

The Role of Cerebral Vascular NF κ B in LPS-induced Inflammation: Differential Regulation of Efflux Transporter and Transporting Cytokine Receptors

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Key Words

Cerebral microvessels • Blood-brain barrier • Neuroinflammation • Lipopolysaccharide • p-glycoprotein • Cytokine receptor • IL15 • TNF

Abstract

Background/aims: The transcription factor NF κ B is a major mediator of lipopolysaccharide (LPS) signaling. We determined the role of NF κ B activation in regulatory changes of the P-glycoprotein (Pgp) drug efflux transporter at the blood-brain barrier (BBB) and proinflammatory cytokine receptors. **Methods:** We treated NF κ B knockout and wildtype mice with LPS or vehicle, obtained enriched cerebral microvessels, and determined target mRNA by qPCR for MDR1a/b, IL15R α , IL2 R α , IL2R γ , LIFR, gp130, and TNFR1/2, and protein expression by western blotting for P-gp, IL15R α , IL2R γ , LIFR, and gp130. **Results:** The effects of LPS on the transporters and cytokine receptors showed differences between wildtype and NF κ B knockout mice, and between mRNA and protein changes. NF κ B not only mediated the LPS-induced increase of MDR1b, IL2R γ , and TNFR2 mRNA in the wildtype mice, but it showed opposite effects by elevating IL15R α and TNFR1 mRNA and decreasing IL2R α in the knockout mice. Although basal

vinblastine uptake was unchanged in the NF κ B knockout mice, LPS induced an increase of the uptake (depressed efflux transport) greater than that seen in the wildtype mice, indicating that NF κ B helps to maintain Pgp efflux transporter function. **Conclusion:** The results show differential involvement of NF κ B signaling in response to LPS at the BBB.

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Introduction

The multidrug resistance (MDR) gene encodes a class of proteins that belong to the ATP-binding cassette (ABC) transporter family. At the level of the blood-brain barrier (BBB), the MDR1b product P-glycoprotein (Pgp) is distributed on the apical membrane of cerebral endothelia, the main constituent of the BBB. Pgp hinders the blood-to-CNS influx of a variety of substrates, including several classes of agents used in the treatment of infection, neurodegeneration, epilepsy, and brain tumors [1]. In cultured RBE4 cerebral endothelial cells treated with tumor necrosis factor α (TNF), we have shown that the MDR1b gene promoter is exclusively regulated by transcriptional activation and nuclear translocation of nuclear factor (NF)- κ B [2, 3]. If this is a universal phe-

nomenon during inflammation *in-vivo*, the targeting of NF κ B activation would have therapeutic potential by increasing central nervous system (CNS) drug delivery. In this study, we determined the regulatory changes of MDR1b in response to lipopolysaccharide (LPS), an endotoxin produced by Gram negative bacteria and commonly used as a proinflammatory stimulus in animal models. The effect of NF κ B was tested by use of knockout (KO) mice deficient in the p50 subunit of NF κ B that is essential for the activation of the NF κ B complex [4, 5].

Besides the drug efflux transporters, neuroinflammation also modulates the level of cytokine receptor expression at the BBB level. This in turn affects the permeation of specific cytokines across the BBB by specific transport systems, or their signaling in endothelia with modulation of CNS function by release of secondary mediators. LPS has been shown to regulate the transport of TNF [6], leukemia inhibitory factor (LIF) [5], and interleukin (IL)-15 [7]. TNF, which can be induced by LPS, shows differential upregulation of the two receptors for LIF, with decreased LIFR (gp190) [8] and increased gp130 in cerebral endothelial cells [9]. TNF and LIF cross the BBB by their respective receptor-mediated transport systems [10-13]. IL15 permeation across the normal BBB is low but it can be activated by LPS [14]. Thus, expression of the probable transporting receptors for these cytokines for blood-to-brain influx will provide a sharp contrast to the efflux transport by Pgp. The differential expression of cytokine receptors in response to LPS, as well as the efflux drug transporters, represents a complex regulatory network at the level of the BBB. Therefore, the involvement of NF κ B signaling in the expression of cytokine receptors was studied in parallel.

Materials and Methods

The studies followed a protocol approved by the Institutional Animal Care and Use Committee. Homozygous p50 NF κ B knockout mice (B6.129P2-Nf κ B1^{tm1Bal}/J, 002849, abbreviated as PKO in this study) [4] were used with their control B6.129PF2/J mice (stock number 100903). All were purchased from the Jackson Laboratory (Bar Harbor, ME), and maintained in group housing (3 - 4 mice/cage) with food and water *ad lib* under a 12 h light-dark cycle at 23 °C, 7:00 - 19:00 being the light period. Food intake, activity level, and body temperature were monitored throughout the study. At the time of tissue harvesting, the mice receiving LPS were in the recovery phase as shown by all of the parameters.

