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A Requirement for Microglial TLR4 in Leukocyte Recruitment into Brain in Response to Lipopolysaccharide

Hong Zhou, Benoît M. Lapointe, Stephen R. Clark, Lori Zbytnuik, and Paul Kubes[1]

To study the mechanisms involved in leukocyte recruitment induced by local bacterial infection within the CNS, we used intravital microscopy to visualize the interaction between leukocytes and the microvasculature in the brain. First, we showed that intra-cerebroventricular injection of LPS could cause significant rolling and adhesion of leukocytes in the brain postcapillary venules of wild-type mice, while negligible recruitment was observed in TLR4-deficient C57BL/10ScCr mice and CD14 knockout mice, suggesting recruitment is mediated by TLR4/CD14-bearing cells. Moreover, we observed reduced but not complete inhibition of recruitment in MyD88 knockout mice, indicating both MyD88-dependent and -independent pathways are involved. The leukocyte recruitment responses in chimeric mice with TLR4-positive microglia and endothelium, but TLR4-negative leukocytes, were comparable to normal wild-type mice, suggesting either endothelium or microglia play a crucial role in the induction of leukocyte recruitment. LPS injection induced both microglial and endothelial activation in the CNS. Furthermore, minocycline, an effective inhibitor of microglial activation, completely blocked the rolling and adhesion of leukocytes in the brain and blocked TNF-α production in response to LPS in vivo. Minocycline did not affect activation of endothelium by LPS in vitro. TNFR p55/p75 double knockout mice also exhibited significant reductions in both rolling and adhesion in response to LPS, indicating TNF-α signaling is critical for the leukocyte recruitment. Our results identify a TLR4 detection system within the blood-brain barrier. The microglia play the role of sentinel cells detecting LPS thereby inducing endothelial activation and leading to efficient leukocyte recruitment to the CNS. *The Journal of Immunology,* **2006, 177: 8103–8110.**

We have known for many years that the brain is "immune privileged" in as much as foreign tissue grafts transplanted into the brain have significantly extended survival (1). This suggests that the immune system has restricted access to the brain (2). Although this immune compromise makes perfect sense from the perspective of trying to prevent collateral damage in the brain in response to various insults, there are instances when very robust innate immune responses occur in the CNS, particularly in response to various cerebral bacterial infections (3–5). A disruption of this delicate balance between infection and inflammation is best exemplified by bacterial meningitis, wherein the response is so profound it not only destroys the pathogen but often also causes profound tissue injury (6). In addition, there is a growing body of evidence that susceptibility, progression, and relapse in chronic CNS diseases is closely associated with a systemic immune response to nonspecific infection (7). Indeed, multiple sclerosis, neurodegenerative diseases such as Alzheimer's disease, prion-related diseases, and various other cerebral diseases may be activated, reactivated, or expedited by infection and more importantly by the innate immune response to infection (8–10). Clearly, a local inflammatory response in the CNS or a systemic inflammatory response to a peripheral infection can have devastating effects in the brain.

Administering the prototypical TLR4 ligand, LPS, i.p. to mice reveals evidence of brain inflammation, partly via activation of peripheral cells, including macrophages, which release cytokines and other proinflammatory molecules that may impinge upon the CNS. This is best exemplified by a clear activation of brain-resident microglia when LPS is given i.p. (10). In addition, there is strong evidence to suggest direct activation of microglia by LPS. TLR4 is predominately expressed on microglia but not oligodendrocytes or neurons (11) and microglia exposed to LPS in vitro have been shown to be potently activated to release IL-1β, TNF-α, and various other proinflammatory molecules (12). More recently, a number of studies attempting to mimic local cerebral inflammation have delivered LPS via intracerebroventricular (i.c.v.)[2] administration and detected robust cytokine and chemokine increases within the brain tissue and more specifically in microglia (13). These studies suggest that microglia are sentinel cells within the brain, poised to detect infectious molecules causing a rapid robust local response.

The mechanism of neutrophil recruitment into the brain in response to local LPS remains poorly understood due to limitations in the ability to detect neutrophil recruitment into the CNS and the lack of an easy way to block microglial function. To date, a microglia-deficient mouse does not exist, and the macrophage-deficient *op/op* mice have only a 30% reduction in microglia (14). In the last few years, a novel microglial inhibitor was identified. In 1998, Yrjanheikki et al. (15) reported that minocycline, a tetracycline derivative, has potent inhibitory functions in microglial activation and in brain inflammation. This has been confirmed and extended by more than 60 studies in the last 5 years. Most importantly, the drug may have efficacy in human CNS disease (16) further underscoring the importance of understanding the mechanisms of action of this drug in brain inflammation.

