Modification Form for Permit BIO-UWO-0263

Permit Holder: Steven Kerfoot

PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOLOGICAL AGENTS.
PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOLOGICAL AGENTS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF.

Approved Personnel
(Please stroke out any personnel to be removed)

Heather Craig (Broughton)

Additional Personnel
(Please list additional personnel and their Biosafety training dates here)

Amy Dang (Sept 7, 2012)
Rajiv Jain (Sept 26, 2012)

	Please stroke out any approved Biological Agent(s) to be removed	Write additional Biological Agent(s) for approval below. Give the full name
Approved Microorganisms	E. coli DH5alpha expressing rat myelin oligodendrocyte glycoprotein (MOG), E. coli DH5alpha expressing mouse MOG	
Approved Primary and Established Cells	Primary: [Rodent] Mouse.	B cell hybridoma (murine) expressing anti-CD32/FcRII (clone 2.4G2)
Approved Use of Human Source Material		
Approved Genetic Modifications (Plasmids/Vectors)		
Approved Use of Animals	Mice	
Approved Biological Toxin(s)		Pertussis Toxin (PTX)
Approved Gene Therapy		
Approved Plants and Insects		

As the Principal Investigator, I have ensured that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.shs.uwo.ca/workplace/newposition.htm

Signature of Permit Holder: _____

Current Classification: 1 **Containment Level for Added Biohazards:** _____

Date of Last Biohazardous Agents Registry Form: Nov 24, 2011

Date of Last Modification (if applicable): May 2, 2012

BioSafety Officer(s)*: _____

***For work being performed at Institutions affiliated with Western University, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to Western University Biosafety Officer.**

Chair, Biohazards Subcommittee: _____ **Date:** _____

Pertussis Toxin:
Pertussis toxin (PTX) is a commonly used reagent in the induction of experimental autoimmune encephalomyelitis (EAE). It is delivered at a dose of 200-250ng in saline i.p. or i.v. Two doses are usually administered, 2 days apart, along with immunization with specific antigen. In our case, it will most commonly be administered alone.

Although the LD50 for mice is listed as 18 ug/kg, in my extensive experience this is likely to be very low. 250ng/mouse roughly translates to half of the LD50 dose, and alone does not have any noticeable effect on the health or behavior or normal mice (see Kerfoot et al, 2004. Journal of Immunology).

As stated on the MSDS, "(PTX), in spite of its name, is not considered hazardous" and no special considerations are required. Typically, 100-200ug of PTX will be in the lab at any given time, and will be stored sterile at 4C.

See attached MSDS.

B cell hybridoma (murine):
Some B cell hybridomas will be maintained in culture and frozen stocks in the lab to act as sources for commonly used antibodies.

Clone 2.4G2 is an antibody directed towards murine CD32 and FcRII and is used as an Fc blocker to prevent non-specific staining in FACS experiments. Soluble antibody will be obtained from hybridoma culture media.

See attached information from the ATCC.

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Product Description

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Organism:	*Rattus norvegicus* (B cell); *Mus musculus* (myeloma) deposited as rat (B cell); mouse (myeloma)
Morphology:	lymphoblast
Source:	**Cell Type: hybridoma:** B lymphocyte;
Cellular Products:	immunoglobulin; monoclonal antibody; against the Fc gamma receptor (FcRII, CD32)
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Applications:	Tested and found negative for ectromelia virus (mousepox). The antibody can be used to block non-specific binding to Fc gamma bearing cells. The antibody reacts with and immunoprecipitates the 50000 dalton to 70000 dalton Fc gamma receptor on macrophages and Fc gamma bearing lymphoid cells.
Tumorigenic:	Yes
Comments:	Tested and found negative for ectromelia virus (mousepox). Animals were immunized with the J774 mouse macrophage cell line. Spleen cells were fused with P3U1 myeloma cells. The antibody reacts with and immunoprecipitates the 50000 dalton to 70000 dalton Fc gamma receptor on macrophages and Fc gamma bearing lymphoid cells.

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The antibody can be used to block non-specific binding to Fc gamma bearing cells.

Propagation:

ATCC complete growth medium: Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, 90%; horse serum, 5%; fetal bovine serum, 5%

Subculturing:

Medium Renewal: Add fresh medium every 2 to 3 days (depending on cell density)
Cultures can be maintained by the addition of fresh medium or replacement of medium. Alternatively, cultures can be established by centrifugation with subsequent resuspension at 1 to 2 X 10 exp5 viable cells/ml.
Maintain cell density between 1 X 10 exp5 and 1 X 10 exp6 viable cells/ml.

Preservation:

culture medium 95%; DMSO, 5%

Related Products:

Recommended Media: Fetal Bovine Serum (ATCC 30-2020)
Recommended Media: Horse Serum (ATCC 30-2040)
Recommended Media: Base medium (without the additional supplements or serum described under ATCC Medium) (ATCC 30-2002)

References:

1631: Unkeless JC. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. J. Exp. Med. 150: 580-596, 1979. PubMed: 90108
1632: Mellman IS, Unkeless JC. Purification of a functional mouse Fc receptor through the use of a monoclonal antibody. J. Exp. Med. 152: 1048-1069, 1980. PubMed: 6158545
1634: Nussenzweig MC, et al. Studies of the cell surface of mouse dendritic cells and other leukocytes. J. Exp. Med. 154: 168-187, 1981. PubMed: 7252426
27575: Yoshikai Y, et al. Clonal expansion of superantigen-reactive T cells is resistant to FK506 in mice with AIDS. J. Virol. 71: 746-749, 1997. PubMed: 8985410
32947: Wilson ME, et al. Local suppression of IFN-gamma in hepatic granulomas correlates with tissue-specific replication of Leishmania chagasi. J. Immunol. 156: 2231-2239, 1996. PubMed: 8690913

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Product #180-181

MATERIAL SAFETY DATA SHEET
Pertussis Toxin
Pertusis Toxin (Salt-Free)

Ingredients:

Each vial contains 50.0 µg of lyophilized pertussis toxin (islet-activating protein). Product 180 also contains 0.01 M sodium phosphate buffer, pH 7.0, with 0.05 M sodium chloride, when reconstituted with 0.5 ml water.

Health Hazard Data:

The LD_{50} in mice is 18 µg/kg i.p. There is no LD_{50} information for humans.

Emergency Procedures:

Pertussis toxin is degraded by the low pH in the gut and is not absorbed. If swallowing occurs, induce vomiting.

If skin pricking should occur, induce bleeding and flush with copious amounts of water.

If i.v. or i.m. injection should occur, consult a physician. Attempt to obtain hyperimmune globulin to pertussis from the CDC. In an adult immunized versus whooping cough, no long term ill effects are likely to result.

