receptors that respond to extracellular nucleotides and nucleosides (1–3). It differs from other members of this family, however, in its relatively low affinity for ATP, the presence of a long C-terminal region that contains several protein-protein interaction domains, and the activation of two membrane conductance states upon receptor ligation. Following brief exposure to ATP a nonselective cation channel is activated, whereas following prolonged or repeated applications of high dose ATP multimeric channels or pores may form that allow passage of solutes as large as 900 kDa (1–5).

Although widely expressed in many cell types, the P2X₇-R has generated much interest in cells of the immune system due to its role in the ATP-dependent processing and release of proinflammatory mediators such as IL-1 and TNF (6–9). It has also been implicated in the activation and maturation of T cells (10, 11), the formation of giant cells (12, 13), the killing of invading microorganisms in macrophages (14–16), the activation of the inducible form of NO synthase (17, 18), and the shedding of L-selectin (CD62L) (19, 20), an adhesion molecule that functions in leukocyte binding to the activated endothelium and in lymphocyte homing to high endothelial venules.

The involvement of the P2X₇-R in regulating factors involved in inflammation has suggested that the loss of function of this receptor might lead to the amelioration of diseases of an inflammatory nature. Consistent with this possibility are the observations in mice in which the gene for the P2X₇-R has been inactivated (P2X₇-R⁻/⁻) (21, 22). As expected, peritoneal macrophages isolated from these animals failed to release bioactive IL-1β following LPS activation and stimulation with ATP (21, 22). However, perhaps of even greater interest was the observation that in vivo the inflammatory response was attenuated in mice made arthritic using anti-collagen Abs and challenge with LPS, with both the incidence and severity of disease being significantly reduced in P2X₇-R⁻/⁻ mice (20). Similarly, in two models of chronic pain, one neuropathic and the other inflammatory in origin, inactivation of the P2X₇-R resulted in significant amelioration of hypersensitivity to both mechanical and thermal stimuli, which, in the chronic inflammatory pain model, correlated with reduced protein, but not reduced mRNA, for IL-1 (22).

The P2X₇-R may also play an important role in regulating inflammatory and immune responses through its cytolytic activity. In several cell types, the channel-to-pore transition is associated with loss of membrane potential and permeabilization of the cell membrane, leading ultimately to cell death (23–25). This ATP-dependent cytotoxicity has been best studied in macrophages, where it has been found that cell death can occur via either a necrotic or an apoptotic process. The picture is more complex in lymphocytes. In B cells, permeability assays indicate the formation of a much smaller pore following activation with ATP than that found in macrophages, and B cells show only a low level of susceptibility to ATP-mediated cytotoxicity (26, 27). In T cells, it has been proposed that cell lysis occurs through a mechanism involving NAD-dependent ribosylation by ADP-ribosyltransferase (ART)³ 2.

³ Abbreviations used in this paper: ART, ADP-ribosyltransferase; adh, adherent; BM, bone marrow; CLL, chronic lymphocytic leukemia; DAPI, 4’,6-diamidino-2-phenylindole; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; KO, knockout; MOG, myelin oligodendrocyte glycoprotein; PF, paraformaldehyde; PI, propidium iodide; sus, nonadherent; WT, wild type.
ART2 is a toxin-related ectoenzyme that is expressed by T cells, and the exposure of mature T cells to NAD induces cell death. Recent studies have shown that ART2-catalyzed ADP ribosylation activates P2\textsubscript{X}\textsubscript{7R}, resulting in calcium flux, shedding of CD6\textsubscript{2L}, cell shrinkage, phosphatidylserine exposure, and propidium iodide (PI) uptake (28, 29). These findings demonstrate that the key mediator of NAD-induced cell death is the P2\textsubscript{X}\textsubscript{7R}, with activation occurring through much lower doses of NAD than what is found with ATP. Of particular interest to the regulation of immune-mediated diseases are the very recent studies that have shown that CD\textsuperscript{4/CD25}\superscript{+} regulatory T cells are particularly sensitive to this mechanism of cell death, resulting in increased levels of CD\textsuperscript{4/CD25}\superscript{+} regulatory T cell activity in P2\textsubscript{X}\textsubscript{7R}\textsuperscript{-/-} mice (30).

In this study, we have explored a role for the P2\textsubscript{X}\textsubscript{7R} in the generation of an autoimmune response by investigating the susceptibility of P2\textsubscript{X}\textsubscript{7R}\textsuperscript{-/-} mice to experimental autoimmune encephalomyelitis (EAE). EAE is an inflammatory demyelinating disease of the CNS that functions as the principal animal model for multiple sclerosis (31). It can be induced in susceptible species by active or adoptive sensitization to myelin Ag, and passive transfer experiments initially implicated a CD\textsuperscript{4} T cell population that expressed a Thl-type cytokine profile as effector T cells (31, 32). However, more recently a newly identified Th cell subset that expresses IL-17 (Th17) has been implicated, with both IFN-\gamma and IL-4 acting as regulatory cytokines (33–35). Activated macrophages, as well as macrophage-derived cytokines such as IL-1 and TNF, have also been shown to participate in disease pathogenesis (36, 37). Depending upon the species used and age at the time of sensitization, as well as the specific myelin Ag chosen, EAE may present as an acute monophasic episode of paralysis or may develop into a more chronic syndrome that shows periods of remission and exacerbation (reviewed in Ref. 31). Lesions within the CNS are most prevalent in the white matter tracts of the lumbar region of the spinal cord and are characterized by edema and perivascular infiltrates of inflammatory cells (32). The extent of myelin loss and axonal degeneration usually reflects the severity of the inflammatory response (32). Using the myelin oligodendrocyte glycoprotein (MOG)-induced model of EAE in the C57BL/6 mouse, we show herein that mice deficient in P2\textsubscript{X}\textsubscript{7R} function are more susceptible to EAE than wild type (WT) mice and show enhanced inflammation in the CNS.