In this study, we systematically examined neutrophil recruitment into the CNS in response to local LPS administration. We

[2] Abbreviation used in this paper: i.c.v., intracerebroventricular; Toll/IL-1 receptor domain-containing adapter inducing IFN-β.

used intravital microscopy to directly visualize the behavior of the leukocytes in the brain microvasculature. Using various knockout mice as well as making chimeric mice expressing the LPS receptor on bone marrow-derived or non-bone marrow-derived cells, we identified that microglia as well as cerebral endothelial cells were activated in response to local LPS. We also determined that minocycline, an established microglial inhibitor, blocked leukocyte recruitment in an unprecedented fashion (as effectively as TLR4 deficiency) in response to intracerebral LPS primarily by blocking production of TNF-α in microglia. Clearly, microglia play a dominant role in the innate immune response to infectious agents in the CNS and minocycline may be able to attenuate or dampen this potentially overexuberant and detrimental response.

Materials and Methods

Animals

C57BL/6 mice were purchased from Charles River Laboratories. TLR4-deficient C57BL/10ScCr mice, CD14$^{-/-}$ mice, TNFR p55/p75$^{-/-}$ mice, and *op/op* mice were all purchased from The Jackson Laboratory. We obtained MyD88$^{-/-}$ mice from Dr. S. Akira (Osaka University, Osaka, Japan). Chimeric mice were made as previously described (17) by transferring the bone marrow cells between wild-type C57BL/6J and C57BL/10ScCr mice. Briefly, recipient mice were exposed to a double irradiation of 525 rad and 8×10^6 bone marrow cells were transferred. After 6 wk of reconstitution, chimeric mice were tested for reconstitution efficiency or used for experiments. Greater than 95% reconstitution efficiency was confirmed by FACS analysis on a control set of chimeric mice made by transferring bone marrow cells from Thy 1.1 to Thy 1.2 mice. All animal protocols were approved by the University of Calgary animal care committee and met the Canadian Guidelines for Animal Research.

Intracerebroventricular LPS injection

Ultra-pure LPS (*Escherichia coli* serotype 055:B5; List Biological Laboratories) was dissolved in sterile saline at a concentration of 1 mg/ml. Mice were anesthetized by i.p. injection of a mixture of 200 mg/kg ketamine and 10 mg/kg xylazine. Then mice were placed into a rodent stereotaxic frame (David Kopf Instruments). The scalp was shaved and a burr hole was drilled 1 mm caudal to bregma and 2.0 mm lateral to the midline. LPS (2 μg) was injected via a Hamilton microsyringe into the ventricle over a 5-min period. This concentration of LPS was identified to induce optimal responses in preliminary experiments and has been used by a number of other laboratories (18, 19) Sham animals received isovolumetric i.c.v. injection of saline. Body temperature was measured using a rectal probe and mice were maintained under anesthesia at $36 \pm 1°C$ using a thermostatic heating blanket throughout the experiment.

Intravital microscopy

Intravital microscopy of the brain microcirculation was performed as previously described (20, 21). Briefly, a craniotomy was performed using a high-speed drill (Fine Science Tools) and the dura mater was removed to expose the underlying pial vasculature. To observe leukocyte/endothelial interactions, leukocytes were fluorescently labeled by i.v. administration of rhodamine 6G (0.5 mg/kg body weight) and observed using a microscope (Axioskop; Zeiss) outfitted with a fluorescent light source. A low light intensified CCD camera (Stanford Photonics) mounted on the microscope was used to project the image to a monitor. Three different postcapillary venules with a diameter between 30 and 70 μm were chosen for observation. Throughout the experiment, the mouse was maintained at $36 \pm 1°C$ and the exposed brain was kept moist with an artificial cerebrospinal fluid. All experiments were recorded onto videotape for later playback analysis. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes. Cells were considered adherent if they remained stationary for at least 30 s.

Immunohistochemical procedure

Animals injected with LPS or saline were deeply anesthetized with a mixture of ketamine and xylazine and perfused through the heart with ice-cold 10% formalin. Brains were removed and fixed in 10% formalin for 1 wk. Thick coronal sections (~2 mm) were taken at ~-1.0 to -3.0 mm from bregma. Formalin-fixed tissues were embedded in paraffin and then cut at 4 μm using a cryostat (CM3050; Leica). The infiltrated neutrophils were detected using dichloroacetate esterase staining (22). The whole brain section was used to count all infiltrated neutrophils. Neutrophils found in the ventricles or meninges were not counted.

Dual-radiolabeled Ab assay for P-selectin expression

P-selectin expression was quantified in the brains of saline- or LPS-injected mice as previously described (20). Briefly, a mixture of 10 μg of ^{125}I-labeled anti-P-selectin (RB40.34) and a dose of ^{131}I-labeled nonbinding Ab (A110-1, anti-keyhole limpet hemocyanin) calculated to achieve a total ^{131}I activity of $4–6 \times 10^5$ cpm were injected i.v. through the jugular vein. The Abs were allowed to circulate for 5 min. The mouse was then perfused with saline through the jugular vein to remove all circulating blood. Tissues were harvested, weighed, and measured for ^{125}I and ^{131}I activity. P-selectin expression was calculated by subtracting accumulated ^{131}I activity (labeled nonspecific Ab) from ^{125}I activity (labeled anti-P-selectin). Data are represented as the percentage of the injected dose of Ab per gram of tissue.