Handling:

Pertussis toxin, in spite of its name, is not considered hazardous. However, as with any biochemical, it should be handled by trained personnel using good laboratory technique. Observe the following practices when working with pertussis toxin: Special care should be taken when working in conjunction with hypodermic needles. Wear protective gloves, avoid contact with cuts or wounds, avoid inhalation, do not mouth pipet, and flush thoroughly any area of the body that comes in contact with this product. Only individuals who were immunized in childhood against whooping cough should work with this product. This product is intended for research purposes only.

Stability:

Stable for months when stored at 4°C. Do not freeze.

Deactivation:

Boil at 100°C for 15 to 30 minutes.

TLR4 Contributes to Disease-Inducing Mechanisms Resulting in Central Nervous System Autoimmune Disease[1]

Steven M. Kerfoot,* Elizabeth M. Long,† Michael J. Hickey,‡ Graciela Andonegui,* Benoit M. Lapointe,* Renata C. O. Zanardo,* Claudine Bonder,* Will G. James,‡ Stephen M. Robbins,† and Paul Kubes[2]*

Environmental factors strongly influence the development of autoimmune diseases, including multiple sclerosis. Despite this clear association, the mechanisms through which environment mediates its effects on disease are poorly understood. Pertussis toxin (PTX) functions as a surrogate for environmental factors to induce animal models of autoimmunity, such as experimental autoimmune encephalomyelitis. Although very little is known about the molecular mechanisms behind its function in disease development, PTX has been hypothesized to facilitate immune cell entry to the CNS by increasing permeability across the blood-brain barrier. Using intravital microscopy of the murine cerebromicrovasculature, we demonstrate that PTX alone induces the recruitment of leukocytes and of active T cells to the CNS. P-selectin expression was induced by PTX, and leukocyte/endothelial interactions could be blocked with a P-selectin-blocking Ab. P-selectin blockade also prevented PTX-induced increase in permeability across the blood-brain barrier. Therefore, permeability is a secondary result of recruitment, rather than the primary mechanism by which PTX induces disease. Most importantly, we show that PTX induces intracellular signals through TLR4, a receptor intimately associated with innate immune mechanisms. We demonstrate that PTX-induced leukocyte recruitment is dependent on TLR4 and give evidence that the disease-inducing mechanisms initiated by PTX are also at least partly dependent on TLR4. We propose that this innate immune pathway is a novel mechanism through which environment can initiate autoimmune disease of the CNS. ***The Journal of Immunology,*** **2004, 173: 7070–7077.**

Multiple sclerosis (MS)[3] is a debilitating disease thought to be mediated by autoreactive T cells against CNS myelin. However, the presence of anti-myelin T cells alone is not sufficient to initiate disease, as evidenced by the fact that myelin-reactive T cells can be isolated from healthy individuals (1–3). In fact, there is a growing body of evidence that environmental factors also play a role in disease development (4–6). This is best exemplified in the development of experimental autoimmune encephalomyelitis (EAE), an animal model of MS. For example, mice transgenic for an anti-myelin TCR do not develop disease when housed in a specific pathogen-free facility despite the presence of large numbers of cells capable of mediating disease (7). In contrast, these mice develop EAE spontaneously if they are either housed under conventional conditions where they are regularly exposed to infectious agents, or if they are injected with pertussis toxin (PTX) isolated from *Bordetella pertussis* (8). Clearly, PTX can function as a surrogate for environmental factors, but how this molecule acts to initiate disease is not yet known. Although many studies have focused on the T cell component of EAE, very few have investigated this other, essential component.

The EAE-enhancing effects of PTX are universal among all species in which EAE can be induced, including mice (9–12), rats (13), and nonhuman primates (14). It is sufficient to initiate disease in animals that would not normally develop disease. For example, inclusion of PTX in EAE induction protocols can render EAE-resistant strains of mice susceptible to disease (12). Indeed, PTX is commonly used in the induction of all EAE models. The prevailing explanation/speculation concerning the action of PTX is that it induces an increase in permeability across the blood-brain barrier (9, 10). This could allow easier passage for T cells across the endothelium. However, visualization of normal brain microvessels revealed that no basal leukocyte/endothelial interactions occur (15, 16), so that a simple increase in permeability would not be sufficient to recruit cells to the vessel wall. We hypothesized that PTX or environmental factors, such as bacterial infections, may sufficiently activate the brain endothelium to establish a proadhesive environment that becomes permissive for T cell recruitment. In addition, we proposed that pattern recognition receptors responsible for the detection of a wide array of infectious agents would potentially also be responsible for the transition from infection to autoimmune disease. Specifically, we hypothesized that the major LPS receptor, TLR4, was the critical molecule via which PTX induces a proadhesive environment within the brain, leading to the development of EAE.

We visualized the brain microvasculature and report for the first time that TLR4 is central to the EAE-inducing effects of PTX by

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[2] Address correspondence and reprint requests to Dr. Paul Kubes, Immunology Research Group, Department of Physiology and Biophysics, University of Calgary, 3330 Hospital Drive Northwest, Calgary, Alberta, Canada T2N 4N1. E-mail address: pkubes@ucalgary.ca

[3] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; LTA, lipoteichoic acid; MOG, myelin oligodendrocyte glycoprotein; PTX, pertussis toxin.

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up-regulating adhesion molecules and initiating leukocyte recruitment, including the recruitment of activated T cells, to the CNS. Although PTX also increased microvascular permeability, it was a result, rather than a cause, of leukocyte recruitment. This study demonstrates an important link between innate immune mechanisms and the initiation of CNS autoimmunity and may lead to important insights into the development of human multiple sclerosis.

Materials and Methods

Abs and reagents

Whole PTX holotoxin, the PTX B-oligomer and ultrapure LPS were purchased from List Biological Laboratories (Campbell, CA). Ultrapure lipoteichoic acid (LTA) was provided by Dr. T. Hartung (University of Konstanz, Konstanz, Germany). Anti-phospho- and anti-total JNK/stress-activated protein kinase, anti-phospho- and anti-total ERK1/2, and anti-phospho p38 were purchased from Cell Signaling Technologies (Beverly, MA). Anti-total IκBα, anti-total p38, and a rabbit anti-mouse HRP secondary Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-P-selectin and nonbinding control Ab (A110-1) were purchased from BD Pharmingen (Ontario, Canada). Anti-CD4 microbeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany), and anti-IL-4 (11B11) was a gift from Dr. C. Weaver (University of Alabama, Birmingham, AL).

