Neuroinflammation facilitates LIF entry into brain: role of TNF

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Pan W, Yu C, Hsuchou H, Zhang Y, Kastin AJ. Neuroinflammation facilitates LIF entry into brain: role of TNF. Am J Physiol Cell Physiol 294: C1436–C1442, 2008. First published April 2, 2008; doi:10.1152/ajpcell.00489.2007.—Leukemia inhibitory factor (LIF) is a proinflammatory cytokine mediating a variety of central nervous system (CNS) responses to inflammatory stimuli. During lipopolysaccharide (LPS)-induced inflammation, blood concentrations of LIF increase, correlating with lethality of sepsis. Circulating LIF crosses the blood-brain barrier (BBB) by a saturable transport system. Here we determine how this transport system is regulated in neuroinflammation. Using transport assays that quantify the influx rate and volume of distribution of LIF in mice, we show that LPS facilitated the permeation of LIF from the blood to the brain without compromising the paracellular permeability of the BBB as determined by coadministration of fluorescein. Concurrently, gp130 (shared by the interleukin-6 family of cytokines), but not gp190 (the specific receptor for LIF) or ciliary neutrophic factor (CNTF-Rx), a unique receptor for ciliary neurotrophic factor that also uses gp130 and gp190, showed increased levels of mRNA and protein expression in cerebral microvessels from the LPS-treated mice. The upregulation of gp130 by LPS was at least partially mediated by vascular tumor necrosis factor receptor (TNFR1) and TNFR2. This was shown by elevated TNFR1 and TNFR2 mRNA and protein in cerebral microvessels after LPS and by the absence of the LPS effect on gp130 in knockout mice lacking these receptors. The results show that neuroinflammation by LPS induces endothelial signaling and enhances cytokine transport across the BBB.

blood-brain barrier; leukemia inhibitory factor; lipopolysaccharide; tumor necrosis factor; nuclear factor-κB; neuroinflammation

Neuroinflammation is a syndrome present in many types of disorders affecting the central nervous system (CNS). A commonly used animal model for neuroinflammation involves induction by lipopolysaccharide (LPS), a prototypical endotoxin that promotes the induction of proinflammatory cytokines by acting on CD14 and toll-like receptor 4 (TLR4). Leukemia inhibitory factor (LIF) may be involved in the effects of LPS. This is shown by enhanced induction of tumor necrosis factor-α (TNF) and interleukin (IL)-6 in response to LPS in LIF knockout mice (35). After intravenous or intracerebroventricular injection, an anti-LIF antibody can reduce LPS-induced plasma adrenocorticotropic (ACTH) release. This suggests that LIF participates in the actions of LPS both in the periphery and in the CNS to induce neuroendocrine changes (34).

LIF and TNF also interact with each other in bacterial infection and sepsis (10). Reciprocal induction and inhibition of the cytokines can occur in a state-dependent manner. Mouse models indicate that exogenous LIF protects against lethal endotoxemia involving a major role for TNF (2, 32). Elevated blood concentrations of LIF, as seen in patients with meningococcemia or septic shock, correlate with higher mortality (31). The role of the CNS response in such actions is not yet clear.

LIF is a member of the IL6 family of cytokines that share the gp130 signal converter. LIF binds specifically to two type-I hematopoietic cytokine receptors, LIFR (gp190) and gp130. LIFR is the specific receptor for LIF and induces LIF signaling by the Janus kinase (JAK)/signal transducer and activator for transcription (Stat) pathway. The gp130 receptor is shared by members of the IL6 family of cytokines. Under basal conditions, gp130 displays a wide distribution in both neurons and glial cells inside the brain (33). Inflammation can further induce gp130 as shown by in situ hybridization. The pattern of gp130 induction differs from that of the IL6 receptor and shows a different response to LPS and IL1β. Specifically, gp130 mRNA expression is somewhat more restricted than that of IL6R and is increased in the organum vasculosum laminae terminalis (a circumventricular organ) and throughout the endothelia of the brain capillaries of LPS-treated rats, but it is not affected by IL1β treatment (30). The induced expression of gp130 mRNA in the microvascular endothelium after LPS stimulation suggests that the BBB may respond to circulating cytokines that bind to gp130.