Intracellular staining

Mice were perfused through the heart with cold PBS, then brains were passed through a wire mesh and incubated with Golgi^{plug} in DMEM medium supplemented with 10% serum. After gradient centrifugation in 70% Percoll (Amersham Biosciences), mononuclear cell preparations were collected and pooled within the same group. Thereafter, cells were incubated on ice for 20 min with FITC-labeled anti-CD11b (M1/70) and PerCP-labeled anti-CD45 (30-F11) or their isotype controls. After washing, the cells were fixed and permeabilized with Cytofix/Cytoperm Plus kit (BD Biosciences). Cells were then stained with PE-labeled anti-TNF-α (XMG1.2) or isotype-control (R3-34) mAbs. All mAbs were obtained from BD Biosciences. Analysis of stained cells was performed with BD FAC-Scan Flow Cytometer (BD Biosciences).

Flow chamber assay

The parallel plate flow chamber assay was adapted from procedures previously described (23). Confluent monolayers of HUVEC were grown on coverslips and then incubated with LPS (0.1 μg/ml), LPS and minocycline (10 μM), or no treatment for 4 h before being mounted in the flow chamber. Blood was collected from healthy subjects into 30 U/ml heparin. Whole blood was perfused across the endothelium at a constant rate of 10 dyne/cm^2 for 5 min, followed by perfusion with HBSS. Interactions were visualized using phase contrast microscopy on an inverted microscope fitted with a video camera and recorded for later analysis. Rolling leukocytes were defined as those traveling slower than free-flowing cells. Adherent leukocytes were defined as those remaining stationary for at least 10 s.

TNF-α ELISA

Mice were killed 4 h after LPS injection. Brains were removed rapidly after decapitation. Tissues were weighed and homogenized in 500 μl of sterile PBS and then centrifuged at 12,000 rpm for 5 min at 4°C. Supernatants were assayed by commercial ELISA kit (BD Biosciences) according to the procedures supplied by the manufacturer.

Statistics

Data in graphs are shown as mean $\pm$ SEM. A one-tailed unpaired Student's *t* test was used for comparisons between samples by Sigmastat 3.0. Statistical differences were regarded as significant with a value of $p < 0.05$.

Results

Local LPS causes profound leukocyte recruitment into the brain

We used intravital microscopy to directly visualize the leukocyte-endothelial cell interaction in the cerebromicrovessels under control condition (Fig. 1A) and following i.c.v. LPS administration (Fig. 1B). Negligible numbers of both rolling leukocytes (Fig. 1C) and adherent leukocytes (Fig. 1D) were noted under control conditions. Four hours after LPS injection, the number of rolling and adherent cells increased >10-fold (Fig. 1, C and D). Entirely consistent with the intravital microscopy data, esterase staining revealed a significant level of neutrophil infiltration into the parenchyma in LPS-injected mice (Fig. 2B) whereas neutrophils were seldom seen in the brain of saline-injected mice (Fig. 2A). The results are quantified in Fig. 2C.

FIGURE 1. Intracerebroventricular LPS injection induces leukocyte rolling and adhesion in the brain postcapillary venules of wild-type mice. Intravital microscopy was performed on C57BL/6J mice 4 h after saline (*A*) or LPS (*B*) injection. The postcapillary venule boundaries were outlined by dotted lines in the image. Leukocyte rolling flux (*C*) and adhesion (*D*) are shown as the mean ± SE. ∗, $p < 0.05$ (vs saline control); $n = 4$ for all groups.
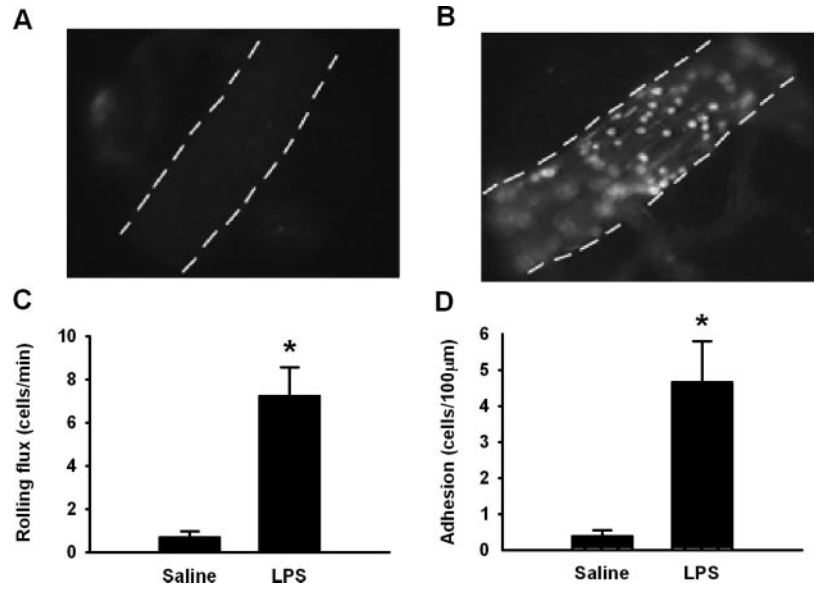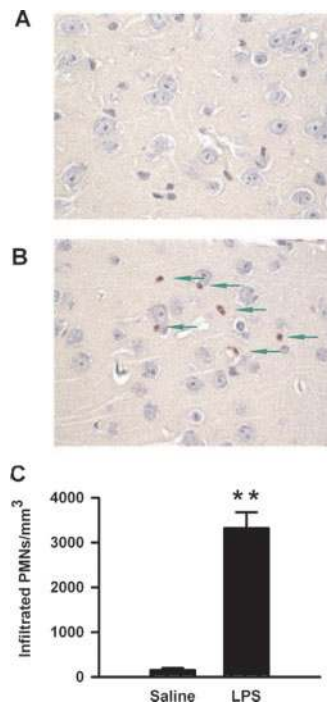
Intracerebroventricular LPS induced leukocyte recruitment via TLR4/CD14 signaling