Mice

C57BL/6 mice were purchased from Charles River Laboratories (Québec, Canada). P-selectin$^{-/-}$ and CD-14-deficient mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/10ScCr, a strain of mice with a naturally occurring deletion of the *Tlr4* gene (17) (referred to in this study as TLR4-deficient mice) were also purchased from The Jackson Laboratory. Animal protocols were approved by the University of Calgary animal care committee and met the Canadian Guidelines for Animal Research.

Intravital microscopy

Intravital microscopy of the brain microcirculation was performed as described in detail previously (15, 16). Briefly, pial vessels in the brain were exposed by removing a piece of the parietal bone and underlying dura mater. Mice were then administered rhodamine 6G (0.3 mg/kg i.v.; Sigma-Aldrich, Ontario, Canada) to label circulating leukocytes. Leukocyte/endothelial interactions were observed using a microscope (Axioskop; Carl Zeiss Canada, Don Mills, Canada; ×10 eyepiece and ×25 objective lens) outfitted with a fluorescent light source (epi-illumination at 510–560 nm using a 590-nm emission filter). All experiments were recorded onto videotape for playback analysis. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes within a given vessel. Leukocytes were considered adherent to the venular endothelium if they remained stationary for 30 s or longer.

Experimental protocol

Mice were injected with 250 ng of PTX in 250 μl of sterile PBS through the tail vein. For some experiments, PTX was heat inactivated by incubation in an 80°C water bath for 20 min. All observations were made 5 h after administration of PTX unless otherwise stated. Twenty micrograms of RB40.34 (blocking anti-P-selectin Ab) in 200 μl of saline was administered i.v.

For experiments using activated T cells, cells were isolated and cultured as described below. Cells were then collected and incubated in 15 ng/ml rhodamine 6G. Intravital microscopy was performed on healthy control mice or mice in the acute stage of EAE. Initial observations were made before administration of labeled T cells to gauge background fluorescence. Cells (1×10^7 in 200 μl of saline) were then administered i.v. and allowed to circulate for 10 min before quantification of leukocyte/endothelial interactions.

Generation of activated T cells

CD4$^+$ T cells were isolated from pooled spleens of DO11.10 mice using mouse anti-CD4 (L3T4) MACS microbeads according to the manufacturer's protocol (Miltenyi Biotec). FACS analysis revealed that isolations routinely resulted in >90% purity of CD4$^+$ cells. Th1 and Th2 cells were generated in vitro from Ag-naive CD4$^-$ T cells as described previously (18). Th1 cells were cultured in Iscove's medium (Invitrogen Life Technologies, Burlington, Canada) at 6.25×10^5 cells/ml in the presence of 3×10^6 cells/ml irradiated splenocytes from BALB/c mice, 5 μg/ml OVA peptide 323–339 (New England Peptides, Gardner, MA), antibiotics, and 10% FCS. To drive cells to a Th1 phenotype, 50 U/ml IL-12 (R&D Systems, Abingdon, U.K.) and 10 μg/ml anti-IL-4 (11B11) were added to the culture medium. Cells were cultured for 5–9 days before use.

Western blots for signaling molecules

Western blots were performed as previously described (19). Briefly, Raw 264.7 cells were plated in six-well tissue culture plates and allowed to grow to 75% confluence. The cells were treated with LPS (50 ng/ml), PTX (500 ng/ml), or B oligomer (500 ng/ml) for the indicated times, then lysed using Nonidet P-40 lysis buffer. LTA, which signals through TLR2, was also administered to demonstrate that TLR4-deficient cells were still capable of mediating intracellular signaling events. Insoluble material was removed from the lysates by centrifugation. Samples were prepared using 2× Laemmli's buffer and were resolved on a 10% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes, which were then blocked by incubation in 5% BSA in TBS plus 0.05% Tween 20 (TBST). The membranes were then incubated at 4°C overnight in the primary Ab, washed for 40 min in TBST, incubated for 1 h in secondary Ab, and washed for another 40 min in TBST. The membranes were developed using an ECL substrate.

For experiments using peritoneal macrophages, cells were isolated by peritoneal lavage with saline from untreated wild-type C57BL/6 or TLR4-deficient mice. Nonadherent cells were removed before treatment with LTA (1 μg/ml) or PTX (500 ng/ml). Western blots were then performed as described above.

Induction of EAE

Nine-week-old C57BL/6 or TLR4-deficient mice were immunized s.c. with 50 μg of a peptide generated from myelin oligodendrocyte glycoprotein (MOG_{35-55}) in CFA (Sigma-Aldrich). A second immunization with MOG peptide in IFA was performed 1 wk later. In some cases mice were immunized with MOG peptide in CFA supplemented with additional *Mycobacterium tuberculosis* (5 mg/ml total). When indicated, 250 ng of PTX (List Biological Laboratories) was injected i.p. along with the first immunization as well as 2 days later. Disease was monitored daily and was scored as follows: 0, no clinical signs; 1, tail paralysis; 2, tail paralysis and hind limb weakness; 3, hind limb paralysis; and 4, complete hind limb paralysis and front limb weakness.

Blood-brain barrier permeability assay

Permeability across the blood-brain barrier was assayed as previously described (20). Briefly, brain microvessels were exposed as described for intravital microscopy (see above). A stainless steel superfusion chamber was then attached to the skull surrounding the window. Artificial cerebrospinal fluid was continuously superfused through the chamber at a constant rate. Permeability of the blood-brain barrier was assessed by measuring the clearance (nanoliters per second) of 70-kDa FITC-dextran from the pial vessels into the suffusate fluid as previously described (20). Experiments were timed to allow for surgical preparation and equilibration before the 5 h point of either PBS or PTX treatment. Exactly 5 h after treatment, FITC-dextran was administered, and permeability was assayed over the following 30 min.

Dual radiolabeled Ab assay for P-selectin expression

P-selectin expression was quantitatively measured in the brain and other tissues of untreated, 5-h PTX-treated mice, and PTX-treated, P-selectin-deficient mice by the dual radiolabeled Ab assay as previously described (15, 16). Briefly, a mixture of ^{125}I-labeled anti-P-selectin (RB40.34) and ^{131}I-labeled nonbinding Ab (A110-1, anti-keyhole limpet hemocyanin) was injected i.v. The Abs were allowed to circulate for 5 min, at which point circulating blood was cleared by perfusing the whole mouse with bicarbonate-buffered saline. Tissues were harvested and weighed, and ^{125}I and ^{131}I activities were measured. P-selectin expression was calculated per gram of tissue by subtracting accumulated ^{131}I activity (labeled nonbinding Ab) from accumulated ^{125}I activity (labeled anti-P-selectin). Data are presented as the percentage of the injected dose of Ab per gram of tissue.

Statistics

Data in figures and tables are shown as the mean ± SEM unless indicated otherwise. Student's *t* test with Bonferroni correction was used for multiple comparisons. Statistical significance was set at $p < 0.05$.