The blood-brain barrier (BBB) allows selective permeation of TNF and LIF under normal and pathological conditions. The BBB consists mainly of specialized cerebral microvascular endothelial cells and constitutes a dynamic regulatory interface mediating the interactions of circulating cytokines with the CNS. The receptor-mediated transport system for LIF facilitates its restricted permeation from blood to brain and spinal cord in normal mice (23). Spinal cord injury causes upregulation of blood-spinal cord barrier uptake of LIF, so that the transport system functions at a higher level (18). The specific patterns and regulatory changes are also seen in studies of TNF transport (16, 17, 20–22, 24). LPS has been shown to induce regulatory changes of other transport systems at the BBB level, including increased insulin influx (36) but decreased leptin influx (14). The results suggest that the BBB responds to LPS challenge by tight control of energy balance and cytokine signals.

Based on the evidence of receptor-mediated transport of LIF, upregulation of microvascular gp130 mRNA in the brain by LPS, and the regulatory changes of the BBB transporters specified above, we therefore formulated the hypothesis that LIF transport across the BBB is subject to regulation by LPS. As the effects of LPS may be mediated directly by activation of TLR4 or by stimulation of macrophage production of TNF and other proinflammatory cytokines, we also determined whether TNF plays a mediatory role. This was tested by...
analysis of BBB transport function in relation to the level of expression of cytokine receptors after LPS treatment and by comparison with BBB transport in knockout mice deficient in TNF receptors or the ligand TNF itself.

MATERIALS AND METHODS

Homozygous TNF knockout and TNF double receptor (p55 and p75) knockout mice were studied along with their strain control B6.129SF2/J. All of these were purchased from the Jackson Laboratory (Bar Harbor, ME). CD1/ICR outbred mice were purchased from Charles River Laboratories (Wilmington, MA). These male mice were studied at age 6–8 wk, and we followed protocols approved by the Institutional Animal Care and Use Committee. Recombinant mouse LIF was purchased from Chemicon (Temecula, CA), labeled with $^{125}$I (Perkin-Elmer, Waltham, MA) by the iodogen method, and purified by elution on columns of Sephadex G-10, resulting in a specific activity of about 70 Ci/g, as described previously (18). The antibodies for LIFR, gp130, TNF receptor (TNFR)1, and TNFR2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The reagents for real-time PCR were purchased from Applied Biosystems (Foster City, CA). Unless specified, other chemicals were obtained from Sigma (St. Louis, MO).

LPS treatment. For mouse studies, LPS (5 mg/kg body wt, from Escherichia coli, catalog no. L6511, Sigma) was dissolved in pyrogen-free sterile normal saline (NS) at 5 mg/ml, and delivered to mice by intraperitoneal injection. The dosing was adopted from reports in the literature (6, 28). The control group was injected with pyrogen-free NS in the same volume. A sample size of 8–10 mice/group was used for transport assays, and 4–6 mice/group were used for either RNA or protein measurement. The mice were observed at least twice by intraperitoneal injection. The dosing was adopted from reports in the literature (6, 28). The control group was injected with pyrogen-free NS in the same volume. A sample size of 8–10 mice/group was used for transport assays, and 4–6 mice/group were used for either RNA or protein measurement. The mice were observed at least twice daily for food intake, weight loss, and general malaise, and care was taken to avoid hypothermia. At 48 h after the single injection, mice were anesthetized for terminal studies (transport assay or tissue collection).

Multiple-time regression analysis. Groups of mice were anesthetized with ethyl carbamate (40% aqueous solution) ip. $^{125}$I-labeled LIF (30,000 cpm/μl) and sodium fluorescein (10% solution) were dissolved in lactated Ringer solution containing 1% bovine serum albumin. A bolus of 100 μl of the mixed tracers was injected into the exposed left jugular vein at time 0. At 1–20 min (different mice for each time point), blood was collected from the right common carotid artery, and the mouse was decapitated immediately afterward. The brain, excluding the pituitary, pineal gland, and hypotalamus, was collected and weighed. The radioactivity of $^{125}$I-LIF in 50 μl of serum and brain was measured in a gamma counter (Wallac, Gaithersburg, MD). The end point was based on our previous results showing that most of the radioactivity in the brain represents intact $^{125}$I-LIF at 20 min rather than degradation products (23). The brain-to-serum ratio of radioactivity was plotted against time of the circulation. The influx rate (slope of the linear regression) and the initial volume of distribution in the brain (the y-intercept of the regression line).

