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The Therapeutic Effects of PJ34, A Selective Inhibitor of Poly(ADP-ribose) Polymerase, in Experimental Allergic Encephalomyelitis are Associated with Immunomodulation

Gwen S. Scott[1], Rhonda B. Kean, Tatiana Mikheeva, Marzena J. Fabis, Jon G. Mabley[2], Csaba Szabó[2] and D. Craig Hooper

Department of Microbiology and Immunology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107.

Running Title: Immunomodulation in EAE by PARP inhibition

Corresponding author: Dr. D. Craig Hooper, Department of Microbiology and Immunology, Thomas Jefferson University, JAH 454, 1020 Locust Street, Philadelphia, PA19107. Tel: 215 503 1774. FAX: 215 923 7745. E-Mail: c_hooper@mail.jci.tju.edu

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Abbreviations: APC, antigen presenting cells; BBB, blood-brain barrier; CFA, complete Freund's adjuvant; CNS, central nervous system; EAE, experimental allergic encephalomyelitis; iNOS, inducible nitric oxide synthase; MBP, myelin basic protein; PARP, poly(ADP-ribose) polymerase; PBS, phosphate buffered saline.

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ABSTRACT

Poly(ADP-ribose) polymerase (PARP) activity has been implicated in the pathogenesis of several CNS disorders. For example, the presence of extensive poly(ADP)ribosylation in CNS tissues from animals with experimental allergic encephalomyelitis (EAE) indicates that PARP activity may be involved in this inflammatory disease process. Using PJ34, a selective PARP inhibitor, we studied the mechanisms through which PARP activity may contribute to the onset of acute EAE. PLSJL mice immunized with myelin antigens were treated with PJ34 and the effects on the progression of EAE and several other parameters relevant to the disease process were assessed. PJ34 exerted therapeutic effects at the onset of EAE that were associated with reduced CNS inflammation and the maintenance of neurovascular integrity. Expression of genes encoding the adhesion molecule ICAM-1, and the inflammatory mediators IFN-γ, TNF-α and iNOS were decreased in CNS tissues from drug treated animals. Administration of PJ34 biased the class of myelin basic protein (MBP)-specific antibodies elicited from IgG2a to IgG1 and IgG2b and modulated antigen-specific T-cell reactivity. Therefore, the mode of action of PJ34 at the onset of EAE is likely mediated by a shift in the MBP-specific immune response from a pro-inflammatory Th1 towards an anti-inflammatory Th2 phenotype.

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Experimental allergic encephalomyelitis (EAE) is an autoimmune disease of the CNS that is widely employed as an animal model of multiple sclerosis. While the etiology of EAE is reasonably well characterized, with the disease generally believed to occur as a consequence of $CD4^+$ T-cell autoreactivity, the cause of multiple sclerosis remains largely undefined. However, several recent reports have proposed that peroxynitrite, a reactive molecule that is rapidly formed when nitric oxide and superoxide combine, is involved in the pathogenesis of both disorders (Hooper et al., 1997; Van der Veen et al., 1997; Cross et al., 1998; Hooper et al., 1998b; Hooper et al., 2000; Scott et al., 2001b).

Particular emphasis has been placed on the ability of peroxynitrite to contribute to the development of multiple sclerosis and EAE by inducing central nervous system (CNS) tissue damage. For instance, peroxynitrite is known to exert toxic effects on a variety of CNS cells, including astrocytes and oligodendrocytes (Endres et al., 1998, Scott et al., 2003) and has been shown to damage CNS myelin *in vivo* (Touil et al., 2001). Peroxynitrite is able to cause cell death through a variety of different mechanisms including tyrosine nitration, lipid peroxidation, and inhibition of mitochondrial respiration (Szabó, 2003). Peroxynitrite may also trigger cell death by inducing DNA strand breakage, resulting in the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) (Szabó, 2003). When activated, PARP catalyzes the attachment of ADP-ribose subunits from its substrate, NAD, onto various nuclear proteins, and excessive activation of the enzyme has been associated with cell death and dysfunction (reviewed in Virag and Szabó, 2002).

PARP activity has been implicated in neurotoxicity and in the pathogenesis of cerebral ischemia and other neurodegenerative disorders (Love et al., 1999; Mandir et al., 1999; Ha and Snyder, 2000; Skaper 2003). Poly(ADP-ribose) residues are present in CNS tissues from animals

with EAE (Scott et al., 2001a). Moreover, administration of PARP inhibitors suppresses disease development in several different EAE models, suggesting that PARP may also contribute to the disease process (Scott et al., 1998, 2001; Chiarugi 2002). However, several widely used pharmacological inhibitors of PARP have been found to also scavenge free radicals (Szabó et al., 1998), which raises the possibility that their therapeutic effects in EAE may not entirely be due to inhibition of PARP. More selective PARP inhibitors that do not have antioxidant properties, including PJ34, a water-soluble phenanthridinone derivative, have recently been developed (Soriano et al., 2001). In an attempt to more precisely determine the relationship between PARP activation and the onset of EAE, we have assessed the effects of PJ34 (N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N, N-dimethylacetamide.Hcl) on the clinical progression of EAE, as well as several other parameters relevant to the disease process. These include blood-brain barrier (BBB) permeability, CNS inflammation, and general aspects of the antigen-specific immune response.

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Materials and Methods

Induction of EAE in PLSJL mice and administration of PARP inhibitor. Female PLSJL mice (8-10 wks old) (The Jackson Laboratory, Bar Harbor, ME) were immunized for EAE as previously detailed (Hooper et al., 1998b). Each animal received 100 μg of myelin basic protein (MBP) in complete Freunds adjuvant (CFA) supplemented with 4 mg/ml *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, MI) s.c. on day 0 followed by 400 ng pertussis toxin (List Biological Laboratories, Campbell, CA) i.p. on days 0 and 2. At least 5 mice were used per treatment group. PJ34 was dissolved in saline and administered orally by gavage to PLSJL mice, at a dose of 10 mg/kg body weight, twice daily, at 9AM and 5PM from day 7 post-immunization. Control EAE-sensitized animals received the saline vehicle alone. The dose of PJ34 was selected from previous *in vivo* studies demonstrating the efficacy and potency of the drug to inhibit PARP activity without any resultant toxicity (Mabley et al., 2001). Animals were monitored twice daily for neurological disease signs by two independent investigators. Signs of EAE were scored on a 7 point severity scale as follows: 0 = normal, 1 = piloerection, tail weakness, 2 = tail paralysis, 3 = tail paralysis plus hind limb weakness, 4 = tail paralysis plus partial hind limb paralysis, 5 = complete hind limb paralysis, 6 = hind and forelimb paralysis, 7 = moribund/dead. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory animals as adopted by the U.S. National Institutes of Health, and were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. Tissues were collected a minimum of 1hr post treatment.

Histology. Sections were prepared from the spinal cords of MBP-immunized, saline and PJ34 treated, both healthy and mice with clinical signs of EAE, 19-20 days post immunization, as well as untreated normal mice. Briefly, mice were anesthetized by i.p. administration of sodium

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pentobarbital (20 mg/kg body weight), transcardially perfused with PBS/heparin (1000 U/l) and PBS, the spinal cord dissected out, and snap-frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA). Sections (15 µm) were stained with Mayer's hematoxylin (0.1%) and eosin Y (1%) and photographed using a Leitz Microlab microscope and Nikon Coolpix 995 digital camera at a magnification of 24x.