Materials and Methods

Animals and reagents

P2\textsubscript{X}\textsubscript{7R}\textsuperscript{-/-} mice back-crossed for 12 generations to C57BL/6 mice were supplied by Dr. C. A. Gabel (Pfizer) (21) and bred at the Albert Einstein College of Medicine (Bronx, NY). WT C57BL/6 mice were obtained from the same source (C57BL6/Nace; Taconic Farms). Successful truncation of the P2\textsubscript{X}\textsubscript{7R} was confirmed by PCR (primer sequences used were as follows: WT, 5'-GCA GCC CAG CCC TGA TAC AGA CAT T-3' and 5'-CCG AGT TGG TGC CAG TGT G-3'; KO, 5'-AGC AGC CCG AGT TGG TGC CAG TGT G-3' and 5'-GGG GGT GGT GGG GGT GGG ATT AGA T-3') (38). All animals were housed and maintained in a federally approved animal facility, and the Animal Care and Use Committee of Albert Einstein College of Medicine approved all protocols.

Induction of EAE

EAE was induced in mice (at 7–9 wk) by s.c. immunization with 300 \(\mu\)g of MOG\textsubscript{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK; Celltek Bioscience) in a 200-\(\mu\)l emulsion of IFA containing 300 \(\mu\)g/ml Mycobacterium tuberculosis H37Ra (Difco) in the lower dorsal twixt, 1 wk apart. The mice received i.p. 500 ng of pertussis toxin (Sigma-Aldrich) on the day of first sensitization and 2 days later. The day after the last injection of MOG was considered day 1. EAE was scored as follows: 0, no symptoms; 1, flophpy tail; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb and hind limb paralysis; and 5, death. In experiments that investigated the role of peripheral mononuclear cells in disease expression, splenocytes (20 \(\times\) 10\(^6\)) from naive WT mice were injected i.p. into P2\textsubscript{X}\textsubscript{7R}\textsuperscript{-/-} mice after lysis of RBCs and extensive washing 6 days before sensitization with MOG.

Bone marrow (BM) chimeras

BM cells were harvested from the long bones of C57BL/6 or P2\textsubscript{X}\textsubscript{7R}\textsuperscript{-/-} mice by flushing with cold PBS. RBCs were removed using RBC lysis buffer (Sigma-Aldrich). Cells were disaggregated by gently cycling through a 26-gauge needle and then through a 70-\(\mu\)m BD Falcon nylon cell strainer (BD Discovery Labware). Cells were washed three times, counted, and transferred i.v. at 6 \(\times\) 10\(^6\) cells recipient. Recipients (3- to 4-wk-old mice) were prepared by one dose of whole body irradiation with 900 rads from a cesium-137 source. BM cells were transferred within 5 h after irradiation. Blood samples were checked for successful engraftment using PCR. Mice were maintained in quarantine for 5–6 wk before EAE sensitization.

Histological analysis

For pathologic analysis of the tissue, EAE mice were anesthetized at indicated times and perfused by intracardiac infusion of 4% parafomaldehyde (PF) in PBS or Trump's solution (4% PF and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4)). The paraffin sections were prepared from 4% PF-fixed tissues and representative sections stained with H&E. The tissues fixed in Trump’s solution were dehydrated and embedded in Epon, and sections were stained with toluidine blue as described previously (39). An inflammatory index was determined by counting the number of lesions per section and multiplying by a factor of 1–5, representing the intensity of the inflammatory infiltrate.

Isolation of CNS infiltrating mononuclear cells

The isolation of CNS mononuclear cells using Percoll gradients was as described previously (39). Mononuclear cells were collected from the 37–70% Percoll interface, washed in medium containing 10% FCS, and counted.

Proliferation assay

Spleen and lymph node cells were isolated by passage through a stainless steel mesh grid in sterilized RPMI 1640. RBCs were lysed with RBC lysis buffer (Sigma-Aldrich), washed twice in 10% FCS/RPMI 1640, and counted. Cells were resuspended in complete RPMI 1640 medium containing 10% FCS, 1% penicillin and streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 20 mM HEPES (all from Invitrogen Life Technologies), and 1% nonessential amino acids (Sigma-Aldrich) at a density of 2 \(\times\) 10\(^5\) cells/ml plated in 96-well plates (200 \(\mu\)l per well), and cultured without or with indicated concentrations of the Ag MOG\textsubscript{35-55}. At day 5, proliferation assays were performed using a cell proliferation ELISA BrdU (colorimetric) kit as per the instruction manual (Roche).

Spleen cell mix and match experiment

The single-cell suspension from spleen was prepared as described in the previous paragraph. Cultured spleen cells were incubated in 96-well tissue culture plates at 37°C for 2 h in a CO\textsubscript{2} incubator to separate adherent cells and cells in suspension. Suspension cells in medium were transferred to another plate, and the adherent cells remaining in the original plate were washed once using warmed fresh medium. After washing, the cells in suspension were transferred to the plates containing adherent cells. The mixture of suspension cells and adherent cells were cultured with or without the indicated concentrations of the Ag MOG\textsubscript{35-55}.