Although TLR4 has been shown to be the only receptor for LPS on macrophage in vitro, whether a TLR4 mechanism also exists beyond the blood-brain barrier and which cell is the sentinel cell in the brain remains unclear. Moreover, there are CD14-dependent and -independent leukocyte responses to LPS in vivo (24). TLR4-deficient mice and CD14 knockout mice had almost no rolling or

adhesion response to LPS treatment (Fig. 3). Downstream of TLR4, there are MyD88-dependent and -independent mechanisms of macrophage activation. Surprisingly, Fig. 3*A* illustrates that significant leukocyte rolling was still evident in MyD88-deficient mice but was reduced (45%), indicating perhaps that expression of rolling molecules (selectins) use both MyD88-dependent and -independent pathway. By contrast, the adhesion appeared to be entirely MyD88 dependent (Fig. 3*B*).

Distinct leukocyte recruitment in the chimeric mice

To begin to narrow the candidate cells responsible for neutrophil rolling and adhesion, chimeric mice were made using bone marrow transplant between mutant and wild-type mice. Irradiated C57BL/10ScCr TLR4-deficient mice reconstituted with bone marrow cells from normal C57 BL/6J donors were termed C57→TLR4 mice and would have wild-type bone marrow-derived cells including


FIGURE 2. Intracerebroventricular LPS injection induces neutrophil infiltration in the brain parenchyma. Esterase staining of brain sections was performed from mice injected with either saline or LPS. *A*, No neutrophil infiltration was observed in saline-treated mice, while (*B*) brain sections from mice injected with LPS show infiltrating neutrophils (arrows pointing) in the parenchyma. *C*, The quantitative analysis of neutrophil infiltration, results are shown as mean ± SE; ∗∗, $p < 0.001$ (vs saline control); $n = 4$ for all groups.
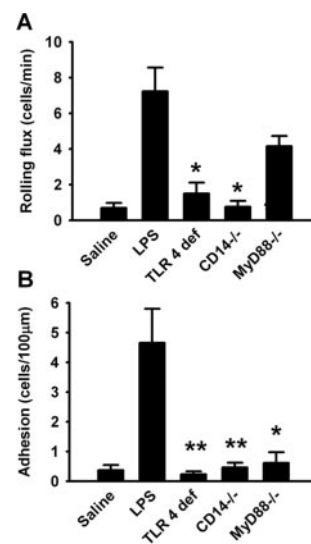
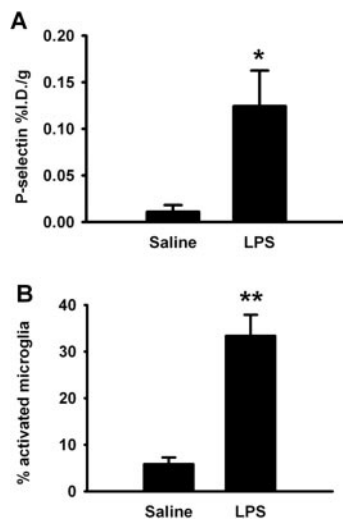FIGURE 3. Intracerebroventricular LPS injection induced leukocyte recruitment via TLR4/CD14 signaling. Control C57BL/6J, TLR4-deficient, CD14$^{-/-}$, and MyD88$^{-/-}$ mice were treated with LPS intraventricularly for 4 h before leukocyte rolling (*A*) and adhesion (*B*) was quantified by intravital microscopy. Results are shown as the mean ± SE. ∗, $p < 0.05$; ∗∗, $p < 0.001$ (vs C57BL/6J control); $n = 4$ for all groups.
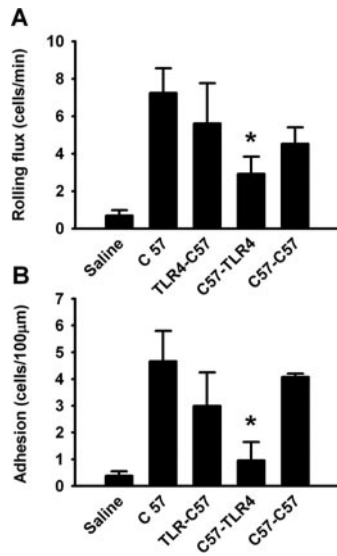
FIGURE 4. Distinct leukocyte recruitment was observed in chimeric mice. Chimeric mice were made by transferring bone marrow cells between TLR4-deficient and wild-type mice. Then C57BL/6J and chimeric mice were injected intraventricularly with LPS. Four hours later, leukocyte rolling (A) and adhesion (B) was quantified by intravital microscopy. C57→TLR4 mice displayed significantly reduced leukocyte recruitment. Results are shown as the mean ± SE. *, $p < 0.05$ (vs C57BL/6J mice); $n = 4$ for all groups.