Results

PTX induces leukocyte recruitment to the brain

We used intravital microscopy of the mouse brain to investigate the effects of PTX on leukocyte recruitment in the cerebromicrovasculature. No leukocyte rolling or adhesion (Fig. 1, *A*, *C*, and *D*) was observed in untreated mice or up to 30 min after the administration of PTX. In contrast, there was a significant induction of leukocyte/endothelial interactions 5 h after PTX injection, which remained elevated over the next 48 h (Fig. 1, *B–D*). Leukocyte adhesion was also induced ~15-fold, but returned to near-control levels within 24 h (Fig. 1*D*). Vehicle (PBS) had no effect on recruitment (data not shown). A dose of 250 ng of PTX/mouse was chosen because this is a common dose used in the induction of EAE. Because maximal recruitment was observed 5 h after administration of PTX, all subsequent experiments were performed at this time point.

PTX-mediated leukocyte recruitment to the brain is P-selectin dependent

We recently demonstrated an important role for P-selectin in leukocyte recruitment to the brain in EAE (16). We therefore hypothesized that PTX may activate the cerebrovascular endothelium to express P-selectin. Mice were administered a P-selectin-blocking Ab at the same time as PTX, and leukocyte/endothelial interactions were observed by intravital microscopy 5 h later. Anti-P-selectin completely blocked leukocyte rolling (Fig. 2*A*) and reduced leukocyte adhesion by 70% (Fig. 2*B*). Identical results were observed when PTX was administered to P-selectin-deficient mice. Acute treatment with anti-P-selectin (5 min) blocked leukocyte rolling, but could not dislodge already adherent cells, consistent with P-selectin-mediating rolling, but not adhesion (16, 21) (data not shown).

P-selectin expression in response to PTX

We used the radiolabeled Ab assay of adhesion molecule expression to measure P-selectin protein in the cerebrovasculature. This quantitative assay is capable of detecting very low levels of P-selectin expression in the CNS that are simply impossible to detect using immunohistochemistry (16). There was no difference in expression between the brains of untreated mice and P-selectin-deficient mice, demonstrating that under basal conditions, P-selectin is not expressed in this tissue (Fig. 2*C*). In contrast, significant amounts of P-selectin protein were measured 5 h after injection of PTX.

PTX induces recruitment of activated T cells to the CNS

EAE develops when myelin-reactive T cells, once activated in the periphery, gain access to the CNS. Activated, but not naive, T cells express functional P-selectin ligand (22), and it has been demonstrated that only activated cells, regardless of their Ag specificity, can gain entry to the CNS (23). To determine whether PTX facilitates the recruitment of activated T cells to the CNS, T cells were isolated and cultured in vitro in the presence of peptide as described in *Materials and Methods*. T cells from mice bearing a transgenic TCR for an OVA peptide were used to generate the numbers of cells required for the experiment. Cells were then fluorescently labeled and transferred i.v. to PTX-treated or untreated mice. Because recruitment is independent of Ag specificity (23), this accurately models the entry of disease-inducing T cells to the CNS. Significant rolling and adhesion of the activated Th1 T cells were observed in PTX-treated, but not control untreated, mice (Fig. 3).

PTX-induced blood-brain barrier permeability is P-selectin dependent

We investigated PTX-induced permeability across the blood-brain barrier. Very little leakage of 70-kDa FITC-dextran was detected in vehicle-treated mice (250 μl of sterile PBS; Fig. 4). In contrast, permeability to 70-kDa FITC-dextran across the blood-brain barrier was >2-fold greater in mice 5 h after i.v. administration of 250 ng of PTX. This is consistent with previous reports that PTX induces increased blood-brain barrier permeability (9, 10). Interestingly, when a blocking anti-P-selectin Ab was administered at the same time as PTX, a treatment that effectively blocks all leukocyte rolling and most adhesion (Fig. 2, *A* and *B*, respectively), the increase in blood-brain barrier permeability was completely eliminated. This suggests that the rolling/adhering leukocytes were responsible for the increased blood-brain barrier permeability. Interestingly, the low level of basal permeability was also eliminated with anti-P-selectin, suggesting that this may also be mediated by a very low number of trafficking leukocytes.

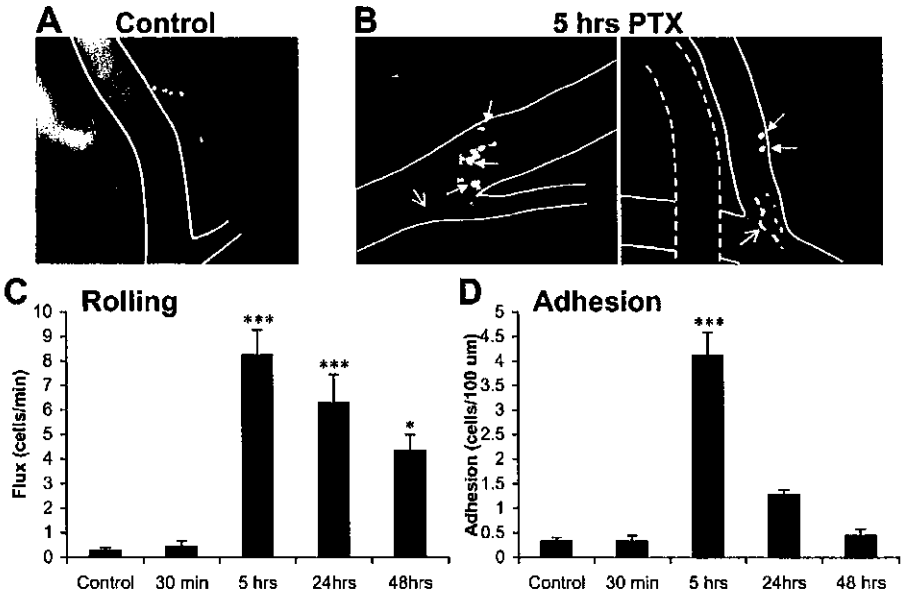

FIGURE 1. PTX induces leukocyte recruitment to the CNS. Intravital microscopy was performed on control mice (*A*) or 5 h after administration of PTX (*B*). Two examples of PTX-treated mice are shown. Solid arrowheads point to examples of leukocytes interacting with the vascular wall. Open arrowheads point to free-flowing leukocytes, which appear as streaks on still frames. The dotted lines in the image on the *right* outline an arteriol, in which no leukocyte recruitment occurs. To determine the time course of PTX-induced leukocyte recruitment, intravital microscopy was performed at various time points after administration of PTX. Leukocyte rolling flux (*C*) and adhesion (*D*) were quantified. Results are shown as the mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (vs control; $n = 4$ for all groups except 30 min ($n = 3$)).