Dendritic cells (DCs) were prepared from adult BM of WT or P2\textsubscript{X}\textsubscript{7R}\textsuperscript{-/-} mice. Briefly, BM cells were harvested from the long bones by insufflation and plated on 24-well plates at a cell density of 2.0 \(\times\) 10\(^5\)/ml in complete DMEM with 10% FCS containing 10 ng/ml recombinant GM-CSF (R&D Systems). Cells were fed every 2 days with complete DMEM containing 10 ng/ml GM-CSF. Cells were harvested after 6 days, and the surface phenotype was confirmed by FACS analysis for expression of cell surface CD11c, MHC II, CD80 (B7.1), and CD86 (B7.2) (BD Pharmingen). CD4 cells were purified from spleens of MOG-sensitized mice using a CD\textsuperscript{4} T cell isolation kit (Milteny Biotec). Cells were plated in 96-well plates at a density of 4 \(\times\) 10\(^4\) CD\textsuperscript{4} T cells and 1 \(\times\) 10\(^5\) DCs in the combinations WT\textsubscript{DC} plus WT\textsubscript{CD4}, WT\textsubscript{DC} plus KO\textsubscript{CD4}, KO\textsubscript{DC} plus WT\textsubscript{CD4}, and KO\textsubscript{DC} plus KO\textsubscript{CD4}. The cells were stimulated with MOG as described above for 3 days.

TUNEL staining

Apoptosis was detected on paraffin sections or cultured cells by an ApoTag peroxidase in situ apoptosis detection kit (Chemicon International)
according to the manufacturer’s instructions. Digital images were collected using an Olympus digital camera and processed using Adobe Photoshop 7.0.1 (Adobe Systems).

**Immunohistochemistry**

Paraffin-embedded sections were deparaffinized and boiled for Ag retrieval in sodium citrate buffer (DakoCytomation) for 20 min. Sections were then incubated with 3% H2O2 for 30 min and blocked in 10% normal goat serum for 1 h at room temperature. Tissues were stained for 2 h at room temperature with the anti-CD3ε Ab (145-2C11; BD Pharmingen) at 1/100 dilution in 5% normal goat serum/PBS. After washing twice in PBS, Cy2-conjugated goat anti-Armenian hamster IgG at 1/200 (Jackson ImmunoResearch Laboratories) was applied for 2 h at room temperature, counterstained for 15 min at room temperature with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) dihydrochloride hydrate/PBS solution, and mounted in aqueous mounting medium (Gel/Mount; Biomeda). Fluorescence microscopy was performed on an Olympus IX70 with ×60 numerical aperture and 1.4 infinity-corrected optics. Images were collected with a Photometrics cooled charge-coupled device camera with a KAF 1400 chip using IPLab Spectrum (Scanalytics).

**Flow cytometry assay**

For annexin V–PI staining, cells were incubated with Annexin V-FITC (BD Pharmingen) and PI (Sigma-Aldrich) in 100 μl of 1 × annexin V binding buffer at room temperature for 15 min. An additional 400 μl of 1 × annexin V binding buffer was then added, and cells were analyzed using CellQuest software in a FACScan cytometer (BD Biosciences).

For ART2.2 and CD3 staining, cells were blocked with purified rat anti-mouse CD16/CD32 (Fcy III/II receptor) mAb in FACS staining buffer (1× PBS containing 1% BSA and 0.05% NaN3) for 20 min on ice. The cells were then incubated with PE-conjugated rat anti-mouse CD3 mAb (IgG2a, Nika102; BD Pharmingen) for 20 min on ice, washed twice with FACS staining buffer, and further incubated with FITC-conjugated mouse anti-rat IgG2a, mAb for 20 min on ice. Cells were washed twice in PBS, fixed in 400 μl of 1% PF, and analyzed using CellQuest software in a FACScan cytometer. Postacquisition analysis was performed using FCS Express software (De Novo Software).

**Cytokine assay**

The protein level of Th1/Th2 cytokines was determined using FAST Quant mouse Th1/Th2 kit (Whatman Schleicher & Schuell) according to the manufacturer’s instructions, and genomic DNA was removed with DNase I. Quantitative PCR was performed as described previously using SYBR Green quantitative PCR master mix (Applied Biosystems) (40). All runs were accompanied by two internal control genes, β-actin and GAPDH. Samples were normalized using a ΔΔ cycle threshold-based algorithm to give arbitrary units representing relative expression levels between samples. The following primers were used: IL-1β (forward) and 5′-TGT CCT CAT CCT GGA AGG TC-3′ (reverse); IL-6, 5′-CGG GAG AGG AGA CTT CAC AG-3′ (forward) and 5′-TCC ACG ATT TCC CAG GCA AC-3′ (reverse); INF-γ, 5′-ACT GGC AAA AGG ATG GTG AC-3′ (forward) and 5′-GCT GAT GGC CTG ATG TTC-3′ (reverse); TNF-α, 5′-ACG GCA TGG ATC TCA AAG AC-3′ (forward) and 5′-GTC GTG GAT GAG GAC CAT GAC GC-3′ (reverse); and GAPDH, 5′-ACC CAT TGG GGA TAG GAA CA-3′ (reverse).

**Statistics**

Statistical analysis was performed using Prism software. Statistical significance of all results was assessed using Student’s t test or a Mann-Whitney U test. A p value of < 0.05 was considered significant.

**Results**

**P2X7R−/− mice show enhanced susceptibility to EAE compared with WT mice**

To define the role of the P2X7R in the regulation of CNS inflammation and demyelination, gender- and age-matched P2X7R−/− and WT C57BL/6 mice were sensitized to develop EAE by active immunization with the MOG35−55 peptide and examined on a daily basis for the clinical expression of disease. Contrary to our expectations given its known role in regulating inflammation, the P2X7R−/− mice developed a more severe form of EAE characterized by higher clinical scores during both the acute and chronic stages of the disease when compared with matched WT mice (Fig. 1). The differences between P2X7R−/− mice and WT mice were statistically significant for both males and females but were particularly evident in females due to the less severe disease in WT mice. However, no difference in the clinical index was noticed between the male and female P2X7R−/− mice. In addition, no differences were noted between WT and P2X7R−/− mice regarding the day of onset, time of peak expression of disease, or incidence (Table I).