Quantitative RT-PCR. Spinal tissues were collected from animals at day 19-20 post-immunization. Mice were anesthetized and transcardially perfused as detailed above and the spinal cord was removed. RNA was isolated from mouse spinal cords using TRIzol B (Gibco Life Technologies, Grand Island, NY) and DNA-free agent (Ambion, Austin, TX) was used to remove DNA contamination. cDNA was synthesized from 5 µg total RNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and dT_{15} primer. An equivalent of 50 ng total RNA was used for the PCR with Taqman PCR core reagent kit (Applied Biosystems, Foster City, CA). Primers and probes were designed using the Web Primer 3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_WWW.CGI). Dual–labelled probes were purchased from Integrated DNA Technologies (Coralville, IA) while oligonucleotide probes were obtained from CyberSyn (Camden, NJ). Primers were synthesized by our in-house Nucleic Acid Facility (Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA). The 5' ends of probes were labeled with reported dyes Hex or 6-Fam, and 3' ends with either BHQ-1 or TAMRA. Probes and primers were as follows: CD4, probe, TGA GGG CAT CGC TGA AGT GCG CCA; 5' primer, GCT CAC CGT CAT CTG CTC TGA; 3' primer, AGG GCC TCC CAC TGT GAT CT. CD8, probe, TGT GTG CGG AGG AGA GCC CGA ATT CA; 5' primer, CAT CCT GCT TCT GCT GGC ATT; 3' primer, TGG GCG CTG ATC ATT TGT GAA A. CD68, probe, CCA GCC CCT CTG AGC ATC TGC CCC A; 5' primer, GTG CTC ATC

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GCC TTC TGC ATC A; 3' primer, GGC GCT CCT TGG TGG CTT AC, CD11b (Mac-1 alpha chain), probe AGA CCC TGT CCG CTC ACG TAT CCG TGC C; 5' primer, GAC CGT CTG CGC GAA GGA GAT A; 3' primer, CGC CTG CGT GTG TTG TTC TTT G. ICAM-1, probe, GCT GCC CAT CGG GGT GGT GAA; 5' primer, CTG CAG ACG GAA GGC AGA TGG T; 3' primer, GAG CTA AAG GCA TGG CAC TGG CAC ACG TA. IFN-γ, probe, ACC TTC TTC AGC AAC AGC AAG GCG; 5' primer, AGC AAC AAC ATA AGC GTC ATT; 3' primer, CCT CAA ACT TGG CAA TAC TCA. TNF-α, probe, CAC ACC GTC AGC CGA TTT GCT ATC; 5' primer, AGG TTC TCT TCA AGG GAC AAG; 3' primer, GCA GAG AGG AGG TTG ACT TTC. iNOS, probe, TGG CCA CCA AGC TGA ACT TGA GCG A; 5' primer, TGG CTA CCA CAT TGA AGA AGC TG; 3' primer, TCT GGC TCT TGA GCT GGA AGA AA. β-Actin probe CCA TCA TGA AGT GTG ACG TTG ACA TCC; 5'primer, CCT TCC TTC TTG GGT ATG GAA; 3' primer, ACA GCA CTG TGT TGG CAT AGA. GAPDH, probe, AGG CCG AGA ATG GGA AGC TTG TCA TC; 5' primer, GGC AAA TTC AAC GGC ACA G; 3' primer, AGA TGG TGA TGG GCT TCC C. Quantitative PCR was performed using a Bio-Rad iCycler iQ Real Time Detection System (Hercules, CA). Data were calculated based on a threshold cycle (Ct) determined as the cycle with a signal higher than that of the background (signal detected in cycles 2-10) plus 10 x it's SD. The values for quantitative PCR are normalized to the expression levels of the housekeeping gene GAPDH in each sample by the calculation: 2 exponent (Ct lowest expresser- Ct test value) divided by the same value determined for GAPDH (Hooper et al., 2001). Results are expressed as a fold-increase in mRNA expression by comparison with the levels detected in tissues from non-immunized controls.

Assessment of BBB Permeability. BBB permeability was assessed using sodium fluorescein as a tracer molecule (Hooper et al., 2000). Each animal received 100 μl of 10 % sodium fluorescein

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(Sigma Chemical Co., St. Louis, MO) in phsophate buffered saline (PBS) i.p. which was then allowed to circulate for 10 min. Mice were anesthetized by i.p. administration of sodium pentobarbital (20 mg/kg body weight). Cardiac blood was collected and animals were transcardially perfused with PBS/heparin (1000 U/l) and PBS. Sodium fluorescein uptake into the spinal cord was determined as detailed previously (5). In brief, spinal cord tissue was homogenized in 7.5 % trichloroacetic acid and centrifuged for 10 min at 10,000 g to remove insoluble precipitates. Following the addition of 5 N NaOH the fluorescence of the sample was determined at excitation 485 nm and emission 530 nm using a Cytofluor II fluorimeter (PerSeptive Biosystems, Cambridge, MA). Serum levels of sodium fluorescein were assessed as described (Hooper et al., 2000). The sodium fluorescein content of the samples was determined by comparison to a series of sodium fluorescein standards (0.125 to 4 μg/ml). Sodium fluorescein uptake into spinal cord tissue is expressed as (μg fluorescence spinal cord/mg protein)/(μg fluorescence sera/μl blood) to normalize values for blood levels of the dye.

***In vitro* assay of MBP-Specific T-cell Reactivity.** Antigen specific T-cell proliferative responses were assessed in lymph node cells, isolated from mice 22 days after immunization with MBP. The spleens and inguinal and axillary lymph nodes were removed from 3 to 5 mice of each treatment group. To test for effects of PJ34 on T cell proliferation *in vitro*, lymph node cells (2.5 x 10^6/ml) were cultured with irradiated (1000 rad) spleen cells (2.5 x 10^6/ml) as antigen presenting cells (APC) in 200 μl volumes in round-bottomed 96-well plates. To assess the effects of *in vivo* administration of PJ34 on T cells and APC *ex vivo*, similar cultures were performed but with viable APC. Culture medium consisted of MEM, alpha modification, (Gibco) supplemented with 4 mM L-glutamine, 25 mM HEPES, 50 μM 2-mercaptoethanol, 10 μg gentamicin and 0.6 %

fresh mouse serum. Cultures were incubated at 37 °C in the presence and absence of MBP (final concentration 10μg). After 72 hours, cultures were pulsed with 1 μCi of methyl-[^{3}H]-thymidine, specific activity 65 Ci/mmol (DuPont-NEN, Boston, MA) for 4 hours. Cells were then harvested on glass fiber filters using a Mach III harvester 96 (Tomtec, Orange, CT). The [^{3}H]-thymidine incorporated into new DNA was estimated by liquid scintillation counting using a 1450 Microbeta Trilux counter (Wallac Oy, Turku, Finland). Results are expressed as cpm of triplicate cultures.