To determine the regulatory changes of MDR1 and cytokine receptors at the mRNA level, four groups of young

adult male mice were studied (n = 6/group). They are wildtype or PKO mice receiving LPS or saline vehicle. LPS (2.5 mg/kg body weight, from *Salmonella enterica* serotype typhimurium, purified by phenol extraction; catalogue no L6511, Sigma, was dissolved in pyrogen-free normal saline (NS) at 5 mg/ml, and delivered to mice by intraperitoneal (ip) injection. The dosing was adapted from various reports in the literature [15-18] and controls were injected with NS in the same volume. 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 with ketamine/xylazine ip and decapitated. Cerebral cortical tissue was obtained for the capillary depletion procedure to obtain enriched microvessels as described previously [5, 19]. The effectiveness of enrichment has been verified by γ -glutamyl transpeptidase activity that showed more than 40-fold increase [20].

Total RNA was extracted from the enriched microvessels with an Absolutely RNA RT-PCR kit (Stratagene, La Jolla, CA), reversely transcribed, and subjected to qPCR by use of gene-specific primers and fluorescent probes (Table 1) on an Applied Biosystems (ABI 7900) analyzer (Foster City, CA). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified for each sample to serve as an internal control. Standard curves for the target genes were generated. The levels of expression of target genes were normalized to that of GAPDH. The effects of strain (wildtype vs KO) and treatment (NS vs LPS) were determined by two-way analysis of variance (ANOVA), followed by the Bonferroni test.

To determine the effect of LPS treatment and NF κ B deletion on the level of Pgp and cytokine receptor protein expression, a separate set of four groups of mice were used (n = 3/group). One sample each in the LPS-treated wildtype and PKO mice was unfortunately lost during processing. The enriched microvessels were obtained as described above, and lysed in protein lysis buffer [radioimmunoprecipitation assay (RIPA) buffer] containing complete protease inhibitor cocktail. After homogenization, sonication, and ultracentrifuge clearance, the protein lysate was collected. Protein concentration was determined by the bicinchoninic acid (BCA) assay. Forty μ g of protein was loaded onto sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). The electrophoresed proteins were transferred to nitrocellulose membranes and probed with antibodies against Pgp, IL15R α , IL2R α , IL2R γ , TNFR1, TNFR2, LIFR, and gp130. After thorough wash, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody and enhanced chemiluminescence reagent, and the signals were developed on X-ray film. The differences between groups was determined by the NIH Image J program for densitometric analysis, and expressed as the signal/ β -actin ratio. There was a single lane defect in the last PKO mouse after NS treatment and the average β -actin signal of the other two mice was used as the substitute value of β -actin for this lane in the final analysis.

For efflux drug transport assays, two groups of mice received ip injection of LPS or NS 48 h earlier (n = 3/group). The mouse was anesthetized, and the chest cavity was exposed to

ligate the descending aorta for perfusion of ^3H -vinblastine through the left cardiac ventricle after lacerations were made in the right atrium and bilateral jugular veins. After 1 min perfusion, the mouse was decapitated to obtain the brain. Solvable was used to dissolve the weighed brain tissue at 55 °C which was further processed by incubation with hydrogen peroxide and Formula 989 scintillation cocktail as previously described [21]. After measurement of the radioactivity of the brain sample and perfusate in a Beckman β -counter, the brain/perfusate ratio of ^3H -vinblastine in each group was determined.

Two-way ANOVA was performed to determine the effects of treatment (LPS vs NS) and strain (PKO vs wildtype) on the level of expression of the efflux transporter and cytokine receptors. One-way ANOVA was performed to determine the difference of western blotting signals and ^3H -vinblastine uptake among the four groups, followed by Tukey's post-hoc test.

Results

Effects of LPS treatment and NF κ B KO on mRNA expression of MDR1 and cytokine receptors

The mRNA for MDR1a differed significantly between the wildtype and PKO mice ($p < 0.005$). However, there was a significant interaction between strain and treatment ($p < 0.05$). Post-hoc analysis showed that LPS induced a significant increase of MDR1a in the wildtype mice ($p < 0.05$) but not in the PKO mice (Fig. 1A). Significant effects of strain and treatment were seen for the mRNA for MDR1b. There was also a significant interaction between the two variables of strain and treatment ($p < 0.0001$). Post-hoc analysis showed a significant effect of LPS on increasing MDR1b in the wildtype mice ($p < 0.001$), but not in the PKO mice (Fig. 1B).

For IL15 α mRNA, there was a significant effect of LPS ($p < 0.001$) and an interaction between treatment and strain ($p < 0.005$). Only the PKO mice showed a significant increase in response to LPS ($p < 0.001$, Fig. 2A). This suggests that NF κ B activity provides tonic inhibition preventing upregulation of IL15 α mRNA. By contrast, there were significant effects of strain ($p < 0.05$) and treatment ($p < 0.05$) on the cognate IL2 α mRNA, and there was an absence of interactions of these two variables. LPS caused a significant reduction of IL2 α mRNA in the PKO mice, but not in the wildtype mice (Fig. 2B). Thus, mice without an active NF κ B complex failed to maintain IL2 α levels after LPS challenge. Since IL15 α and IL2 α mRNA showed opposite changes, we further determined the level of the co-receptor IL2 γ . There were significant effects of strain and treatment ($p < 0.001$), and there were significant interactions between the two variables ($p < 0.001$). Only the wildtype