**P2X7R−/− mice show more severe pathology in the CNS**

In both WT and P2X7R−/− mice, white matter lesions typical of acute EAE were observed in the spinal cord (Fig. 2, A–D). These lesions consisted of perivascular inflammatory infiltrates of mononuclear and polymononuclear cells and evidence of demyelination and axonal degeneration (Fig. 2, C and D). To determine whether the cellular profile of these inflammatory infiltrates differed between WT and P2X7R−/− mice, we performed a FACs analysis of cells isolated from the spinal cord. Spleen and lymph node cells were examined in parallel. No differences were noted for any of these cell types in spleen and lymph node preparations. Similarly, in the CNS cellular infiltrates no differences were noted in the percentage representation for CD4+, CD8+, B220+, and Gr1+ cells between the two groups of mice. However, statistically significant increases in the percentage of CD45hiCD11bhi macrophages were detected in infiltrates from the spinal cord of P2X7R−/− mice (Table II).

Analysis of lesion distribution showed that in the P2X7R−/− mice the lesions were more extensive than those present in WT mice (see areas marked in red in Fig. 2, A and B). The most striking difference between WT and P2X7R−/− mice was the more extensive involvement of the brain and cerebellum in P2X7R−/− mice, areas of the CNS not typically involved to any great extent in EAE (Fig. 2, F–K). As shown in Fig. 2G (arrows) and at higher power in Fig. 2I, the brain and cerebellum showed numerous small perivascular lesions predominantly associated with white matter tracts. These lesions were much less frequent in the WT mice (Fig. 2F), and similar small vessels were not usually inflamed in WT brains (Fig. 2H). An increase in lesion frequency was also detected in the cerebellum (Fig. 2, compare K with J). Quantification of lesions in these tissues showed a significant increase in their number in P2X7R−/− mice (Fig. 2L).

**Spleen cell transfer partially reverses disease exacerbation in P2X7R−/− mice**

The P2X7R is known to be widely expressed in both the peripheral immune system as well as in cells endogenous to the CNS (5, 42). As a first step in exploring whether loss of P2X7R function that resulted in disease exacerbation was due to its role in cells of the...
immune system or cells in the CNS, we transferred spleen cells between WT and P2X<sup>-/-</sup> mice and sensitized these mice for EAE. As shown in Fig. 3A, P2X<sup>-/-</sup> mice receiving WT spleen cells showed an acute disease course that closely resembled that found in WT mice who had been transferred with WT spleen cells. However, during the chronic phase of the disease these mice showed clinical scores that fell between those noted in the WT and P2X<sup>-/-</sup> mice. These data suggest that, during the acute phase of the disease, increased susceptibility to EAE likely reflects a role for this receptor in the response of immune cells to sensitization.

**Radiation of BM chimeras confirmed a role for P2X<sub>7</sub>R<sup>-/-</sup> leukocytes in disease exacerbation**

To establish further the critical role of the P2X<sub>7</sub>R in the response of immune cells to Ag, we established BM chimeras. In these mice, enhanced susceptibility to EAE was noted in WT host animals receiving P2X<sup>-/-</sup> BM cells when compared with WT host animals receiving WT BM cells (Fig. 3B). Similarly, EAE was more severe in P2X<sup>-/-</sup> animals receiving P2X<sup>-/-</sup> BM cells compared with P2X<sup>-/-</sup> host animals receiving WT BM cells (Fig. 3C). These data confirmed the important role of BM-derived cells in defining the enhanced susceptibility of P2X<sup>-/-</sup> mice to EAE.

**Analysis of spleen cell response to MOG peptide**

To further investigate a role for the P2X<sub>7</sub>R in spleen cell responses to the MOG peptide, a proliferation assay was performed using cells isolated from animals that had been sensitized 7 days previously. The data showed that both spleen and lymph node cells isolated from P2X<sup>-/-</sup> mice proliferated more vigorously to the MOG peptide than did cells isolated from WT mice (Fig. 4A). The P2X<sub>7</sub>R is known to regulate proinflammatory cytokine levels and cell death in both lymphocytes and macrophages (6–9, 14, 16). To test for possible differences in cytokine responses between WT and P2X<sup>-/-</sup> mice, the extent of apoptosis present in these cultures was determined using TUNEL staining. TUNEL-positive cells were numerous in WT and P2X<sup>-/-</sup> cultures incubated without Ag. However, in cultures stimulated with MOG, fewer lymphocytes were TUNEL-positive in spleen cells derived from P2X<sup>-/-</sup> mice compared with those from WT mice (Fig. 4B). To determine whether this effect was due to the absence of a cytotoxic factor such as TNF from macrophages or to the loss of a cytotoxic response in lymphocytes, we transferred nonadherent (sus) spleen cells from WT mice (WT-sus) onto adherent (adh) cells from WT (WT-adh) and P2X<sup>-/-</sup> (KO-adh) mice and vice-versa (KO-sus and WT-adh); these cells were then cultured for 5 days with MOG peptide. Control cultures consisting of WT plus WT or P2X<sup>-/-</sup> plus P2X<sup>-/-</sup> were prepared in parallel. As shown in Fig. 4D, the difference in proliferation between WT and P2X<sup>-/-</sup> mice was found to reside predominantly within the nonadherent cell population, with only a minor effect being contributed by the adherent cells. To provide a more quantitative analysis of this effect, the experiment was then repeated and data were calculated as the fold increase over controls. Cumulative data for three additional experiments using 20 μg/ml MOG peptide gave values of ×5.5 for WT-adh plus KO-sus, ×1.7 for KO-adh plus WT-sus, ×2.8 for WT-adh plus WT-sus, and ×7.1 for KO-adh plus KO-sus (WT-adh plus KO-sus vs KO-adh plus WT-sus; p < 0.01, n = 7).