macrophages, lymphocytes, and neutrophils but TLR4-negative parenchymal cells including microglia and endothelial cells. Although microglia originate from bone marrow early in life, they are insensitive to irradiation and turnover is very slow (25). Therefore,

FIGURE 5. Intracerebroventricular LPS injection induced both microglial and endothelial activation in vivo. A, P-selectin expression was observed on the brain endothelium induced by i.c.v. LPS injection. Mice were injected with LPS intraventricularly. Four hours later, P-selectin expression was quantified using a dual-radiolabeled Ab method. Significant P-selectin expression was observed in the LPS-treated mice. B, Intracerebroventricular LPS injection induced microglial activation in the brain. Mice were injected with LPS intraventricularly. Four hours later, mononuclear cells were first stained with FITC-labeled CD11b and PerCP-labeled CD45 followed by intracellular staining with PE-labeled anti-TNF-α mAb. Microglial populations were identified as CD45low and CD11b positive. The quantitative frequency of stained microglia of saline- and LPS-injected mice were then analyzed by flow cytometry. Results are shown as mean ± SE. *, $p < 0.05$; **, $p < 0.001$ (vs saline injected mice); $n = 4$ for all groups.
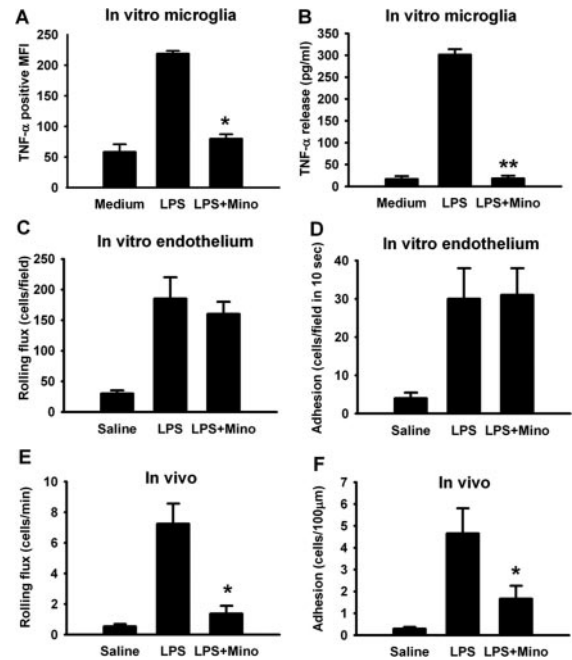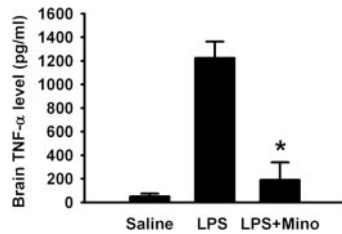

FIGURE 6. Minocycline inhibited the LPS-induced leukocyte recruitment but not direct activation of endothelium. Microglia were activated directly with LPS in vitro and minocycline was cocultured with microglia. A, Using flow cytometry, intracellular TNF-α was measured. B, ELISAs were performed to measure the TNF-α level in the supernatant. C and D, Using a flow chamber assay, we measured the effects of minocycline on endothelium activation induced directly by LPS. Minocycline could not directly inhibit the rolling (C) and adhesion (D) induced by direct endothelium activation. Using intravital microscopy, we showed that minocycline could significantly inhibit the leukocyte rolling (E) and adhesion (F) in the postcapillary venules. All the results are shown as the mean ± SE. *, $p < 0.05$; **, $p < 0.001$(vs C57BL/6J control); $n = 4$ for all groups.

this group of cells is not included when we refer to bone marrow-derived cells. C57→TLR4 mice displayed significantly reduced rolling and adhesion (Fig. 4). Clearly, activation of non-bone marrow-derived cells was required to cause leukocyte recruitment into the brain. Irradiated C57BL/6J mice receiving bone marrow from TLR4-deficient mice were termed TLR4→C57 mice. These chimeric mice with TLR4-positive microglia and endothelial cells, but TLR4-deficient circulating cells, displayed similar levels of rolling and adhesion as control chimeric mice. Clearly, residential cells in the CNS including microglia and/or endothelium were sufficient to cause the leukocyte recruitment. Also shown in Fig. 4, leukocyte recruitment responses in chimeric control mice made by transferring bone marrow cells between C57BL/6J mice were comparable to normal C57BL/6J mice, suggesting that bone marrow transplantation itself had no effect on the LPS-induced leukocyte recruitment.