After measurement of the radioactivity, the brain and serum samples were dissolved in 1 and 0.2 ml of Solvable (Perkin-Elmer, Boston, MA), respectively, and digested for 40 h at 50°C in the dark. The relative fluorescent intensity of 200 μl of the solution was measured in a fluorescent microplate reader (Spectramax Gemini, Molecular Devices, Sunnyvale, CA), with Solvable as the blank control. The excitation wavelength was 485 nm, the emission wavelength was 538 nm, and the cutoff was 530 nm. The brain-to-serum ratio of fluorescence, corrected by brain weight for volume variation during digestion, was plotted against time of the circulation. After measurement of the radioactivity, the brain and serum samples were dissolved in 1 and 0.2 ml of Solvable (Perkin-Elmer, Boston, MA), respectively, and digested for 40 h at 50°C in the dark. The relative fluorescent intensity of 200 μl of the solution was measured in a fluorescent microplate reader (Spectramax Gemini, Molecular Devices, Sunnyvale, CA), with Solvable as the blank control. The excitation wavelength was 485 nm, the emission wavelength was 538 nm, and the cutoff was 530 nm. The brain-to-serum ratio of fluorescence, corrected by brain weight for volume variation during digestion, was plotted against time of the circulation. The difference between the LPS-treated and control group was compared with Prism GraphPad statistical software as mentioned above.

RNA extraction and real-time PCR. Microvessel-enriched fractions from the cerebral cortices of the mice were obtained by the capillary depletion method as described previously (19). Total RNA was extracted from the microvessels with an Absolutely RNA RT-PCR kit (Stratagene, La Jolla, CA). Reverse transcription and real-time PCR were performed with the one-step core PCR kit from Applied Biosystems (Foster City, CA). The primers and fluorescent probes for LIFR (gp190), the LIF coreceptor gp130, TNFR1 (p55), TNFR2 (p75), and the housekeeping gene GAPDH are listed in Table 1. Standard curves for the target genes were generated. The levels of expression of cytokine receptors were normalized to that of GAPDH, and group means were compared by analysis of variance (ANOVA).

Western blot analysis. Microvessel-enriched fractions obtained by capillary depletion were lysed at 4°C in a modified radioimmunoprecipitation (RIPA) buffer in the presence of a complete protease inhibitor cocktail, as described previously (39). Protein concentration in the supernatant was determined by bicinchoninic acid assay (Pierce, Rockford, IL). Forty micrograms of protein were electrophoresed, transferred to nitrocellulose membranes, and probed with primary antibodies against LIFR, gp130, receptor for ciliary neurotrophic factor (CNTF)-Ro, TNFR1, TNFR2, and the housekeeping gene β-actin (Santa Cruz Biotechnology), followed by horseradish peroxidase-conjugated secondary antibody. The signal was developed with an enhanced chemiluminescence kit (Pierce). The signal intensity was quantified with National Institutes of Health Image J software and normalized to that of β-actin in the same samples. Group means are presented with their standard errors in the bar graphs, and significant changes were evaluated by ANOVA with SPSS software.

RESULTS

LPS increases BBB permeability to LIF without affecting permeability to fluorescein. Two groups of CD1 mice were studied. $^{125}$I-LIF and sodium fluorescein were delivered by intravenous bolus injection at time 0, and mice were studied