Analysis of MBP-specific antibodies in ELISA. Serum samples were collected from mice on day 22 post-immunization and antibody specificity and isotype were assessed in solid phase ELISA. Plates were coated overnight in a humidified chamber with 2μg/ml MBP diluted in PBS. The plates were washed with PBS containing 0.05 % Tween 20 and blocked for 1 hr with 1 % bovine serum albumin in PBS before the addition of serum samples. Samples were diluted 1:100 in PBS and titrated 2-fold down the plate. Following a 2 hr incubation at room temperature, plates were washed with PBS containing 0.05 % Tween 20 to remove any unbound primary antibody. Bound antibody was detected by the addition of either alkaline phosphatase-conjugated rat anti-mouse IgG1 (1:2000) (Pharmingen, San Diego, CA), Cappel™ alkaline phosphatase-conjugated rabbit anti-mouse IgG2a (1:2000) (ICN Biomedicals Inc., Costa Mesa, CA), Cappel™ alkaline phosphatase-conjugated rabbit anti-mouse IgG2b (1:2000) (ICN Biomedicals) using *p*-nitrophenyl phosphate (Sigma Chemical Co) in 0.1 M glycine buffer as a substrate. Absorbance was read at 405 nm in a microplate spectrophotometer (Bio-Tek, Winooski, VT). Data is expressed as A_{405}.

Statistical Analysis. Results are expressed either as the mean ± SEM for n observations, or the mean ± SD. Differences in the disease incidence and mortality were analyzed using the Chi-square Test. Evaluation of significant differences in disease onset, EAE severity, mRNA levels, BBB permeability and T-cell proliferation were performed using the Kruskal-Wallis test with post-hoc Mann-Whitney U-test. In all tests $p<0.05$ was considered significant.

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Results

PJ34 Suppresses the Development of Clinical Signs of EAE in PLSJL Mice

To focus our analyses of the effects of PJ34 treatment on the later stages of the clinical progression of EAE, as opposed to the initiation of the MBP-specific immune response, PJ34 was orally administered to MBP-immunized PLSJL mice beginning on day 7 post-immunization. In this study, saline-treated animals displayed signs of EAE from day 11 post-immunization onwards, with 95% eventually developing clinical signs of the disease (Fig. 1). The disease was lethal in 65% of the saline-treated mice by day 20 post-immunization (Fig. 1). While some PJ34-treated mice demonstrated disease signs beginning at the same time as saline-treated controls, day 11 post-immunization, the mean day of onset in the majority of PJ34-treated animals that developed EAE was delayed by several days (mean day of disease onset: 14 ± 0.6 saline group, versus 17 ± 1.0 PJ34 group, $p<0.001$, Mann Whitney U-test). Moreover, the incidence of EAE was significantly reduced in animals treated with PJ34, as only 13 out of 30 of these mice exhibited disease signs, compared to 19 out of 20 animals in the saline group ($p<0.001$) (Fig. 1a). Correspondingly, administration of PJ34 markedly decreased the mean severity of EAE in PLSJL mice ($p<0.001$) (Fig. 1b) as well as the overall disease mortality ($p<0.01$) (Fig. 1c). A significant therapeutic effect was also noted when only mice showing clinical signs of disease from the control and PJ34-treated groups were compared (data not shown). When treatment was discontinued at day 21, 4/5 of the PJ34-treated mice that had not previously developed EAE developed mild disease by day 28 (average score 3; no lethality), and 2/5 of the treated mice that had shown mild disease during the treatment phase suffered relapses.

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CNS Inflammation is Reduced in the Spinal Cord Tissues of MBP-immunized Mice Treated with PJ34

The pathogenesis of EAE is thought to be associated with the migration of autoreactive T-cells into CNS tissues (Hartung and Rieckmann, 1997). Several reports have demonstrated that PARP-1 is involved in promoting cell recruitment in other disease models (reviewed in Virag and Szabó, 2002). Thus, it is conceivable that PJ34 exerts therapeutic effects in EAE by preventing inflammatory cells from accumulating in the CNS. Since the inflammation seen in the EAE model studied here is primarily associated with discrete lesions in the spinal cord (Hooper et al., 2000). As can be seen in Figure 2, there appears to be a reduction in the extent of lesion formation in the spinal cords of MBP-immunized, PJ34-treated mice regardless of whether or not they develop clinical signs of EAE. No lesion activity was detected in spinal cords from PJ34-treated mice that remained healthy (Fig. 2). It is difficult to obtain a quantitative and qualitative assessment of the CNS inflammatory response by immunohistochemistry. Therefore to further test the possibility that PJ34 treatment has an impact on the CNS inflammatory response in EAE, we used real-time quantitative RT-PCR to assess the levels of mRNAs specific for CD4, CD8, as well as CD68 and CD11b (Mac-1) in spinal cord extracts as markers of infiltrating $CD4^+$ T-cells, $CD8^+$ T-cells, and microglia/macrophages respectively (Fig. 3). Compared to those of non-immune mice, CD4 mRNA levels were markedly increased in the spinal cord tissues of MBP-immunized, saline treated mice. CD68 and CD11b as well as, to a lesser extent, CD8 mRNAs were also elevated by immunization. Administration of PJ34 reduced the levels of CD4 and CD68 mRNAs detected in the spinal cord tissues of immunized mice. However, spinal cord levels of CD8 and CD11b mRNAs were not altered by drug treatment.

It may be expected that the reduction of inflammatory cell accumulation in spinal cord tissues by PJ34 treatment would also limit the expression in the CNS of genes associated with

inflammation, such as IFN-γ and inducible nitric oxide synthase (iNOS). The results presented in Figure 4 demonstrate that IFN-γ mRNA levels become elevated in the spinal cord tissues of PJ34-treated animals but to a lesser extent than in control mice with EAE (Fig. 4). A similar pattern was obtained for the more moderate elevations in iNOS mRNA levels detected in spinal cord tissues from the different groups. Only low levels of IL4 message that did not significantly differ between the groups were detectable in the spinal cords of control mice with EAE and their PJ34 treated counterparts (data not shown).

PJ34 Treatment Limits BBB Breakdown in MBP-Immunized Mice

Functional changes in the BBB, including reduced integrity, have been associated with several neurological conditions, including multiple sclerosis and EAE (De Vries et al., 1997). Enhanced BBB permeability not only enables toxic substances that are normally excluded to enter the CNS from the circulation, but also facilitates inflammatory cell infiltration into CNS tissues. For example, maintenance of BBB integrity in MBP-immunized PLSJL mice prevents CNS inflammation as well as the development of clinical signs of EAE (Hooper et al., 2000; Kean et al., 2000). Both mice with a targeted deletion of the PARP-1 gene and rats given the PARP inhibitor 3-aminobenzamide have reduced levels of BBB disruption resulting from pneumococcal meningitis compared to controls (Koedel et al., 2002). Thus it is possible that PJ34 administration prevents CNS inflammation in EAE through an effect on the BBB. We therefore assessed BBB permeability in MBP-immunized mice treated with PJ34 or saline by measuring the uptake of sodium fluorescein into their spinal cord tissues (Fig. 5). At 20 days post-immunization, when clinical signs of EAE had developed in MBP-immunized mice given saline, sodium fluorescein levels in the spinal cord tissues of these animals were significantly increased compared to tissues from non-immunized animals ($p<0.001$). This evidence of a

marked loss of neurovascular integrity was not seen in MBP-immunized mice treated with PJ34. The level of uptake of sodium fluorescein into the spinal cord tissue of MBP-immunized, PJ-34 treated mice did not significantly differ from that detected in similar tissue from non-immunized mice (Fig. 5).