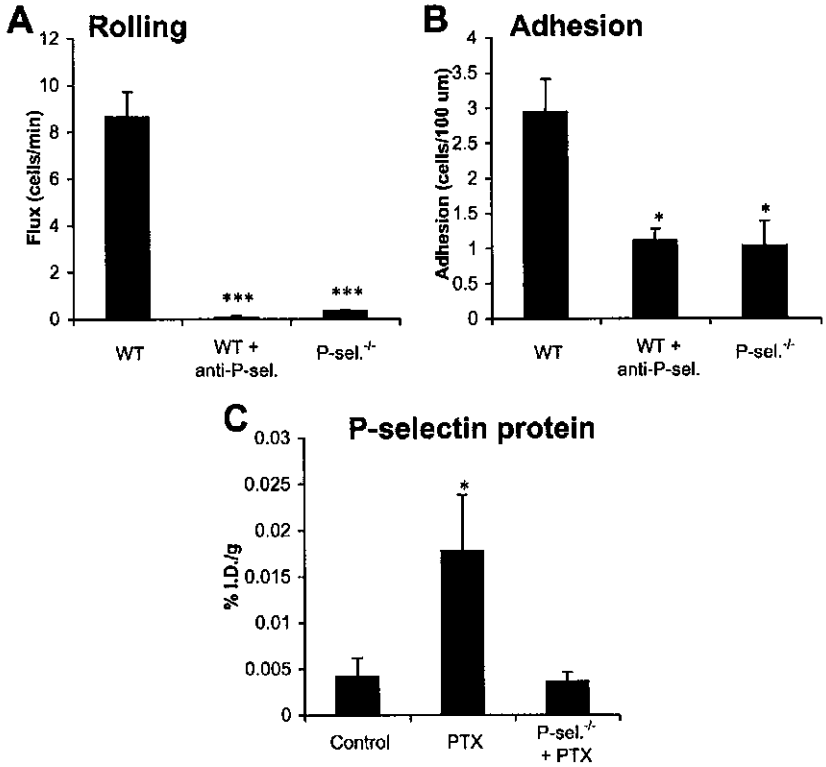

FIGURE 2. PTX-mediated leukocyte recruitment to the brain is P-selectin dependent. Inhibition of leukocyte rolling (*A*) and adhesion (*B*) by pretreatment with anti P-selectin was determined. RB40.34 was administered at the same time as PTX, and intravital microscopy was performed 5 h later. Intravital microscopy was also performed on PTX-treated, P-selectin-deficient mice. Results are shown as the mean ± SEM. *, $p < 0.05$; ***, $p < 0.001$ (vs wild type (WT); $n = 3$). *C*, Quantitative measurement of P-selectin protein in the brain of untreated WT, PTX-treated WT, and PTX-treated P-selectin$^{-/-}$ mice. Results are shown as the mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (vs control; $n = 7$ for control; $n = 6$ for PTX; $n = 5$ for P-selectin$^{-/-}$ plus PTX).

CD14 and TLR-4 are required for PTX-induced leukocyte recruitment

It has been suggested that PTX may share a putative receptor with LPS (24), which we now know includes the GPI-linked protein CD14 and TLR4. To determine whether CD14 or TLR4 plays a role in PTX-induced leukocyte recruitment to the CNS, intravital microscopy of the cerebromicrovasculature was performed on CD14- and TLR4-deficient mice. In the absence of TLR4, PTX was unable to induce leukocyte rolling or adhesion (Fig. 5, *A* and *B*, respectively) in the cerebrovasculature. A >60% reduction in leukocyte rolling was observed in CD14-deficient mice compared with controls (Fig. 5*A*); however, the decrease in adhesion was not significant (Fig. 5*B*).

To confirm that the effects of PTX were not the result of contaminating endotoxin, intravital microscopy was performed on mice treated with PTX that had been incubated at 80°C for 20 min to denature proteins, but not endotoxin. No induction of leukocyte/endothelial interactions was observed (data not shown), demonstrating that LPS contamination was not responsible for the TLR4-dependent activity of PTX.

To determine whether the PTX holotoxin was required to induce leukocyte/endothelial interactions in the cerebromicrovasculature, mice were treated with either whole PTX or the B oligomer fragment of PTX, which is thought to bind to the cell surface, but has no enzymatic activity itself (25). In contrast to treatment with the PTX holotoxin, the B oligomer was unable to induce leukocyte recruitment in the CNS (Fig. 5, *C* and *D*), implying that the complete toxin was required to activate cerebrovascular endothelium.

PTX induces an intracellular signal through TLR4

Members of the IL-1R family, which includes the TLRs, activate a number of signaling pathways, including the NF-κB pathway, the JNK pathway, and the ERK and p38 MAPK pathways (reviewed in Ref. 26). To determine whether PTX induced the activation of similar pathways, the presence of phosphorylated JNK, p38, and ERK1/2 and total IκB in cell lysates of TLR4$^+$ RAW 264.7 cells was assayed by Western blot. Phosphorylated JNK, p38, and ERK1/2 could not be detected in the lysates of unstimulated RAW 264.7 cells (Fig. 6*A*). Stimulation with LPS resulted in rapid phosphorylation of p38, ERK1/2, and JNK, peaking within 15 min of stimulation. Similarly, total IκB protein degradation was complete at 15 min. PTX induced similar signaling in RAW 264.7

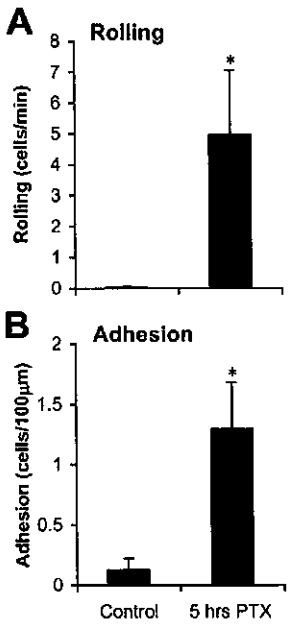

FIGURE 3. PTX induces the recruitment of activated T cells to the CNS. Fluorescently labeled, activated Th1 T cells were administered i.v. to untreated control or PTX-treated mice. T cell rolling (*A*) and adhesion (*B*) were quantified by intravital microscopy. Results are shown as the mean ± SEM. *, $p < 0.05$ (vs control; $n = 4$ for control; $n = 3$ for 5-h PTX).