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<th>Table 1. Real-time PCR primers and probes</th>
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<td>Primers (5′ → 3′)</td>
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LIFR, leukemia inhibitory factor receptor; TNFR, tumor necrosis factor receptor; 6-FAM, 6-carboxy-fluorescein.
1–20 min later, each mouse representing one time point. The brain-to-serum ratios of 125I-LIF and sodium fluorescein over-exposure time were used to calculate the influx rate and volume of distribution in the brain. The control mice injected intraperitoneally with NS 48 h earlier showed an influx rate of 0.26 ± 0.05 μL·g⁻¹·min⁻¹ and an initial volume of distribution 15.15 ± 0.83 μL/g (n = 10 mice/group). The LPS-treated mice (5 mg/kg) had an influx rate of 0.69 ± 0.07 μL·g⁻¹·min⁻¹ and initial volume of distribution 17.98 ± 1.09 μL/g in the brain 48 h later (n = 8 mice/group). The difference between these influx rates was significant (P < 0.001) (Fig. 1A). By contrast, there was no significant influx of sodium fluorescein from the blood to the brain in either group, despite a relatively high volume of distribution (Fig. 1B). This indicates that fluorescein is retained in the cerebral vasculature without crossing the BBB to reach brain parenchyma, and that LPS treatment did not cause a substantial disruption of the BBB at this time to increase the paracellular permeation of fluorescein.

**LIPS increases gp130 expression of both mRNA and protein to a greater extent than LIFR expression.** Groups of mice (n = 6–7 mice/group) treated with LPS or vehicle were used to obtain cerebral microvessels for mRNA and protein analyses. In microvessels from mice treated with LPS 48 h earlier, there was a significant increase in the level of mRNA for gp130 (P < 0.05). The increase was not significant for LIFR (Fig. 2A). The increase in gp130 mRNA expression was paralleled by elevated protein signals shown by Western blot analysis (P < 0.01) (Figs. 2, B and C). Thus LPS caused transcriptional activation of gp130.

LIF, CNTF, and cardiotoxin-1 are closely related members of the neuropoietic cytokine family. They share the receptors LIFR and gp130 to induce receptor phosphorylation by associated JAK tyrosine kinases and STAT3 activation. CNTF requires an additional subunit (CNTF-Rx) for receptor binding and activation (5). To determine whether LPS induces specific changes of LIF receptors, we also measured the level of expression of CNTF-Rx. There was no significant difference of signal intensity of CNTF-Rx in the cerebral microvessels obtained after NS or LPS treatment (n = 5/group) (Fig. 2D).

**LPS induces a concurrent increase of the receptors for TNF (TNFRI and TNFRII).** The same samples used for LIF receptor mRNA and protein analyses described above were used for these analyses. In enriched microvessels of LPS-treated mice, the mRNA for both TNFRI and TNFRII was significantly increased (Fig. 3A). Western blot analysis showed that the proteins of both TNF receptors were also increased after LPS treatment (Fig. 3B). Densitometry analysis of the signals indicated that the increase was significant (P < 0.05) (Fig. 3C).

**Lack of increase of gp130 in TNF receptor knockout mice.** The significant (P < 0.001) weight loss occurring 48 h after injection of LPS in the TNF ligand knockout mice and TNF receptor knockout mice was similar to that seen in the control strain, indicating that all groups of mice mounted an inflammatory response (Fig. 4A). TNF knockout mice and TNF receptor knockout mice did not respond to LPS treatment with a change in LIFR mRNA (Fig. 4B). Although this is similar to that seen in the control strain shown above, the pattern of gp130 mRNA was strikingly different. The significant upregulation by LPS of gp130 mRNA in cerebral microvessels seen in the control strain was not present in TNF ligand knockout and TNF receptor knockout mice (Fig. 4C). The level of protein expression showed similar changes as observed for mRNA. LPS caused a significant increase of the gp130 signal in B6 mice but not in the TNF (TKO) or TNF receptor (RKO) knockout mice (Fig. 4, D and E).