PJ34 Treatment Partially Inhibits the Expression of TNF-α and ICAM-1 in the Spinal Cord Tissues of MBP-Immunized Mice

Another aspect of the functional changes in the BBB seen in a CNS inflammatory response is the upregulation of adhesion molecules on neurovascular endothelial cells. The enhanced expression of adhesion molecules, such as ICAM-1, at the BBB is known to make an important contribution to cell trafficking into the CNS in EAE. Previous studies have demonstrated that PARP effects the expression of various adhesion molecules, including ICAM-1 (Zingarelli et al., 1998; Sharp et al., 2001). PARP has also been implicated in the regulation of several pro-inflammatory molecules, including TNF-α, a known inducer of ICAM-1 (Dobbie et al., 1999). Conceivably, the reduced inflammatory cell infiltration observed in CNS tissues from PJ34 treated mice could be a consequence of the modulation of adhesion molecules, such as ICAM-1, at the BBB. We have therefore assessed the effects of PJ34 administration on the expression of TNF-α and ICAM-1 specific mRNA in spinal cord tissues following MBP-immunization (Fig. 6). Levels of both TNF-α and ICAM-1 mRNAs, were strongly elevated in the spinal cords of saline-treated, MBP-immunized mice by comparison with non-immune controls (Fig. 6). TNF-α and ICAM-1 mRNAs levels were also elevated in spinal cords from PJ34 treated mice, but to a significantly lesser extent (Fig. 6).

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PJ34 Effects T-Cell Reactivity *in vivo* but not *in vitro*

Previous studies have suggested that PARP inhibition may be immunomodulatory. For example, PARP inhibitors have been shown to interfere with lymphocyte proliferation in response to mitogenic stimulation (Broomhead and Hudson, 1985; Weltin et al., 1995). Therefore the reduction in CNS inflammatory processes in MBP-immunized mice treated with PJ34 could conceivably result from an effect on the T-cell response to MBP. To investigate this possibility, we first assessed the effects of PJ34 on the proliferation of lymphocytes in response to MBP *in vitro*. T-cells were isolated from the lymph nodes of MBP-immunized mice and cultured with APC and MBP in the presence of increasing concentrations of PJ34 (Fig. 7). PJ34, at concentrations up to 1μM, had no effect on the MBP-specific T-cell proliferative response. Thus PJ34 is unlikely to have a direct inhibitory effect on either APC function or the capacity of T-cells to proliferate in response to antigen.

To determine whether an action of PJ34 may influence the development of antigen-specific immunity *in vivo*, T-cells and APC obtained from MBP-immunized mice treated with PJ34 or saline were compared for their capacity to respond to MBP *in vitro* (Fig. 8). The secondary *in vitro* MBP-specific T-cell proliferative response of lymphocytes isolated from PJ34 treated mice was significantly lower than those of similar cells obtained from animals receiving saline (Fig. 8). To gain further insight into the nature of this difference, we compared the MBP-specific proliferative responses of mixtures of T-cells and APC from saline and PJ34 treated MBP-immunized mice (Fig. 8). A significant reduction in the MBP-specific proliferative response was only evident in cultures containing T-cells from PJ34 treated mice. No defect in the capacity to present MBP was apparent in APC obtained from animals that had been receiving PJ34.

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Administration of PJ34 Biases the T-cell Response towards a Th2 Phenotype

The results from our *ex vivo* examination of T-cell reactivity together with the analyses of cell invasion and factor production in CNS tissues imply that PJ34 administration may modulate some aspect of T-cell function *in vivo*. The reduction in CNS inflammation observed in PJ34-treated mice suggests that one possibility may be a selective reduction in the Th1 helper cell reactivity that primarily mediates the cellular, inflammatory class of immune response which is generally associated with the pathogenesis of EAE. If this is the case, a consequence of PJ34 administration may be a bias towards Th2 activity, believed to protect against the development of EAE (Bettelli and Nicholson 2000). Since Th1 responses predominantly elicit IgG2a antibodies while Th2 responses produce higher levels of IgG1 in mice (Hooper et al., 1998a), we assessed whether PJ34 treatment influences the pattern of isotypes of MBP-specific antibodies following immunization with MBP. The results, shown in Figure 9, indicate that PJ34 treatment biases the MBP-specific antibody response towards IgG1 and IgG2b and against IgG2a indicating that the ratio of Th1 to Th2 activity has likely been reduced.

Discussion

Evidence that PARP activity contributes to the development of a number of CNS disorders has been accumulating over the past few years. For instance, a number of studies have examined the role of PARP in the evolution of cerebral ischemia (reviewed in Virag and Szabó, 2002) and, more recently, PARP activity has been implicated in the pathogenesis of bacterial meningitis and traumatic brain injury (Koedel et al., 2002; LaPlaca et al., 2001). We have demonstrated the presence of poly(ADP-ribose) residues in CNS tissues from animals with EAE,

indicating that PARP activity may be involved in this disease process (Scott et al., 2001a). Consistent with this observation, we and others, have established that PARP inhibitors are therapeutic in different EAE models (Scott et al., 1998, 2001; Chiarugi, 2002).

In the past, PARP was thought to contribute to disease pathogenesis primarily by inducing cell death (Szabó, 2002a). However, more recently it has been recognized that PARP activity may also modulate the transcription and translation of genes involved in inflammation (Szabó, 2002b; Kraus and Lis, 2003). In the case of EAE, inflammatory processes trigger changes in BBB function that are central to the infiltration of cells into CNS tissues and, ultimately, pathogenesis of the disease (Hooper et al., 2000; Kean et al., 2000). Consequently, PARP likely mediates pathogenic effects in EAE through multiple mechanisms. In the present study we assessed the effects of PJ34, a selective PARP inhibitor, on various parameters of the disease process *in vitro* and *in vivo* to more specifically probe the mechanisms through which PARP activity contributes to the onset of acute EAE. Despite the fact that an MBP-specific immune response was allowed to progress for 7 days before treatment commenced, PJ34 exerted therapeutic effects in a PLSJL mouse model of EAE over the time period studied. Overall, not only were both the incidence and severity of disease significantly curtailed, but also where EAE developed in treated animals, it was delayed in onset. Nevertheless, a small proportion of the PJ34 treated animals developed severe disease signs with approximately 20% mortality occurring by day 20 post-immunization.

Based on evidence that PARP activity promotes inflammation (Szabó, 2002b; Kraus and Lis, 2003; Chiarugi and Moskowitz, 2003) it is not surprising that PARP inhibitors reduce CNS inflammation in EAE (Scott et al., 1998, 2001; Chiarugi, 2002). In agreement with these findings, PJ34 treatment reduced lesion formation and the accumulation of mRNAs specific for the T-cell marker CD4 in the spinal cords of MBP-immunized mice. The apparent decrease in

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T-cell invasion was paralleled by reduced levels of mRNAs specific for several inflammatory mediators implicated in the disease pathogenesis, including TNF-α, IFN-γ and iNOS. However, PJ34-treated mice segregated into two groups, one without clinical signs of EAE and a second with moderate signs of disease. When these two groups were compared for expression of the various mRNAs, healthy versus sick PJ34-treated animals differed significantly in the levels of mRNAs specific for TNF-α, IFN-γ, ICAM-1, and CD68, while both groups were lower in CD4 mRNA levels than control-treated animals (data not shown). When PJ34-treated mice developed EAE, the levels of mRNAs for TNF-α, IFN-γ, ICAM-1, and CD68 were not significantly different from those of saline treated mice (data not shown).