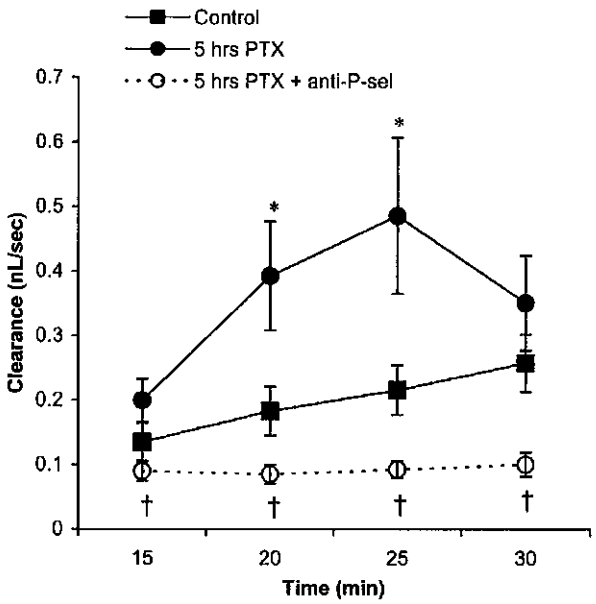

FIGURE 4. Increased permeability across the blood-brain barrier is dependent on leukocyte/endothelial interactions. Blood-brain barrier permeability to FITC-dextran was assayed in control mice and in mice treated with PTX or a combination of PTX and RB40.34. Results are shown as the mean ± SEM. *, $p < 0.05$ (vs control); †, $p < 0.05$ (vs PTX and anti-P-selectin (anti-P-sel); $n = 5$ for control; $n = 4$ for PTX and PTX plus anti-P-selectin).

cells, although p38 and ERK1/2 phosphorylation as well as IκB degradation were slightly delayed and did not peak until 60 min after treatment. JNK phosphorylation could only be detected at 60 min. The B oligomer of PTX was completely unable to induce intracellular signaling, consistent with the leukocyte recruitment data.

Similar experiments were performed in freshly harvested peritoneal macrophages from wild-type C57BL/6 mice. As in the RAW 264.7 cells, JNK, p38, and ERK1/2 were all activated in response to PTX; however, the kinetics were faster, because peak phosphorylation was observed 15 min after treatment. IκB protein degradation occurred with similar kinetics (data not shown). Interestingly, although LPS and PTX activated the same signaling events, PTX-mediated signaling was delayed compared with that mediated by LPS in both RAW 264.7 (Fig. 6*A*) cells and peritoneal macrophages (data not shown).

To determine whether TLR4 is required to mediate PTX-induced intracellular signaling, peritoneal macrophages were isolated from wild-type and TLR4-deficient mice (Fig. 6*B*). These cells were treated with either PTX or LTA (which signals through a TLR other than TLR4) and were included as a positive control to demonstrate that intracellular signaling was not grossly impaired in TLR4-deficient cells. ERK1/2 phosphorylation as well as IκB degradation were entirely dependent on TLR4, because PTX was completely unable to induce these events in peritoneal macrophages isolated from TLR4-deficient mice. Phosphorylation of JNK and p38 still occurred, but to a lesser extent compared with that in wild-type macrophages (Fig. 6*B*), demonstrating that although the majority of signaling is mediated through TLR4, PTX can induce some events through a TLR4-independent pathway.

Induction of EAE by PTX is dependent on TLR4

To determine whether the disease-inducing activity of PTX was dependent on TLR4, as is the case for its capacity to induce intracellular signaling and activity on leukocyte recruitment, EAE in C57BL/6 wild-type and age-matched TLR4-deficient mice was investigated. Wild-type mice treated with PTX first showed signs of disease ~12 days after the first immunization with MOG (Fig. 7). Disease progressed over the next 4–5 days, at which time symptoms stabilized. Mice that did not receive PTX did not develop any sign of disease. Disease incidence was substantially lower in TLR4-deficient mice, in that only 45% of these mice developed disease compared with 95% of wild-type mice (Fig. 7*A*). For those TLR4-deficient mice that did develop disease, TLR4 deficiency did not have any impact on disease severity or time of onset, implicating TLR4-mediated mechanisms in the initiation, but not the subsequent development, of EAE.

Interestingly, EAE initiation in TLR4-deficient mice was not evenly distributed across groups of animals over the course of these studies. Indeed, in some groups none or only a single mouse (of five) developed any symptoms (an example is shown in Fig. 5*B*), whereas in other groups there was no difference between TLR4-deficient and wild-type mice (an example is shown in Fig. 7*C*). At all times, disease in both strains remained dependent on PTX (not shown). Both these results were observed in multiple trials. Although no pattern, factor, or mechanism could be identified to predict the outcome of any particular trial, this suggests that the mechanisms required to induce disease are the cumulative effects of PTX and the environment. Therefore, under some circumstances TLR4-independent mechanisms induced by a combination of PTX and the environment were sufficient to induce disease.

In a final series of experiments, wild-type mice were immunized with MOG in CFA supplemented with sufficient *M. tuberculosis* to overcome the requirement for PTX. When the same protocol was applied to TLR4-deficient mice, these animals were capable of developing disease, suggesting that TLR4 is required for PTX-induced mechanisms of disease induction (data not shown).

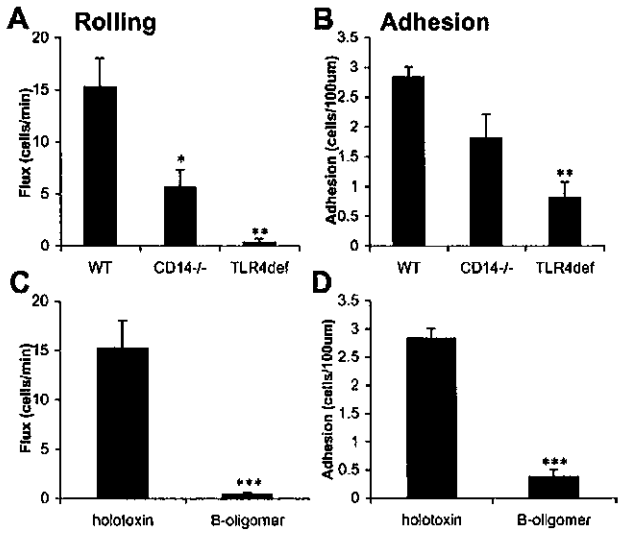

FIGURE 5. PTX-induced leukocyte recruitment is dependent on TLR4 and is partially dependent on CD-14. Intravital microscopy was used to observe leukocyte rolling (*A*) and adhesion (*B*) in PTX-treated, wild-type, CD14-deficient, and TLR4-deficient mice. Results are shown as the mean ± SEM. *, $p < 0.05$; **, $p < 0.01$ (vs wild type; $n = 4$ for wild-type and $CD14^{-/-}$ mice; $n = 3$ for $TLR4^{-/-}$ mice). PTX-induced leukocyte recruitment to the CNS is dependent on the enzymatically active A promoter. Intravital microscopy was used to investigate leukocyte rolling (*C*) and adhesion (*D*) after administration of either whole PTX or PTX B oligomer. Results are shown as the mean ± SEM. ***, $p < 0.0001$ (vs holotoxin, as determined by two-tailed t test; $n = 4$ for holotoxin; $n = 7$ for B oligomer).