LPS injection induced both endothelial and microglial activation

A key event in leukocyte recruitment is the ability of the endothelium to express selectins to allow for leukocyte rolling. To test whether endothelium in the CNS was activated after LPS injection, P-selectin expression in the brain was measured using a quantitative radiolabeled Ab assay. As shown in Fig. 5A, although very little P-selectin expression was noted in the brain microvasculature of saline-injected control mice (not different from P-selectin knockout mice) (20), a >10-fold increase in P-selectin expression was observed in the LPS-treated mice. Clearly, i.c.v. LPS injection induced strong endothelium activation in the brain.

FIGURE 7. Minocycline inhibited the TNF-α expression in the brain of LPS-injected mice. TNF-α concentration was measured in the brain extracts from either control or LPS-injected mice by ELISA. All the results are shown as the mean ± SEM. Statistical differences were indicated by ∗, $p < 0.05$ (vs LPS-injected group); $n = 4$ for all groups.

Activated microglia are potent secretors of proinflammatory cytokines such as TNF-α and IL-1β. To test whether LPS treatment activated microglia in vivo, we applied saline or i.c.v. LPS to animals, waited 4 h, and then gated on microglia (CD11b-positive CD45^{low} population) as previously described (26) and examined the TNF-α-expressing capability of microglia. Very few TNF-α-producing microglia were detected in saline-injected control mice, while 30–40% of microglia from LPS-treated animals were expressing elevated levels of TNF-α (Fig. 5B).

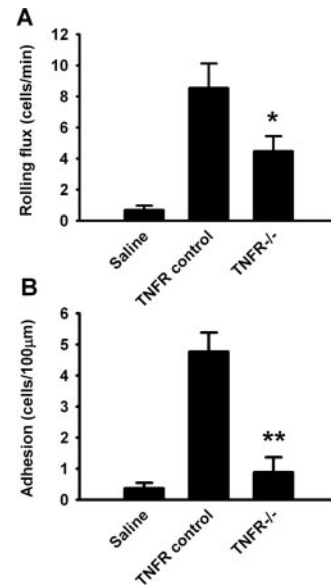*LPS-induced leukocyte recruitment is dependent on microglial activation*

Minocycline has been reported by many groups to be an effective inhibitor for microglial activation (27, 28). To first determine whether minocycline inhibits microglia and/or endothelial activation, either microglia (Fig. 6, A and B) or endothelium (Fig. 6, C and D) were directly stimulated with LPS. Minocycline completely blocked TNF-α synthesis (Fig. 6A) and release (Fig. 6B) from LPS-treated microglia. By contrast, minocycline did not reduce leukocyte rolling (Fig. 6C) or adhesion (Fig. 6D) in vitro when endothelium was directly stimulated with LPS. Fig. 6, E and F, demonstrates that minocycline treatment dramatically reduced leukocyte rolling and adhesion in vivo. In fact, the number of rolling and adherent leukocytes decreased by 80 and 70%, respectively, relative to LPS-injected mice that received no minocycline treatment.

TNF-α levels in the brain were also measured by ELISA in the mice with or without minocycline administration (Fig. 7). We observed a huge increase in TNF-α with LPS stimulation that was entirely inhibited by minocycline. This was most intriguing as the minocycline was given i.p. while the LPS was given i.c.v., sug-

Table I. *LPS i.c.v. injection activated multiple organs in the periphery*

P-Selectin Expression (% I.D./g)	Saline	LPS i.c.v.
Lung	0.16	5.13**
Heart	0.01	2.38*
Liver	0.10	9.08**
Pancreas	0.04	1.59**
Spleen	0.28	2.90**
Stomach	0.04	1.70***
Muscle	0.00	0.89**
Skin	0.02	1.05**
Brain	0.01	0.12*
Spinal cord	0.01	0.41*
Bone marrow	0.27	4.24***
Kidney	0.00	1.15**

^{a} Mice were treated with i.c.v. LPS for 4 h. Then P-selectin expression in the brain was measured using a quantitative radiolabeled Ab assay. Data are expressed as the mean ± SEM from four mice in each group. ∗, $p < 0.05$, ∗∗, $p < 0.01$, ∗∗∗, $p < 0.001$.

FIGURE 8. TNFR knockout mice revealed reduced leukocyte recruitment after LPS injection. Intravital microscopy was performed on either TNFR p55/p75 double knockout mice or control mice with a syngeneic background 4 h after LPS injection. Leukocyte rolling flux (A) and adhesion (B) were quantified by intravital microscopy. Results are shown as the mean ± SE. ∗, $p < 0.05$; ∗∗, $p < 0.001$(vs saline control); $n = 4$ for all groups.

gesting the minocycline rapidly crosses the blood-brain barrier. We also examined whether minocycline had an effect on macrophage activation in the periphery. Interestingly, peritoneal macrophages were activated in vivo following i.c.v. LPS. We noted that peritoneal macrophages were not able to synthesize TNF-α in the presence of minocycline (data not shown). It should be noted from this experiment that i.c.v. administration of LPS leads to extracerebral (i.p.) activation of macrophages clearly demonstrating that a systemic response does occur. Indeed, P-selectin expression was also increased in most tissues following i.c.v. injection of LPS (Table I).