BBB breakdown is a characteristic feature of EAE and is coupled with an influx of immune cells, fluid and protein into the CNS. In the PLSJL mouse model studied here, cell invasion into CNS tissues appears to be promoted by the loss of BBB integrity as a consequence of the production of peroxynitrite (Hooper et al., 2000; Kean et al., 2000). Changes in BBB function are therefore an integral part of the CNS inflammatory response and may, at least in part, be mediated through the pro-inflammatory actions of PARP. In other disease models PARP activity has been proposed to alter leukocyte migration through modifying the expression of adhesion molecules (Zingarelli et al., 1998; Sharp et al. 2001). Consequently, inhibiting PARP activity in EAE may modulate inflammatory cell migration by altering adhesion molecule expression. Indeed, our results indicate that administration of PJ34 to MBP-immunized mice reduces the CNS expression levels of the adhesion molecule ICAM-1. Based on studies in different models of pneumococcal meningitis, it has also been proposed that PARP may directly modulate neurovascular integrity, possibly by inducing endothelial cell cytotoxicity (Koedel et al., 2002). This is unlikely to be the case in EAE because enhanced BBB permeability is accompanied by elevated ICAM-1 expression, likely by neurovascular endothelial cells, and cell

invasion into the CNS tissues is highly selective rather than non-specific. We therefore consider that the effects of PJ34 on BBB function in EAE are more likely to be an indirect consequence of the modulation of the inflammatory response by PARP inhibition.

If the predominant effects of PARP inhibition with PJ34 on the genesis of BBB functional changes and CNS inflammation in EAE are manifested by a change in the nature of the inflammatory response it may be expected that the immune response to MBP is altered. It is noteworthy in this regard that extensive poly(ADP-ribose) formation occurs in the lymph nodes following myelin-immunization in a rat model of EAE (Chiarugi, 2002). In addition to the effects of PARP on inflammatory processes, there is evidence that PARP activity may modulate immune cell function (Broomhead and Hudson, 1985; King et al., 1989; McNerney et al., 1989; Weltin et al. 1995; Chiarugi, 2002). However there is some controversy as to whether or not PARP inhibitors directly interfere with T-cell reactivity which may be explained by differences in the experimental approaches and PARP inhibitors utilized (Broomhead and Hudson, 1985; McNerney et al., 1989; Weltin et al. 1995; Chiarugi, 2002). For example, several widely used pharmacological inhibitors of PARP have also been found to scavenge free radicals in addition to preventing enzyme activity (Szabó et al., 1998). We failed to detect any effect on antigen specific proliferative responses of T-cells from MBP-immunized mice when PJ34 was added to the culture medium. However, the *in vitro* proliferative response of cells from MBP-immunized mice treated with PJ34 was significantly reduced by comparison with those from MBP-immunized control animals. The results of cell mixing experiments suggest that, in this case, the major effect of PJ34 treatment *in vivo* may be manifested at the level of T-cell function. In view of the fact that T-cells play a pivotal role in the pathogenesis of EAE, it is quite possible that the therapeutic effects of PJ34, which include maintaining neurovascular integrity and reducing parameters of CNS inflammation, may, in fact, be due to modulation of T-cell activity.

Given that PJ34 treatment was not begun until 7 days following immunization with MBP, we considered it more likely that the MBP-specific immune response may have been modified as opposed to suppressed. A bias from a largely Th1 inflammatory response to a less inflammatory Th2 response would be consistent with a reduction in PARP-mediated pro-inflammatory processes as well as the effects of PJ34 treatment on the clinical course of EAE. Th2 cells produce cytokines with anti-inflammatory properties and their activity has been linked to disease remission in EAE (Bettelli and Nicholson, 2000). Furthermore, altering the immune response in EAE towards a Th2 phenotype has been shown to change disease progression (Bettelli and Nicholson, 2000). Since the Th bias of an immune response is reflected by the antibody isotypes that are elicited, we assessed MBP-specific antibody titres to establish whether the effects of PJ34 treatment in EAE may be mediated through modulation of T-cell activity. The switch from predominantly IgG2a to IgG1 and IgG2b antibody isotype production confirms that a bias in the MBP-specific T-cell response from Th1 to Th2 occurs in PJ34-treated mice. We consider it likely that PARP activity promotes the Th1 response, through its known effects on the expression of pro-inflammatory genes (Chiarugi, 2002; Chiarugi and Moskowitz, 2003). We therefore propose that PARP inhibition is primarily therapeutic in EAE by selectively interfering with the Th1 inflammatory response that is pathogenic in this autoimmune disorder. PARP inhibition in EAE is therefore manifested in a reduction in most aspects of CNS inflammation including the loss of BBB integrity. However, PARP activation has also been implicated in neurotoxicity (Virag and Szabó, 2002; Skaper, 2003). Consequently, inhibiting PARP may also be beneficial in EAE by maintaining neuronal cell viability thus preventing functional deficits as well as reducing the release of new auto-stimulatory antigens. It is noteworthy in this regard that when PJ34-treatment is terminated, MBP-immunized mice often develop clinical signs of EAE.

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Further experiments are required to determine if a therapeutic effect can be maintained by continued PJ34 treatment.

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Footnotes

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[1] Current address: Department of Biochemical Pharmacology, Barts and The London, Queen Mary's School of Medicine and Dentistry, Charterhouse Square, London, UK, EC1M 6BQ.

[2] Inotek Pharmaceuticals Corporation, 100 Cummings Center, Beverly, MA 01915.

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Figure Legends

Fig. 1. PJ34 suppresses the development of clinical signs of EAE in MBP-immunized PLSJL mice. PLSJL mice (n≥20 per group) were immunized subcutaneously with MBP and orally dosed, twice daily, with PJ34 (10mg/kg body weight) (open squares) or saline (closed squares) from day 7 post-immunization as detailed in Materials and Methods. Mice were monitored for signs of EAE and the results for all mice, both healthy and sick, are presented as A. % incidence of disease, B. mean disease score ± SEM, and C. % mortality; *$p<0.05$, compared to saline group by the Mann-Whitney U-test.

Fig. 2. Administration of PJ34 limits CNS inflammation in the spinal cords of MBP-immunized mice. MBP-immunized PLSJL mice were orally dosed, twice daily, with PJ34 (10mg/kg body weight) or saline from day 7 post-immunization onwards. At days 19-20 post-immunization, treated animals (saline with EAE, PJ34 with and without clinical signs of EAE) and non-immunized control mice were perfused and their spinal cords removed for histological analysis as detailed in Materials and Methods. Sections from the spinal cords of a minimum of 3 mice per treatment group were stained with hematoxylin-eosin and representative sections from the lumbar region are shown: A/ untreated normal; B/ MBP-immunized, saline-treated with EAE; C/ MBP-immunized, PJ34-treated, healthy; D/ MBP-immunized, PJ34-treated, with EAE. In B, arrows indicate representative lesions that were common throughout all three spinal cords studied. In D, the arrow indicates an area of mild perivascular inflammation. Several similar areas were detected in each of the spinal cords from PJ34-treated mice that developed clinical signs of EAE.