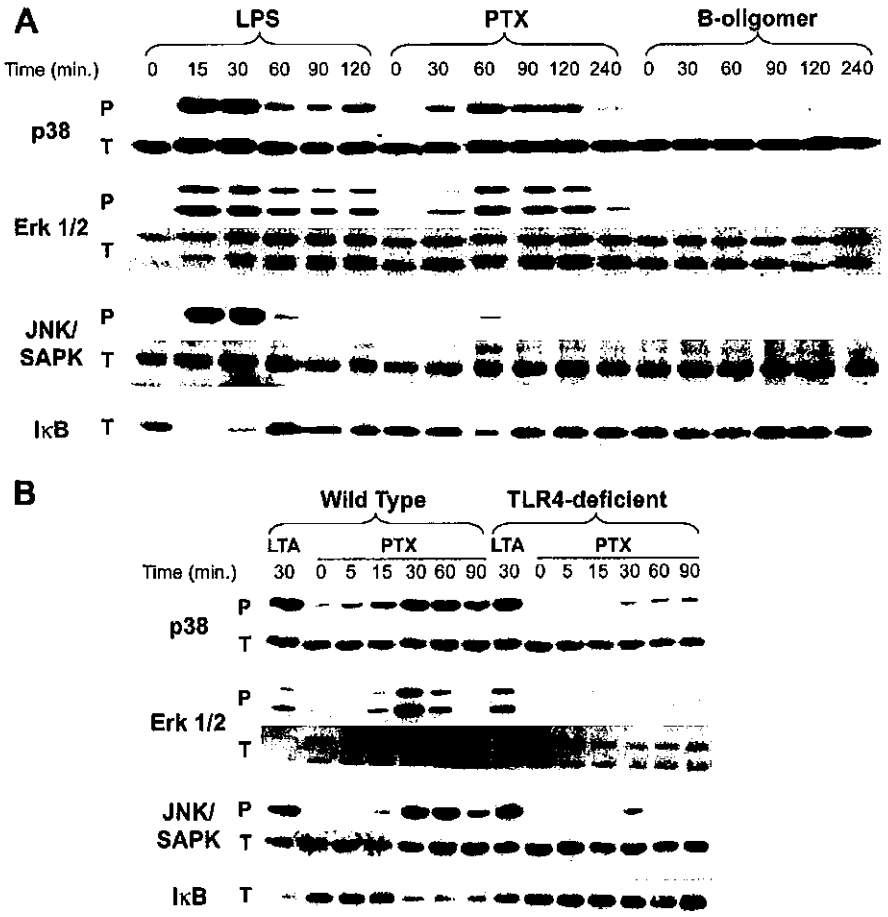

FIGURE 6. *A*, PTX-induced intracellular signaling events in TLR4$^+$ RAW 264.7 cells. Phospho- (P) and total (T) p38, ERK1/2, and JNK were assayed by Western blot of lysates from cells treated for various lengths of time with LPS, PTX, or B oligomer. IκB degradation was assayed by Western blot of total protein. *B*, PTX-induced intracellular signaling is largely dependent on TLR4. Peritoneal macrophages were isolated from wild-type or TLR4-deficient mice and treated with LTA or PTX. Signaling events were assayed as described above.

Discussion

There are three major findings in this study. First, we identify TLR4 as a pathway through which PTX contributes to the induction of EAE, implicating innate immune mechanisms in the development of CNS autoimmune disease. Second, PTX induces the recruitment of leukocytes to the CNS through the activation of cerebrovascular endothelium to be proadhesive. This was also dependent on TLR4. Finally, PTX-induced permeability across the blood-brain barrier is a secondary byproduct of leukocyte recruitment and can be prevented by blocking leukocyte/endothelial interactions. To date, blood-brain barrier permeability has been thought to be the mechanism by which PTX facilitates access of the immune system to the CNS. Each of these points will be discussed below.

Our studies demonstrate for the first time that TLR4 is a receptor for PTX, and that PTX is largely dependent on TLR4 to induce intracellular signaling events. Indeed, PTX induced the activation of a number of intracellular signaling pathways, including degradation of IκB and phosphorylation of JNK, which would, respectively, result in activation and translocation to the nucleus of the transcription factors NF-κB and AP-1 (26). This is significant, because these two factors are very important to the transcription and subsequent production of many inflammatory factors, including murine P-selectin (27) and other adhesion molecules. Intracellular signaling was nearly abolished in the absence of TLR4, as was leukocyte recruitment in the CNS as observed by intravital microscopy. Combined, these observations clearly demonstrate an important role for TLR4 in PTX-mediated events both in vitro and in vivo. The fact that the enzymatic subunit of PTX was required to induce these events suggests either that the entire intact molecule is required to activate TLR4 to initiate signaling and/or that the ADP-ribosylation activity of PTX may also be necessary.

The contribution of TLR4 to EAE induction implicates innate immune processes in the development of CNS autoimmune disease. It is well known that the local environment, in particular the presence of infectious agents, can have a tremendous impact on the course of disease. Importantly, this also appears to be the case for human disease, because exposure to various childhood infections has been linked to MS (28), and infections can exacerbate ongoing disease (6). In the animal model, PTX appears to act as a surrogate for environmental conditions by inducing the same mechanisms that are required for disease development. Indeed, Goverman et.al (7) reported that either PTX or a "dirty" environment induced EAE in their anti-myelin TCR transgenic mice. However, our observation that under some circumstances PTX could induce EAE independently of TLR4 suggests that additional factors, probably environmental (or PTX itself), are able to act through other mechanisms such as other TLRs to induce disease. The central role of the innate immune system in the initiation and regulation of a subsequent Ag-specific immunity is becoming increasingly apparent (29, 30). It seems logical that this would also extend to the dysregulation of immunity to an autoantigen. However, to date this has remained largely unaddressed, and investigations of the initiation of autoimmune disease have instead focused nearly exclusively on the T cell.