LPS-induced leukocyte recruitment is dependent on TNFR signaling

To determine whether the increased levels of TNF-α in the brain induced leukocyte recruitment in response to LPS, we used TNFR p55/p75 double knockout mice. These mice have no response to TNF-α (29). A significant reduction in both neutrophil rolling and adhesion was noted in TNFR knockout mice (Fig. 8). Once again, a significant leukocyte rolling response was still seen; rolling was reduced by only 45% in TNFR knockout mice while leukocyte adhesion was again reduced by a substantially greater value (90%) compared with neutrophil recruitment observed in the control mice.

Discussion

Despite growing evidence of the potential importance of leukocyte recruitment in CNS inflammation as a result of both local and systemic infection, there are few studies that attempt to define how leukocyte recruitment can be initiated into the brain. It is well-appreciated that the initial step in leukocyte recruitment requires detection of bacteria or bacterial products which activate sentinel cells and/or endothelium. Sentinel cells including macrophage and mast cells can release proinflammatory factors which could also activate endothelium. The endothelium up-regulates adhesion molecules including selectins that allow leukocytes to roll and integrin ligands that permit firm adhesion. Once adhesion occurs the
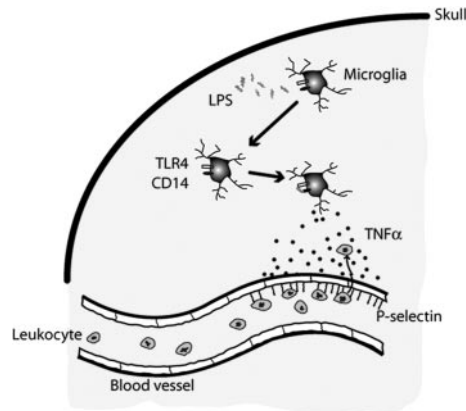
FIGURE 9. Microglia detected bacterial products and were rapidly activated and releasing TNF-α, which activates endothelium to express adhesion molecules and allows leukocytes to roll, adhere, and enter the brain parenchyma.

leukocytes can emigrate into the surrounding tissue. In this study, we report that microglia via TLR4 appear to be the dominant sentinel cells detecting bacterial products, releasing TNF-α which activates endothelium and allows leukocytes to roll, adhere, and emigrate.

(Fig. 9) Our data also suggest that minocycline, a well-recognized inhibitor of microglia can disrupt this process and dramatically attenuate the inflammatory response in CNS.

The CNS has both microglia and resident macrophages which includes perivascular macrophages, meningeal macrophages, choroids plexus macrophages that have all been reported to be activated by LPS in culture. Moreover, there is no specific inhibitor that would necessarily inhibit activation of one vs another cell type. However, it has clearly been shown that bone marrow transplant causes very significant replenishment of the macrophage population in the brain while the microglial population is not replenished significantly even after 12 mo (25, 30). Making use of this fact, we transplanted wild-type mice with TLR4-deficient bone marrow, so that all the macrophages and circulating leukocytes were no longer responsive to LPS. However, this procedure did not prevent the inflammatory response to LPS. By contrast, wild-type bone marrow transplanted into TLR4-deficient mice was not sufficient to recruit leukocytes following i.c.v. injection of LPS. In these mice, all of the parenchymal cells including microglia, astrocytes, oligodendrocytes, and neurons would retain responsiveness to LPS. However, Lehnardt et al. (31) reported that isolated microglia express TLR4 and bind LPS in vitro. Astrocytes, oligodendrocytes, and neurons do not appear to bind LPS or express detectable levels of TLR4. Moreover, it has been shown in culture that when LPS binds microglia, large amounts of proinflammatory molecules including TNF-α are released. In fact, sufficient amounts of proinflammatory molecules are released to cause oligodendrocytes and neuron bystander injury in culture (31). Finally, minocycline has been purported to inhibit microglial activation (27, 28) and in our study inhibited leukocyte recruitment into the brain. Therefore, taking together our data and that of the aforementioned studies, it is quite likely that the microglia are responsible for the leukocyte recruitment.

However, it should be noted that endothelium-lining blood vessels also express all of the machinery necessary to detect LPS, including TLR4 and CD14 (24, 32). Moreover, endothelial cultures can be activated by LPS to induce leukocyte rolling and adhesion without the requirement of any sentinel cells (macrophages, microglia, etc.). Indeed, we have reported that endothe-

lium was necessary to induce leukocyte recruitment into lungs in response to systemic LPS (17). In this study, local administration of LPS into the brain did lead to endothelial activation (P-selectin expression) leading to leukocyte rolling and adhesion. The endothelium is not replenished by bone marrow transplant so that the leukocyte recruitment occurred when both microglia and endothelium were TLR4 positive. Therefore, based on these observations, it is possible that LPS is directly stimulating the endothelium. There are two experiments that suggest that LPS is not directly activating the endothelium but rather activating microglia that release TNF-α to activate endothelium. First, the leukocyte recruitment was inhibited in TNF-α p55/p75 knockout mice and TNF-α was primarily made by the microglia. Second, minocycline inhibited TNF-α production in microglia in vitro, inhibited TNF-α release in vivo, and blocked leukocyte recruitment. However, minocycline was not able to inhibit LPS-induced activation of endothelium. Indeed, when endothelium was treated with minocycline there was absolutely no inhibition of the LPS response in vitro. Clearly, it is the inhibition of microglia rather than endothelium by minocycline that prevents the LPS responses.