Fig. 3. Administration of PJ34 limits the appearance of mRNAs specific for inflammatory cells in the spinal cords of MBP-immunized mice. MBP-immunized mice were treated as described in the legend to Fig. 2. At days 19-20 post-immunization treated animals and non-immunized

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control mice were perfused and the expression levels of mRNA specific for CD4, CD8, CD11b and CD68 in spinal cord tissues assessed by quantitative RT-PCR. Data is expressed as mean ± SEM fold increase in specific mRNA level compared to non-immunized mice, where n= 5 to 6 mice per group. Statistical significance was determined by the Mann Whitney U-test; *$p<0.05$, **$p<0.01$ compared to the non-immunized group; $^{\dagger\dagger}p<0.01$ compared to the EAE-saline group.

Fig. 4. Administration of PJ34 to MBP-immunized mice inhibits the expression of inflammatory mediators in the spinal cord. MBP-immunized PLSJL mice were orally dosed, twice daily, with PJ34 (10mg/kg body weight) or saline from day 7 post-immunization and at day 19-20 post-immunization spinal cord IFN-γ, and iNOS mRNA levels were determined by quantitative RT-PCR. Data is expressed as mean ± SEM fold increase in specific mRNA level compared to non-immunized mice, where n= 5 to 6 mice per group. Statistical significance of the differences was assessed by the Mann Whitney U-test; **$p<0.01$, compared to the non-immunized group.

Fig. 5. PJ34 treatment prevents BBB permeability changes in EAE. MBP-immunized PLSJL mice were orally dosed, twice daily, with PJ34 (10mg/kg body weight) or saline from day 7 post-immunization and BBB permeability was assessed at day 20 post-immunization by measuring the uptake of sodium fluorescein into spinal cord tissues. Sodium fluorescein uptake is defined as (μg fluorescence spinal cord/mg protein)/(μg fluorescence sera/μl blood) and data is expressed as the mean sodium flourescein uptake ± SEM, where n=8 to 12 mice per group. Statistical significance was determined by the Mann Whitney U-test; ***$p<0.001$ compared to the non-immunized group; $^{\dagger\dagger\dagger}p<0.001$ compared to the EAE-saline group.

Fig. 6. Expression levels of TNF-α and ICAM-1 mRNA are decreased in spinal cord tissues of MBP-immunized mice treated with PJ34. MBP-immunized PLSJL mice were orally dosed, twice daily, with PJ34 (10mg/kg body weight) or saline from day 7 post-immunization. At day 19-20

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post-immunization spinal cord ICAM-1 and TNF-α mRNA levels were determined by quantitative RT-PCR. Data is expressed as mean ± SEM fold increase in specific mRNA level compared to non-immunized mice, where n= 5 to 6 mice per group. Statistical significance of the differences was assessed by the Mann Whitney U-test; ***$p<0.001$ compared to the non-immunized group; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ compared to the EAE-saline group.

Fig. 7. PJ34 does not effect MBP-specific T-cell proliferation *in vitro*. Lymph node cells, isolated from healthy PLSJL mice immunized 15 days previously with MBP in CFA, were cultured with an equal number of irradiated unselected spleen cells as APC's in the presence (hatched bars) or absence (clear bars) of MBP (10 μg/ml) and increasing concentrations of PJ34 (1 to 1000 nM). T-cell proliferation was determined by a 4 hr pulse with [^{3}H]thymidine. Data is expressed as mean cpm ± SD of triplicate samples.

Fig. 8. PJ34 treatment of mice inhibits the *ex vivo* MBP-specific proliferation of their T-cells. Groups of 5 PLSJL mice were immunized with MBP in CFA. Animals were orally dosed, twice daily, with PJ34 (10mg/kg body weight) or saline from day 7 post-immunization and at day 22 post-immunization their spleens, inguinal and axillary lymph nodes were removed. Lymph node cells (T-cells) were cultured with an equal number of unselected spleen cells (APC) in the presence (grey bars) or absence (clear bars) of MBP (10 μg/ml). T-cell proliferation was determined by a 4hr pulse with [^{3}H]thymidine. Data is expressed as mean cpm ± SD of triplicate samples. Statistical differences were assessed by the Mann-Whitney U-test; ***$p<0.001$ by comparison with MBP-stimulated cultures containing T cells and APC from the saline-treated group.

Fig. 9. PJ34 treatment alters the isotype of the MBP-specific antibody response. MBP-immunized PLSJL mice were orally dosed, twice daily, with PJ34 (10mg/kg body weight) (open

circles) or saline (closed circles) from day 7 post-immunization and serum samples obtained at day 22 post-immunization. Serum was also obtained from normal, non-immune mice (open squares). The serum antibody response to MBP was assessed in solid phase ELISA as described in Materials and Methods using alkaline phosphatase-conjugated secondary antibodies specific for IgG isotypes IgG1, IgG2a, and IgG2b and *p*-nitrophenyl phosphate as a substrate. Absorbance was read at 405 nm in a microplate spectrophotometer. Data is presented as mean ± SD of serum samples from 6 mice per group.

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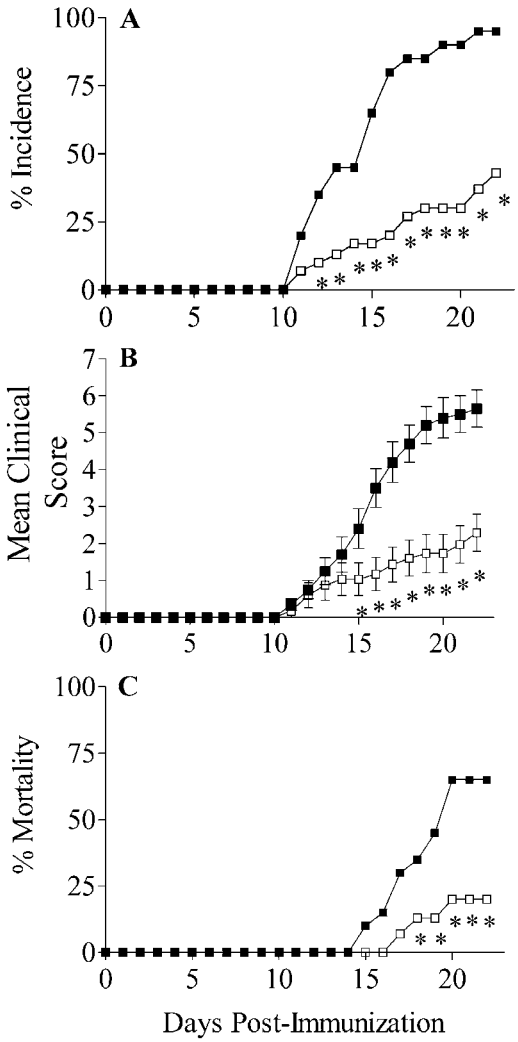
JPET #63214 Fig. 1