Leukocytes are recruited from the circulation through a well-characterized cascade of events, where they first tether to and then roll along the blood vessel wall before firmly adhering to and transmigrating across the endothelial layer into the surrounding tissue (31). This process is entirely dependent on the expression of

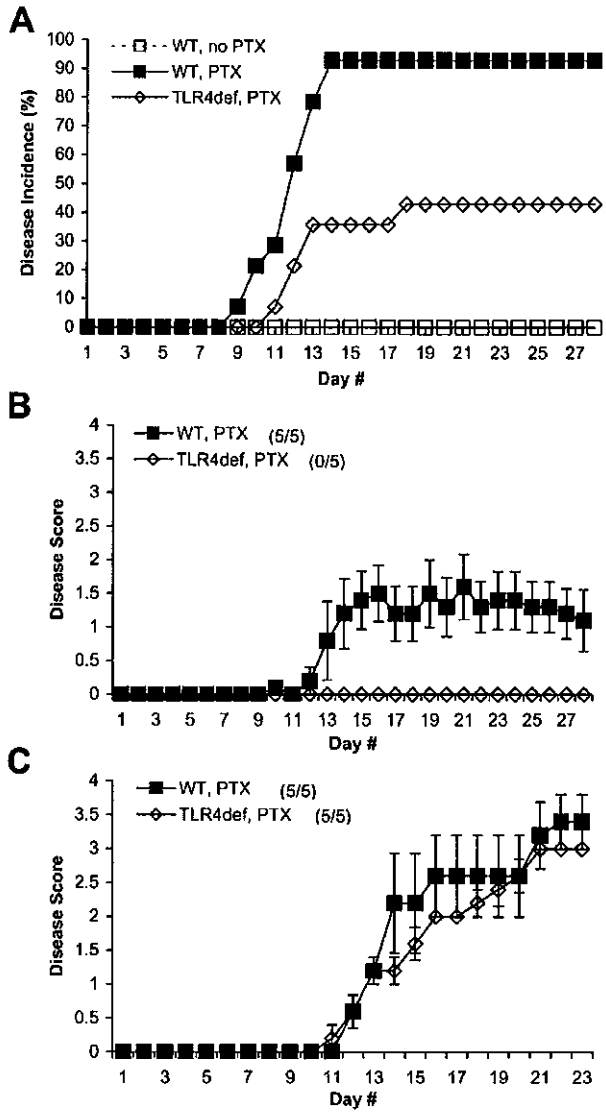

FIGURE 7. PTX acts through TLR4 to induce EAE. Wild-type C57BL/6 or age-matched TLR4-deficient mice were immunized with MOG peptide in CFA along with PTX as described in *Materials and Methods*. *A*, Total combined disease incidence in C57BL/6 or TLR4-deficient mice. Mice not receiving PTX did not develop disease. The cumulative disease incidence over the course of disease is shown. The distribution of disease incidence was not even across experiments. One example where TLR4-deficient mice did not develop symptoms (*B*) and one where all TLR4-deficient mice developed symptoms (*C*) are shown.

adhesion molecules by endothelial cells lining the lumen of the blood vessel. In the current study, adhesion molecule expression was very low to absent in the microvasculature of the CNS under normal conditions, consistent with previous findings (15, 16). Therefore, for PTX to mediate leukocyte recruitment to the CNS, its primary mechanism would have to be to induce the expression of adhesion molecules. Indeed, P-selectin protein could be detected in the CNS of PTX-treated mice, and leukocyte/endothelial interactions could be blocked with a P-selectin-blocking Ab, clearly demonstrating that PTX acts to activate endothelium.

It has been known for some time that treatment with PTX results in increased permeability across the blood-brain barrier (9, 10). It has since been regularly stated that PTX "opens up" the blood-brain barrier, allowing immune cell access to the CNS and subsequent development of autoimmune disease. In this study we demonstrate that the PTX-mediated increase in permeability across the blood-brain barrier could be completely prevented by blocking leukocyte/endothelial interactions with an adhesion molecule-blocking Ab. Therefore, the effects of PTX on permeability are indirect and secondary to its primary mechanism of leukocyte recruitment, which is through the expression of adhesion molecules. This is not without precedent, because anti-adhesion therapy has been shown to prevent permeability across the blood-brain barrier after ischemia/reperfusion injury (32) and mast cell degranulation (33).

There are two potential mechanisms through which PTX may induce autoimmune disease. The first is to facilitate the entry of anti-myelin T cells to the CNS through endothelial activation. Recent studies have identified P-selectin as critical to mediating the recruitment of disease-inducing T cells to the CNS (34–36). Our demonstration that PTX induces P-selectin expression and the recruitment of activated Th1 T cells to the CNS implicates PTX in this initial and critical step in the development of EAE. In another study, PTX alone induced encephalopathy characterized by a massive mononuclear inflammation in transgenic mice with CNS-specific expression of the chemokine MCP-1 (37). This demonstrates that PTX can indeed facilitate leukocyte access to the CNS in an Ag-independent model of CNS inflammation. However, it is not clear whether endothelial activation and leukocyte recruitment are the primary mechanisms by which PTX induces EAE.

A second disease-inducing mechanism for PTX has been proposed by studies demonstrating that it is also an adjuvant, acting on dendritic cells to drive T cell responses toward a Th1 phenotype (38, 39). Our demonstration that PTX induces signaling through TLR4 may explain these observations, because TLR4 on dendritic cells has been identified as important for the development of a Th1 response (40). PTX has been demonstrated to induce EAE in a spontaneous disease model in a myelin-specific TCR transgenic mouse (8). In a recent study, in vivo administration of CpG DNA or, to a lesser degree, LPS was also demonstrated to have disease-inducing activity in a similar transgenic model of EAE (41). This was attributed to the activation of dendritic cells through TLR9 and TLR4, respectively, to break self tolerance and activate the autoimmune, anti-myelin T cell response. This study strongly supports our hypothesis that activation of TLRs is an important inducer of autoimmune disease. Indeed, in our model it may be that at least part of the contribution of PTX to disease is simply to bolster the Th1 adjuvant effect of the immunization protocol. However, in the study just described, PTX was by far superior in inducing EAE in their model compared with CpG DNA or LPS (41). Therefore, PTX may act through multiple and potentially cumulative pathways to induce disease.

In conclusion, very little is known about the requirements for the initiation of CNS autoimmune disease. Most work has focused on the activation of autoreactive T cells; however, it is clear that other mechanisms are also required. PTX is able to induce these mechanisms and is therefore very important to the development of EAE. We identify a role for TLR4 in this process and in EAE development, linking innate inflammatory mechanisms to the development of CNS autoimmune disease.

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TOXIN USE RISK ASSESSMENT

Name of Toxin:	Pertussis Toxin
Proposed Use Dose:	0.25 µg
Proposed Storage Dose:	200 µg
LD $_{50}$ (species):	18 µg

Calculation:

18 µg/kg x 50 kg/person

Dose per person based on LD $_{50}$ in µg = 900

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = ***90***

Comments/Recommendations:

Storage (200 µg) exceeds safe calculation (90 µg).