To address the possible mechanism for the difference in proliferation and apoptotic activity between WT and P2X<sup>-/-</sup> mice, we assayed culture supernatants for cytokines and NO production from the mixed cell transfer experiment shown in Fig. 4D. No significant differences in the levels of IL-2 or IL-10 were detected in these culture supernatants (Fig. 4F). However, for IL-6, IFN-γ, IL-13, and NO, levels were significantly lower for cultures containing P2X<sup>-/-</sup> lymphocytes as compared with the supernatants from cultures containing WT lymphocytes. Furthermore, these data correlated with the enhanced proliferation of P2X<sup>-/-</sup> cells to Ag (Fig. 4D). IFN-γ production was also found to be dramatically decreased in supernatants from the P2X<sup>-/-</sup> spleen cells cultured with MOG (20 μg/ml) shown in Fig. 4A (see Fig. 4C).
We then investigated whether differences in cytokine levels between WT and P2X$_7$R$^{-/-}$mice could be detected in vivo during the acute phase of EAE. To test for these differences, we performed real-time PCR and protein array assays on spinal cord homogenates harvested on day 9. As shown in Table III, significantly reduced levels of mRNA and protein for IL-6 and IFN-γ were noted in P2X$_7$R$^{-/-}$mice compared with WT mice, whereas no differences in protein for IL-2 and TNF-α were detected. Thus, the in vivo data support the result of the in vitro culture studies showing reduced IL-6 and IFN-γ in P2X$_7$R$^{-/-}$mice with EAE. The data also show that, in agreement with the known role for the P2X7R in the processing and release of IL-1 (7), protein but not

Table II. Cellular composition of leukocytes in WT and P2X7R$^{-/-}$mice

<table>
<thead>
<tr>
<th></th>
<th>CD4$^+$</th>
<th>CD8$^+$</th>
<th>B220$^+$</th>
<th>CD45$^+$ CD11b$^+$</th>
<th>Gr-1$^+$</th>
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<td>Spinal cord</td>
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<tr>
<td>WT</td>
<td>34.3 ± 2.6</td>
<td>9.7 ± 0.7</td>
<td>9.1 ± 1.8</td>
<td>20.8 ± 1.3</td>
<td>22.1 ± 5.5</td>
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<tr>
<td>P2X7R$^{-/-}$</td>
<td>28.3 ± 1.7</td>
<td>9.3 ± 1.9</td>
<td>11.3 ± 2.2</td>
<td>26.5 ± 0.3*</td>
<td>29.2 ± 2.4</td>
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<td>Lymph node cells</td>
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<td>WT</td>
<td>31.5 ± 3.5</td>
<td>22.2 ± 1.6</td>
<td>48.8 ± 1.8</td>
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<tr>
<td>P2X7R$^{-/-}$</td>
<td>27.7 ± 0.4</td>
<td>18.5 ± 0.9</td>
<td>51.1 ± 0.9</td>
<td>8.8 ± 0.5</td>
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<td>Spleen cells</td>
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<tr>
<td>WT</td>
<td>24.2 ± 0.4</td>
<td>13.8 ± 0.2</td>
<td>48.6 ± 3.2</td>
<td>13.7 ± 4.8</td>
<td>14.7 ± 3.8</td>
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<tr>
<td>P2X7R$^{-/-}$</td>
<td>22.0 ± 0.7</td>
<td>11.2 ± 1.1</td>
<td>56.7 ± 2.4</td>
<td>9.5 ± 0.4</td>
<td>9.6 ± 0.7</td>
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*All values are presented as the mean ± SD percentage of each cell type (n = 3 per group).

*, p < 0.05 (between WT and P2X7R$^{-/-}$ animals).
similarly, analysis of IFN-γ levels (Fig. 4E) and NO production (data not shown) from these cultures of purified CD4 cells showed no difference between WT and P2X7R−/− mice. These data show that reduced IFN-γ production is not an intrinsic property of Ag-reactive T cells in the P2X7R−/− mice.

The incidence of apoptosis in the CNS of sensitized animals is higher in WT than in P2X7R−/− mice

The experiments described above suggested that the cytolytic activity of the P2X7R in lymphocytes plays a role in regulating EAE. To determine whether a difference in cells undergoing cell death could be detected in vivo between WT and P2X7R−/− mice, cells were isolated from the CNS on day 7, stained for annexin V and PI, and analyzed by FACS. The lymphocyte gate was determined by staining for CD3. The results (Fig. 5A) showed that more cells displayed evidence of cell death in samples derived from the CNS of WT animals than from P2X7R−/− animals. Cumulative data from four additional experiments for both the total mononuclear cell population isolated from the CNS (day 7), as well as for cells within the lymphocyte gate, are shown in Fig. 5C.

We then performed TUNEL staining on sections derived from animals sensitized 11 days earlier. In agreement with previously published data (43), in both the brain and spinal cord the majority of apoptotic cells in these tissues were localized to perivascular infiltrates. In these tissues, more TUNEL-positive cells were detected in inflammatory lesions in WT animals than in P2X7R−/− animals (Fig. 5E). Quantification of these data is shown in Fig. 5F. Furthermore, CD3 and DAPI immunohistochemistry staining in WT EAE brain tissues confirmed that apoptotic cells within the perivascular cuffs included CD3+ T cells (Fig. 5G).

It has been proposed that recovery from the acute clinical episode in EAE is associated with apoptotic events in activated lymphocytes in the CNS (44). Therefore, to determine whether any differences were detected between WT and P2X7R−/− mice during this phase of EAE, we used the same assays described above on samples harvested on day 14 postsensitization. The results showed no difference in the percentage of cells undergoing cell death as determined by FACS analysis (Fig. 5B) or TUNEL staining (Fig. 5G) at this time point.