**Lack of change in basal LIF influx in mice without TNF or its receptors.** Since the above results indicated the involvement of TNF and/or its receptors in the regulatory changes of gp130 expression after LPS treatment, we further determined whether TNF affects the basal entry of LIF across the BBB in naïve mice. Groups of TNF ligand and receptor knockout mice were studied with the appropriate strain background control (B6.129SF2/J). Multiple-time regression analysis was performed at different time intervals between 1 and 20 min after intravenous injection of 125I-LIF. The control mice had an influx rate of 0.28 ± 0.07 μL·g⁻¹·min⁻¹ (n = 12), which is comparable to the value obtained in the LPS study shown in Fig. 1A. The double-receptor knockout mice had an influx rate of 0.15 ± 0.06 μL·g⁻¹·min⁻¹ (n = 8), and the TNF ligand
knockout mice had an influx rate of $0.31 \pm 0.05 \mu l \cdot g^{-1} \cdot min^{-1}$ ($n = 9$). The difference among the groups was not significant ($P > 0.05$) (Fig. 5).

DISCUSSION

We chose to study LIF transport after LPS injection based on compelling evidence from the literature, indicating that LIF is a marker for the severity of septic shock and that LIF plays a pivotal role in CNS inflammation and autoimmune diseases. In the spinal cord, LIF is a potent proinflammatory cytokine, as...
shown by overexpression (12) and knockout studies in a seizure model (9). LIF promotes oligodendrocyte survival after spinal cord injury (13) and limits demyelination and oligodendrocyte loss in mouse models of experimental autoimmune encephalomyelitis (3, 4). Because inflammation and autoimmunity can also be neuroprotective (8, 27), the fine balance necessary to achieve an adequate inflammatory response in the CNS without exacerbation of inflammation and cell death may be readily achieved by regulatory changes of BBB transport.

LPS binds to the CD14/TLR4/MD2 receptor complex, induces transcriptional activation, and eventually stimulates production of proinflammatory cytokines. The proinflammatory cytokine LIF appears to have a beneficiary role in counteracting LPS-induced endotoxic shock (35), suggesting its potential to provide negative feedback in exaggerated host defense. At the BBB level, we show here that LPS can cause a significant increase in the permeability of LIF from the blood to brain. Thus the CNS responds to LPS challenge by increasing the availability of blood-borne LIF, which has a relatively low basal permeation in normal mice (23).

Partial disruption of the BBB leads to increased paracellular permeability to tracers like albumin; this can be influenced by the dose of LPS and time after its administration. The inflammatory response to LPS involves a syndrome of sickness behavior, including changes in body temperature, weight, locomotor activity, exploration, and food intake, and it is accom-

Fig. 4. Differential microvascular responses to LPS treatment in the induction of LIF receptors in the TNF ligand knockout (TKO, n = 5 mice/group), TNF receptor double knockout (RKO, n = 4 mice /group), and background strain control mice (n = 7 mice /group). All three types of mice showed significant weight reduction in response to LPS (A). The mRNA for LIFR showed no significant changes (B). The mRNA for gp130 was increased significantly only in the B6 mice but not in the TKO or RKO mice (C). Concurrently, there was an increased signal of gp130 in the B6 mice only, shown by Western blot analysis (D). The significant increase was also shown by densitometric analysis (E). *P < 0.05; **P < 0.01; ***P < 0.005.
panned by elevated inflammatory cells, cytokines, neuroendocrine changes, and microgliosis. An LPS-induced increase of BBB permeability to radioactively labeled albumin has been observed after three consecutive doses of LPS (3 mg/kg ip in lactated Ringer solution), 8 h apart, before examination of CD1 mice at 24 h. However, a single injection of 3 mg/kg did not induce an increase of albumin uptake 8 h later (15). The decreased BBB permeation of putitary adenylate cyclase activating peptide (15) and leptin (14) after LPS, but increased BBB permeation of insulin (36), also supports the specificity of the regulatory changes.

In our study a fluorescent tracer, which is much smaller than albumin, was used to detect any subtle alterations of the BBB. Fluorescein is a molecule of only 376 Da that serves as a sensitive marker for paracellular permeability. It is also a substrate for the efflux transporters multidrug resistance protein (MRP)-2 and organic anion transporter (OAT)-3 (29). In rats with diabetes mellitus induced by streptozotocin, the permeation of fluorescein is decreased after 4 min of in situ brain perfusion, probably related to activation of efflux transporters (7). Although NF-κB mediates upregulation of multidrug resistance protein 1 (mdr1) by TNF (37, 38) and LPS (Yu et al., unpublished observations), LPS did not increase fluorescein influx in our study. Thus LIF enters the brain in mice treated with LPS by specific upregulation of its transport system at the BBB level.