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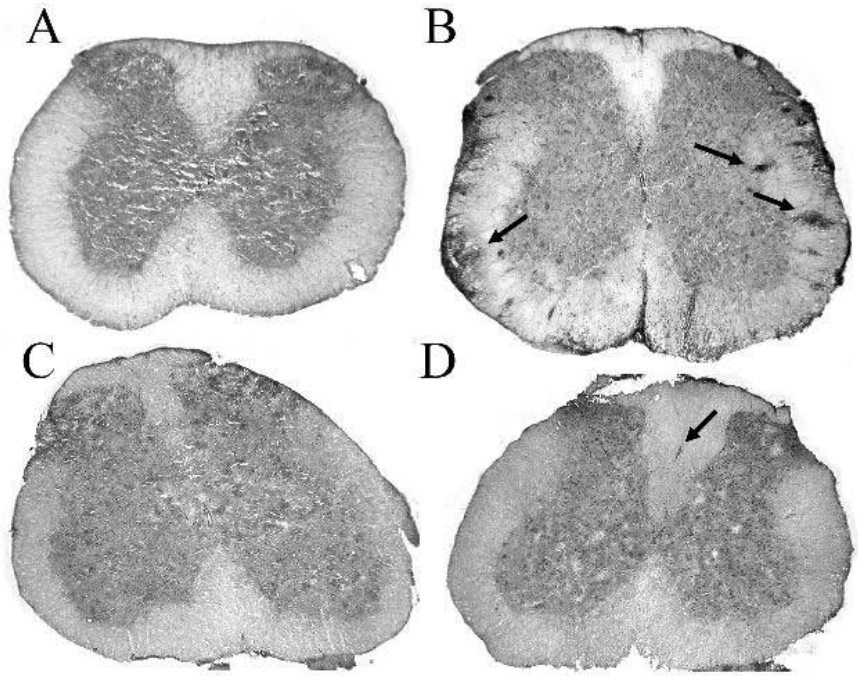
JPET #63214 Fig. 2



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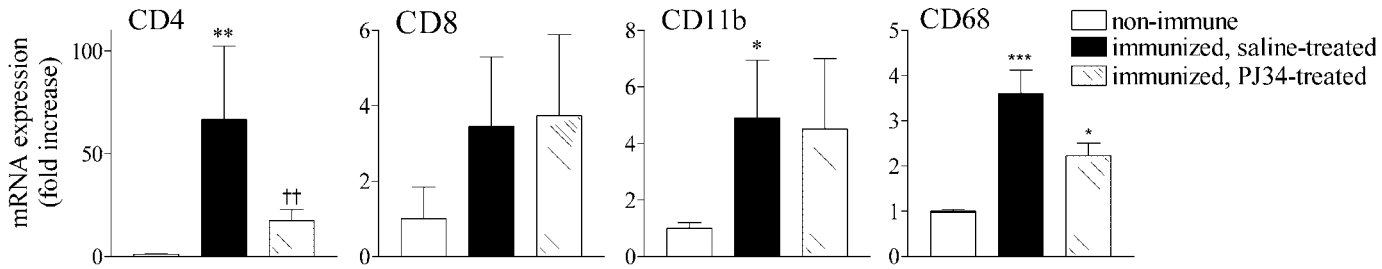

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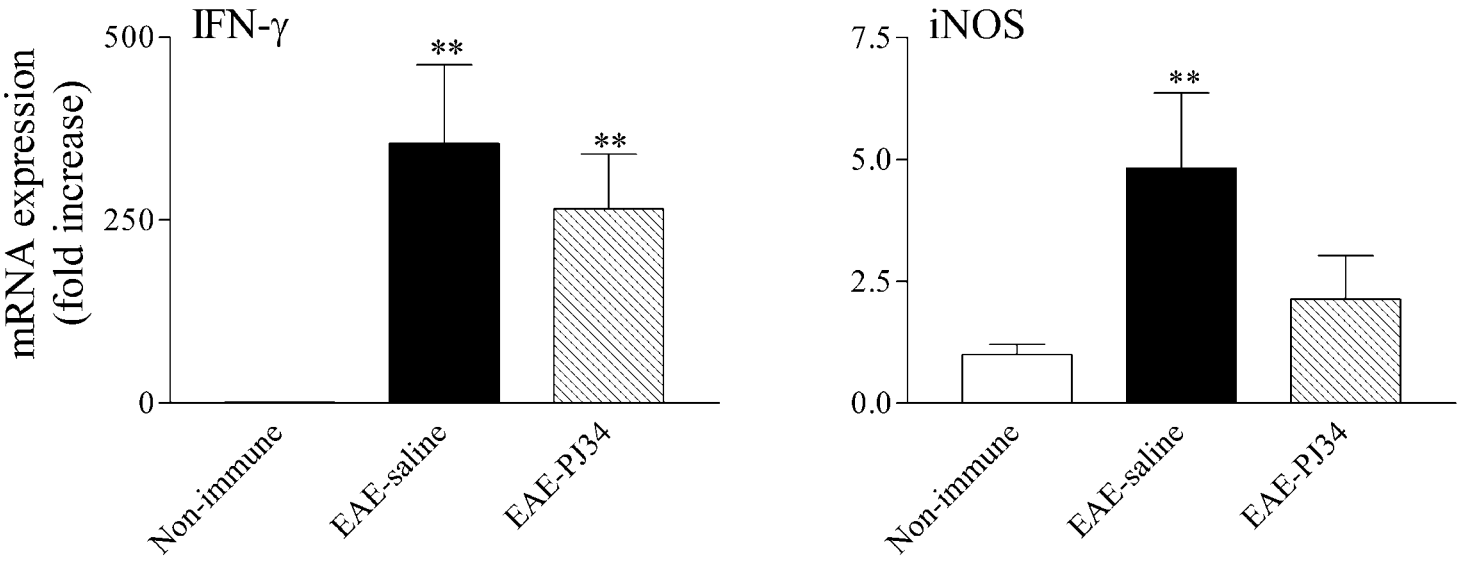