FACS analysis of lymphocyte expression of Art2.2 in EAE

It has been shown that in lymphocytes the P2X7R can mediate apoptosis induced by extracellular NAD through an interaction with ART 2.2 (28). This enzyme is known to be expressed only in T cells and to be shed upon activation (45). To explore the possibility that ART2.2 expression might correlate with apoptotic activity in lymphocytes in these mice, we assessed ART2.2 expression by FACS in lymphocytes isolated from the CNS, spleen, and lymph nodes 7 and 14 days postsensitization with MOG. The results showed no difference in the percentage of lymphocytes that expressed ART 2.2 between WT and P2X7R−/− mice (Fig. 6). However, in cells isolated from the CNS, the percentage of CD3+ T cells that expressed ART 2.2 was lower than in spleen and lymph node populations. This difference was particularly striking in males for both day 7 and day 14. This would suggest that in males more of the lymphocytes in the CNS are in an activated state than in females.

Discussion

In this study we have shown that mice lacking a functional P2X7R are more susceptible to MOG-induced EAE than their WT counterparts. Clinical expression of disease was more severe during...
both the acute and the chronic phases of the disease, and pathological examination of the tissues showed that the typical inflammatory and destructive lesions in the CNS were more widespread in P2X<sup>7</sup>R<sup>−/−</sup> mice than in WT mice. Of particular note was the more extensive involvement of the brain. EAE is known to initially affect the more distal regions of the cord and to involve more proximal regions with time and increased severity. Therefore, more numerous perivascular lesions in the brain reflect a more severe inflammatory process in the P2X<sup>7</sup>R<sup>−/−</sup> mice. Radiation BM chimeras showed that increased susceptibility to EAE resided within the BM-derived cells, indicating that radiation-resistant cells within the CNS did not confer susceptibility to EAE in the P2X<sup>7</sup>R<sup>−/−</sup> mice, at least for the acute phase of the disease. In vitro, proliferation assays and TUNEL staining showed that spleen and lymph node cells from P2X<sup>7</sup>R<sup>−/−</sup> mice had a higher proliferative activity in response to MOG peptide and that lymphocytes in these cultures showed less evidence of apoptosis. Mixed transfer experiments of spleen adherent and nonadherent cells indicated that adherent cells were not a major source of factors regulating lymphocyte proliferation and/or cell death. Annexin V/PI staining of CNS-infiltrating cells also revealed that there were fewer apoptotic cells in the CNS of P2X<sup>7</sup>R<sup>−/−</sup> mice than in that of WT mice, and TUNEL staining of sections of brain and

**FIGURE 4.** Immune response to MOG in spleen and lymph node cells of WT and P2X<sub>7</sub>R<sup>−/−</sup> mice. A. Spleen and lymph node cells were harvested on day 7 from WT and P2X<sub>7</sub>R<sup>−/−</sup> mice and stimulated with MOG<sub>35-55</sub> peptide at the indicated concentrations. Proliferation was determined using BrdU (n = 7 mice per group; *, p < 0.05). B, TUNEL staining was performed to determine the cytotoxic response in spleen cells cultured as in A. The majority of the apoptotic cells in these cultures were lymphocytes. C, IFN-γ levels, as determined by ELISA, in supernatants harvested from spleen (sp) cells cultured with or without MOG (20 μg/ml) from experiment in A. D, Spleen cells were prepared from WT and P2X,R<sup>−/−</sup> mice sensitized with MOG<sub>35-55</sub> Peptide. Nonadherent spleen cells were transferred from WT mice onto adherent cells from P2X<sub>7</sub>R<sup>−/−</sup> mice and vice versa. Control cultures consisting of WT, WT and P2X<sub>7</sub>R<sup>−/−</sup>, and P2X<sub>7</sub>R<sup>−/−</sup> were prepared in parallel. Cells were cultured with 25 μg/ml MOG<sub>35-55</sub> peptide for 5 days, and a proliferation assay was performed using BrdU. One of four experiments is shown. E, IFN-γ production of culture supernatants from the mix and match cultures using purified DC (dc) and CD4 (cd4) cells was measured using an ELISA kit. F, Cytokine and NO production of culture supernatant harvested from the experiment shown in C was determined using FAST Quant mouse Th1/Th2 kit and the Griess reaction.
spinal cord gave the same result. These data are the first to show that the P2X7R regulates T cell-mediated inflammation of the CNS and support an important role for this receptor in lymphocyte homeostasis.

The contribution of the P2X7R to inflammation and the immune response was first recognized following the identification of its role in the processing and release of IL-1 (3, 5). Consistent with this function for the P2X7R, we noted that whereas mRNA for IL-1 did not differ in spinal cord homogenates from P2X7R−/− mice and WT mice, protein assays showed a significant reduction in IL-1 levels in the P2X7R−/− mouse. In many cell types, activation with IL-1 leads to the induction of IL-6; thus, the reduced levels of both mRNA and protein detected in spinal cord homogenates for IL-6 in the P2X7R−/− mice would also be consistent with a reduction in the levels of IL-1 in these mice. Both IL-1 and IL-6 are well recognized as proinflammatory cytokines, are elevated in the early stages of EAE, and are considered risk factors for EAE (46–48); therefore, the reduced levels of both IL-1 and IL-6 in the P2X7R−/− mouse are at variance with their known role in this disease. However, these cytokines also have immunosuppressive functions, and their expression in the CNS has been shown to assist in recovery and repair following toxic or traumatic insults to the brain (49–51). Neuronal/axonal injury has only recently been recognized as a component of clinical expression of EAE, and both IL-1 and IL-6 may contribute to the recovery from the acute clinical episode by offering protection against destruction of these tissues. Thus, in common with TNF and IFN-γ, IL-1 and IL-6 likely play complex roles in the clinical and pathologic expression of EAE (47, 48, 52).