Minocycline, a tetracycline derivative, has provided some remarkable neuroprotective results in animal models of human neurodegenerative diseases including Huntington's disease (33, 34), Parkinson's disease (35), and Alzheimer's disease (36). Minocycline also has protective effects against spinal cord injury (37), ischemia/reperfusion injury (15), brain trauma (38), and against white matter including oligodendrocyte injury induced by LPS in the developing neonatal brain (39). Its efficacy may rely on its surprisingly effective ability to penetrate the blood-brain barrier (40) and its potent anti-inflammatory properties related to microglial activation (41). Indeed, minocycline has been reported to block LPS-induced microglial release of cytokines, inducible NO synthase production, and oxidant production in vitro (27, 28). Our data provide further information on minocycline's modus operandi. First, minocycline does not have any direct antiadhesive properties for leukocytes inasmuch as LPS-induced leukocyte-endothelial interactions were not altered in vitro. Moreover, minocycline does not have inhibitory activity for all cell types, because endothelial activation was not affected nor was astrogliosis in another study (15). However, our data do suggest that the effects of minocycline may extend beyond the effects on microglia. Indeed, minocycline blocked peritoneal macrophage activation by LPS in our study. Because microglia are thought to be derived from a monocyte/macrophage lineage that homes to the CNS during development (30, 42), it is tempting to propose that minocycline has general inhibitory effects on macrophage lineage cells. This, however, does not confound our data as we have shown in chimeric mice that TLR4-positive macrophages were not sufficient to recruit leukocytes.

Proinflammatory cytokine expression is increased in the CNS after LPS treatment. Indeed, we observed a significant increase in TNF-α in the LPS-treated brain and it has been widely accepted that this cytokine avidly activates the endothelium to cause an increase in adhesion molecule expression and leukocyte recruitment. Indeed, we have previously reported that systemic administration of TNF-α causes significant leukocyte rolling and adhesion in the brain vasculature (43). We used the TNFR p55/p75 double knockout mice in the current study and observed a significant reduction but not a complete block of leukocyte rolling and adhesion in the brain microvasculature, consistent with the view that TNF-α activated the endothelium and induced subsequent leukocyte rolling and adhesion. The lack of complete block of leukocyte recruitment in TNFR p55/p75 double knockout mice suggests that TNF-α might not be the only signaling mediator for microglia

activating the endothelium in brain postcapillary venules. Indeed, microglia have been shown to respond to invading viruses, bacteria, or brain injury by synthesizing many cytokines including IL-1β as well as chemokines which could further contribute to recruitment of leukocytes from the circulation (12).

A very interesting observation in this study was the fact that CD14 and TLR4 deficiency completely blocked LPS-induced leukocyte rolling and adhesion, whereas the MyD88-deficient mice had no leukocyte adhesion but significant leukocyte rolling in response to LPS. It is now clear that downstream of TLR4 are two coadapters MyD88 and Toll/IL-1 receptor domain-containing adapter inducing IFN-β (TRIF) (44, 45). Although MyD88 appears to stimulate NF-κB-dependent TNF-α production, TRIF stimulates IFN regulatory factor and IFNs (46). However, TRIF is also able to stimulate NF-κB, however, this appears to occur in a delayed fashion (47). Our data would suggest that both an MyD88-dependent and an MyD88-independent pathway exist in microglia and that the MyD88-dependent pathway leads to TNF-α synthesis and activation of leukocyte adhesion. By contrast, the MyD88-independent pathway appears to contribute more to the leukocyte rolling. Interestingly, TNFR p55/p75 double knockout mice had almost no adhesion, but rolling was still apparent, suggesting that TNF-α and related adhesive mechanisms may be produced by the MyD88-dependent pathway whereas other mediators may be produced by the MyD88-independent pathway which also contribute to the synthesis of selectins and perhaps other molecules responsible for rolling.

In summary, it is clear that the parenchyma of the brain has a sentinel system comprising of microglia that detect bacterial products and can rapidly release cytokines that activate the endothelium to recruit leukocytes. Although it is extremely important to remove infectious agents, if the inflammatory response is too robust, bystander neuron injury can also occur and often this overexuberant inflammatory response may be more devastating than the infection per se. Our study also demonstrates that minocycline can significantly reduce the inflammatory response, raising the possibility that this drug could be used to modulate or dampen the inflammatory response if and when necessary.

Disclosures

The authors have no financial conflict of interest.

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