To further determine the mechanisms of upregulation of LIF transport by LPS, we measured the level of expression of LIF receptors in enriched cerebral microvessels. LPS mainly increased the expression of gp130, both at the mRNA and protein levels. This indicates a transcriptional activation. The findings contrast with the lack of significant increase of LIFR (gp190) in the same samples. Thus LPS differentially affected the expression of the two receptors for LIF. Since CNTF uses an additional receptor subunit CNTF-Rα in addition to LIFR and gp130, we also determined the level of CNTF-Rα protein expression. There was no significant change in cerebral microvascular CNTF-Rα, shown by Western blot analysis, after LPS treatment. This further indicates the selectivity of gp130 upregulation.

The correlation of elevated gp130 and LIF transport suggests that gp130 in cerebral microvessels mediates the increased transport of LIF across the BBB. This in vivo observation differs from our in vitro results in RBE4 cerebral microvessel endothelial cells, in which binding and endocytosis of LIF were reduced after TNF treatment accompanied by decreased LIFR and increased gp130 (40, 41). Since the cell studies involved stimulation with TNF, rather than LPS, and a shorter duration of treatment than received by the mice, the discrepancy probably reflects the complexity of dynamic changes in vivo. This prompted us to examine the role of the TNF system in LPS-induced upregulation of LIF transport.

In cerebral microvessels from CD1 mice, LPS induced both TNFR1 and TNFR2 mRNA and protein 48 h after treatment. To determine whether the TNF system plays a role in mediating LPS-enhanced LIF transport, we used knockout mice lacking either functional TNF receptors or TNF itself and compared the induction of LIF receptors with the respective strain background control (B6.129S2/J). Double TNF receptor knockout mice have elevated serum concentrations of TNF and increased resistance to LPS-induced lethality, an effect mainly conferred by the p55 receptor (TNFR1) (26). The p75 receptor TNFR2 plays an important role in vasculopathy, as human transmembrane TNF/p75TNFR transgenic mice show prominent cerebral endothelial activation, meningeal inflammation, and vessel fibrosis with vasculitis eventually leading to vessel occlusion and multifocal infarct (1). TNF knockout mice show a deficiency in humoral immune responses, with an absence of splenic primary B cell follicles and inadequate formation of follicular dendritic cell networks and germinal centers. These mice are resistant to the systemic toxicity of LPS at the usual doses (25). Our LPS treatment regimen was effective in the TNF ligand and TNF receptor knockout mice as well as in the control strain, shown by reduction of body weight and selective induction of cytokine receptors. However, the significant increase of gp130 mRNA and protein expression was no longer present when the knockout mice were treated with LPS. This indicates a critical role of TNF in mediating the increased transport of LIF after LPS treatment. The opposing changes of LIF transport in the wild-type mice treated with LPS and RBE4 cells treated with TNF, therefore, may suggest that the regulation of LIF in LPS-treated mice also involves secondary mediators in addition to TNF.

It was further shown, however, that the TNF system did not play a significant role in the basal permeation of LIF across the BBB. By comparison with the control strain, the influx rate of blood-borne 125I-LIF was not significantly different in mice lacking TNF or both of its receptors. It is possible, of course, that compensatory mechanisms occurred in these knockout mice. Since we used homozygote breeding rather than littermate controls, caution also needs to be taken in interpretation of the results. Nonetheless, TNF pretreatment in wild-type mice also failed to affect LIF permeation across the BBB 6 h later (unpublished observations).

In summary, LPS caused a significant increase of basal LIF transport across the BBB in mice 48 h after its administration. This upregulation was mainly mediated by gp130 and modified by TNF. It was absent in TNF ligand knockout mice and in TNF receptor knockout mice. The effects of LPS on gp130 were at least partially mediated by its subsequent induction of TNF. From the known roles of LIFR and gp130 in the transport...
of LIF across the mouse BBB, we conclude that neuroinflammation specifically affects the availability of blood-borne cytokines to the CNS.

GRANTS

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REFERENCES