Our data strongly implicate loss of apoptotic activity in lymphocytes as a contributing factor to disease exacerbation in the P2X7R−/− mouse. In the EAE model, several studies have proposed that Ag-induced cell death in the CNS contributes to the recovery from the acute phase of the disease (44), and both Fas/FasL interactions (53–55) and p53 (56) have been implicated in this process. The observation that the frequency of apoptotic events in the CNS only differed between P2X7R−/− and WT mice during the early phases of the disease suggests that P2X7R-dependent apoptosis may be one of several death-inducing pathways that become activated in the inflamed CNS, forming part of a complex immunoregulatory process that evolves over the course of the disease. The mechanism by which the P2X7R regulates apoptosis in lymphocytes requires further investigation, but decreased apoptotic activity of P2X7R−/− lymphocytes correlated with reduced levels of IFN-γ and NO in supernatants from Ag-activated cultures. Reduced levels of IFN-γ were also noted in spinal cord homogenates from P2X7R−/− mice with EAE when compared with WT controls. These data are consistent with several previous studies that have clearly linked a role for IFN-γ and NO as regulatory factors in EAE (57–59). In this scenario, high levels of NO are generated by the inducible form of NO synthase, which is potently activated in macrophages, as well as in other cell types, by IFN-γ (59). NO then acts to induce T cell apoptosis and to block T cell proliferation, thus functioning to limit the expansion of autoreactive T cells.

However, we did not detect any difference in the MOG-induced proliferative activity of WT and P2X7R−/− lymphocytes when purified DC and CD4 cells were used to perform the mix and match culture experiments. Similarly, no difference in the production of IFN-γ and NO was detected (Fig. 4E, and data not shown). These data indicate that P2X7R−/−CD4+ T cells from MOG sensitized animal are fully capable of making IFN-γ in response to MOG when separated from other spleen cells. Thus, the decreased production of IFN-γ found in P2X7R−/− spleen cells in the in vitro cultures (Figs. 4, C and F), as well as in the spinal cord homogenates (Table III), suggest the presence of a regulatory cell(s) or factor that inhibits the production of IFN-γ. In this regard, it is of interest to note that P2X7R−/− mice have recently been reported to have enhanced FoxP3 and CD4+CD25+ regulatory T cell activity (30), and our unpublished data for FoxP3 expression in P2X7R−/− EAE mice is consistent with this finding, suggesting no role for this type of T regulatory cell in this process. However, whether some other possible regulatory cell is involved in this processing, e.g., CD8+ T cells, B cells, γδ T cells, or NK cells, remains to be determined.

Decreased apoptosis of lymphocytes in P2X7R−/− mice also likely reflects a direct role for the P2X7R in mediating cell death. In most cell types, ligand binding to the P2X7R results in two conductance states: following brief exposure to ligands such as ATP a nonselective cation channel is activated, but following prolonged or repeated exposure to high dose ATP a large macrochannels pore is formed. These changes result in transmembrane ion flux (particularly influx of Ca2+, Na+, and eflux of K+), cell swelling, vacuolation, and death by both necrotic and apoptotic means. There are multiple potential sources of high levels of ATP in the inflamed CNS; however, it could be questioned whether ATP is the specific ligand involved in these experiments. In mice, a single base pair substitution, T1352C (P451L), has been detected in the P2X7R genome that dramatically affects receptor function. This mutation lies within a region of the C-terminal cytoplasmic tail that bears significant homology with a TNFR1 death domain, as well as to a fragment of the Src homology 3-binding protein (60, 61). Different strains of mice carry different alleles of the P2X7R, with the L451 allele conferring a reduced sensitivity to ATP-induced channel activity and pore formation (60, 61). C57BL/6 mice express this allele, and T cells from these mice show reduced apoptotic activity to ATP when compared with T cells from BALB/c

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Table III. Cytokine mRNA and protein expression in spinal cord homogenates from EAE mice

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<thead>
<tr>
<th></th>
<th>IL-1β</th>
<th>IL-6</th>
<th>TNFα</th>
<th>IL-2</th>
<th>IFN-γ</th>
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<td></td>
<td></td>
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<tr>
<td>WT</td>
<td>179 ± 14</td>
<td>25 ± 2</td>
<td>58 ± 11</td>
<td>NDa</td>
<td>26 ± 3</td>
<td>NDa</td>
</tr>
<tr>
<td>P2X7R−/−</td>
<td>152 ± 15</td>
<td>16 ± 2*</td>
<td>62 ± 26</td>
<td>NDa</td>
<td>9 ± 0.8***</td>
<td>NDa</td>
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<tr>
<td>Protein</td>
<td></td>
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<tr>
<td>WT</td>
<td>808 ± 96</td>
<td>851 ± 111</td>
<td>265 ± 20</td>
<td>267 ± 28</td>
<td>305 ± 33</td>
<td>96 ± 10</td>
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<tr>
<td>P2X7R−/−</td>
<td>348 ± 54***</td>
<td>416 ± 55**</td>
<td>232 ± 29</td>
<td>217 ± 33</td>
<td>211 ± 33</td>
<td>89 ± 22</td>
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a Data are expressed as mean ± SD of the relative expression levels for four animals per group harvested on day 9.
b Quantitative PCR.
c Not done.
*p, p < 0.05 (between WT and P2X7R−/− animals).
**+, p < 0.01 (between WT and P2X7R−/− animals).
***+, p < 0.001 (between WT and P2X7R−/− animals).
mice. More recently, an alternative P2X7R-dependent cell death pathway in T cells has been proposed that is induced by NAD acting as a substrate for the cell surface ART2 (28). Binding of covalently attached ADP-ribosyl groups to the P2X7R results in pore formation and rapid cell death following exposure to much lower concentrations of NAD than of ATP (29). These data prompted us to study ART2 expression in CD3ε T cells isolated from the CNS, spleen, and lymph node at varying times in the disease process (Fig. 6). No differences were observed between WT and P2X7R−/− mice in any of these samples. However, strikingly lower percentages of CD3ε ART2+ cells were isolated from the inflamed CNS than from spleen and lymph nodes, especially in males. Because ART2 is rapidly shed from the T cell surface following activation, these data indicate that a much higher percentage of T cells in the CNS are activated compared with lymphocytes in peripheral lymphoid organs and further suggest that the