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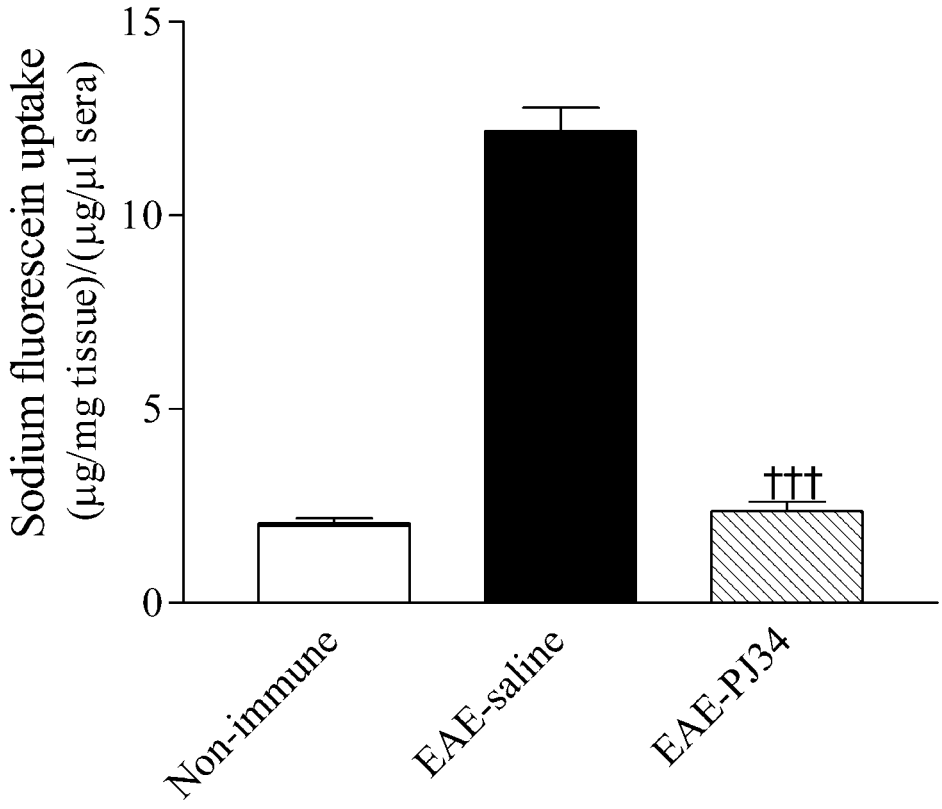

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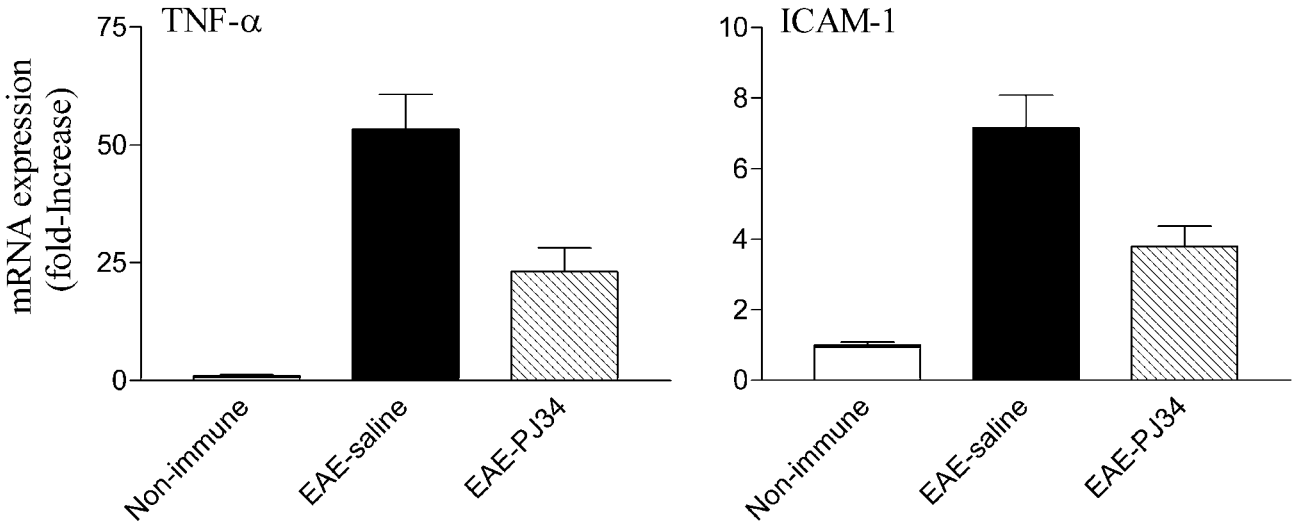
JPRT #63214 Fig. 6



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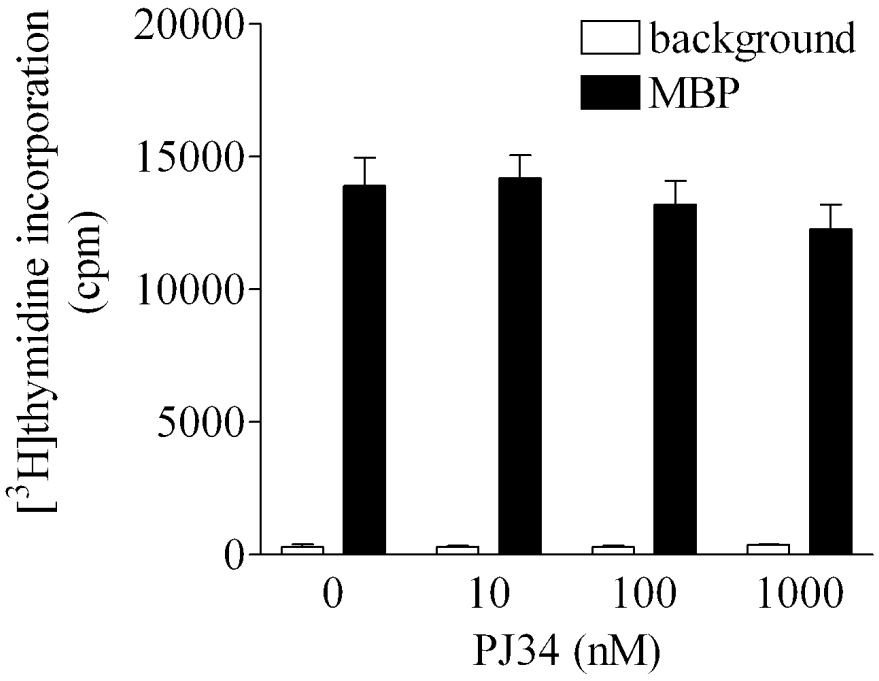
JPET #63214 Fig. 7



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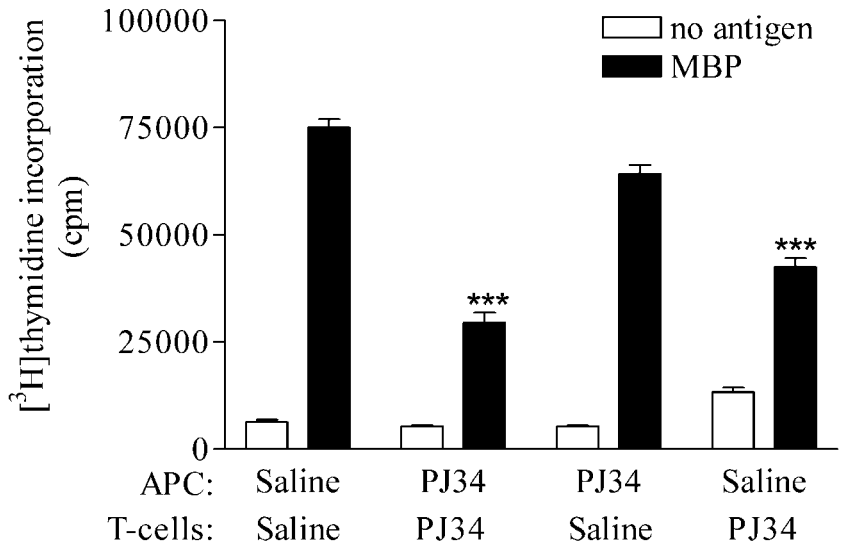
JPET #63214 Fig. 8



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JPET #63214 Fig. 9