FIGURE 5. The incidence of apoptosis in the CNS of sensitized mice is higher in WT mice than P2X7R−/− mice. A and B. Infiltrating mononuclear cells were isolated from the CNS of P2X7R−/− and WT mice on day 7 (A) and day 14 (B) post-sensitization with MOG, and cells were stained for annexin V and PI. The lymphocyte gate was determined by staining for CD3. C and D. Cumulative FACS data for CNS infiltrating mononuclear cells stained for annexin V and PI on day 7 (C) and day 14 (D). Each symbol represents data from one mouse. Data were significantly different between P2X7R−/− mice and WT for day 7 but not for day 14 (p < 0.04). E, TUNEL staining was performed on brain or spinal cord sections derived from animals sensitized 11 days previously. F, The cumulative data for the percentage of TUNEL-positive inflammatory cells for tissue harvested on days 11 and 14. Asterisk denotes values significantly different from the P2X7R−/− mice: **, p < 0.001, *, p < 0.05, n = 6 per group. H, Immunofluorescence staining of CD3ε and DAPI is shown in an inflammatory lesion from brain sections of a WT EAE mouse. Red arrows indicate the apoptotic cells with condensed chromatin that are double-stained for CD3ε.
more severe disease noted in males may reflect a higher and more prolonged state of activation of CD3+ cells in the CNS than that found in females.

Interestingly, although the clinical course of EAE was more severe in male WT mice than in female WT mice, we found little difference in disease activity between P2X,R−/− males and P2X,R−/− females. These data suggest that there may be an interaction between sex steroids and signaling via the P2X7R. Consistent with this hypothesis, it has been shown in vitro that 17β-estradiol and estrogen regulate receptor activation when assessed by electrophysiological methods (62, 63), and studies in vivo that have addressed a role for this receptor in bone formation and resorption found a greater reduction in bone formation in male P2X7R−/− mice vs WT mice (64). These results suggest that certain sex related molecules may regulate the function of the P2X7R and that, in the absence of this receptor, this effect disappears.

It should be noted here that the more severe disease seen in males in these experiments is at variance with previously published data for C57BL6 mice where no difference in susceptibility to EAE between males and females was noted (65, 66) but is consistent with sex differences in susceptibility to EAE in other strains of mice (66). Although we have no explanation for this result at the present time, we did observe that the C57BL/6N Tac mice, when sensitized side-by-side with C57BL/6J mice, were less sensitive to MOG-EAE induction. Thus, our data likely reflect a genetic drift that has occurred in these substrains over the extended length of time that these mice have been inbred, as has been noted previously for BL6 mice (67).

Like the mouse, several polymorphisms have also been detected in the human P2X7R that affect receptor function (68, 69). The best studied of these is the A1513C (E496A) variant, which also resides within a region of the C-terminal tail that shows homology with the TNFR1 death domain (68). Individuals with the 1513C genotype show a loss-of-function for the P2X7R with respect to both pore formation and cytokine production (68–71). Several studies have also suggested an association between this polymorphism and patients with B cell chronic lymphocytic leukemia (CLL) (72–76). At the present time the exact contribution that this receptor plays to disease expression in CLL remains to be resolved; but, as has been cogently discussed by Di Virgilio and Wiley (76), the data could equally well be interpreted to support either a growth promoting activity for the receptor, a loss of apoptotic activity, and/or a loss of regulation of adhesion molecules important in facilitating B cell exit from the peripheral circulation. Nevertheless, taken together these data support a role for this receptor in clinical manifestations of disease in patients with CLL.

Understanding the pathogenesis of inflammation is a central issue in furthering our understanding of a wide range of human diseases. Activation of the inflammatory response is recognized to be an essential component of an effective defense against microbial infections but also raises the danger of inducing irreversible tissue damage, as well as possibly activating autoreactive bystander T cells (28). The P2X7-R is emerging as a central regulator of the inflammatory process, but little is known about how this receptor functions in different models of inflammation in vivo. The data presented here, along with the data previously reported by Labasi et al. (20) and Chessell et al. (22), show that the P2X7-R differentially regulates inflammation depending upon the underlying mechanisms involved, i.e. it attenuates disease elicited by a combination of Ab and the TLR4 ligand LPS (20) and chronic inflammatory and neuropathic pain (22), but it exacerbates disease mediated by CD4+ T cells. It is of particular interest to note that these four models were tested in the same C57BL/6 mouse strain that expresses a receptor with low affinity for ATP; therefore, it will be of further interest to test different models of inflammation in P2X7-R−/− mice back-crossed onto other strains of mice. Because certain polymorphisms in the human P2X7-R have also been found to have functional consequences and to associate with disease, this mouse model provides a valuable tool for furthering our understanding of the complex role of the P2X7-R in inflammation.

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Disclosures
The authors have no financial conflict of interest.

References