Gene	Sequences
MDR1a	Forward primer: 5'-ACC CCC GAG ATT GAC AGC TA Reverse primer: 5'-AAT TGC ACA TTT CCT TCC AAC A Probe: 5'-6-FAM-AGC ACG CAA GGC CTA AAG CCG AA -TAMRA
MDR1b	Forward primer: 5'-GGA GCA GAA GTT TGA AAC CAT GT Reverse primer: 5'-GTG TGC TTT CTT CAT CGC ATT T Probe: 5'-6-FAM-TGC CCA GAG CTT GCA GGT ACC ATA CA -TAMRA
IL15 α	Forward primer: 5'-CCA GGC CAT TCC TGT GTT G Reverse primer: 5'-GAT GTC AGC ATG CTC AAT AGA TAC G Probe: 5'-6-FAM-CCG GGC ACC ACG TGT CCA CC-TAMRA
IL2 α	Forward primer: 5'-AGT GAG ACT TCC TGC CCC ATA A Reverse primer: 5'-ATG TCT CCG TCA TTG CAG TTG T Probe: 5'-6-FAM-CAC CAC AGA CTT CCC ACA ACC CAC AG -TAMRA
IL2 γ	Forward primer: 5'-GAA GCT GGA CCG AAC TAA TAG TGA A Reverse primer: 5'-CTC CGA ACC CGA AAT GTG TAC Probe: 5'-6-FAM-GAA CCT AGA TTC TCC CTG CCT AGT GTG GA-TAMRA
IL1 β	Forward primer: 5'-AGT GGC TGT GGC TGT CAT TGT Reverse primer: 5'-TGG GTA GAA TGT TTC CTT AAT CCA TT Probe: 5'-6-FAM-CGAGCACTCTTGTCTATCGGAAGC-TAMRA
gp130	Forward primer: 5'-CCA GCA ACG AGG AGA ATG AGT Reverse primer: 5'-TGT GCA CCA CAG TGG AGT ACT G Probe: 5'-6-FAM-TCA GAG CAC CGC CAG CAC GG-TAMRA
TNFR1	Forward primer: 5'-CCG ACT TGG TGT ACA TAG CTT TTC T Reverse primer: 5'-GGT ACA TCT CCC TGC CAC TCA Probe: 5'-6-FAM-CCG CCG AGG ACT GCC TGA GC-TAMRA
TNFR2	Forward primer: 5'-GGA TGA GCA GGT CCC CTT CT Reverse primer: 5'-CTC TGC AGT GTC TCT GTA GTC TCA CA Probe: 5'-6-FAM-CAG GAG GAG TGT CCG TCT CAG TCC CC-TAMRA
GAPDH	Forward primer: 5'-TGT GTC CGT CGT GGA TCT GA Reverse primer: 5'-CCT GCT TCA CCA CCT TCT TGA Probe: 5'-6-FAM-CCG CCT GGA GAA ACC TGC CAA GTA TG-TAMRA

Table 1. Primers and probes for qPCR.

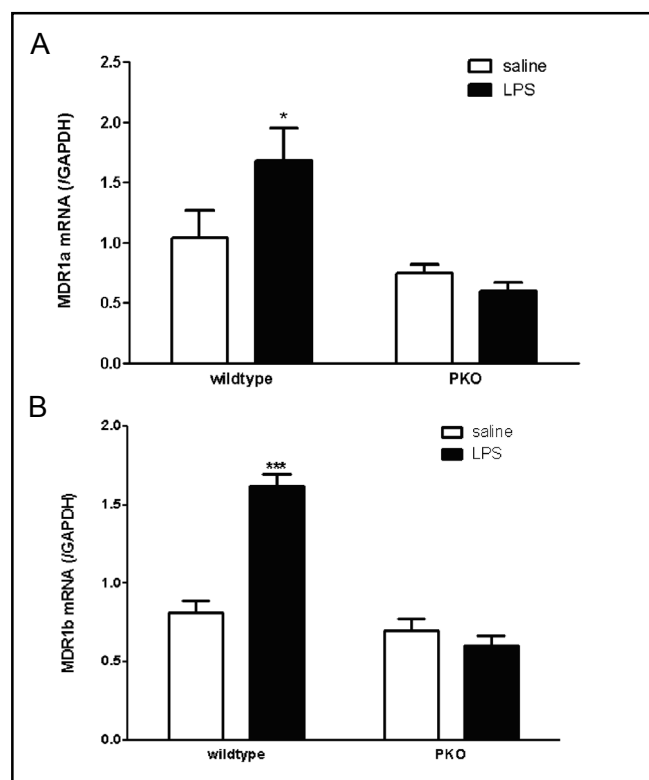


Fig. 1. The effects of LPS and PKO on efflux drug transporter expression. (A) MDR1a mRNA was increased in cerebral microvessels in the wildtype mice 48 h after LPS treatment (2.5 mg/kg ip). PKO did not affect the basal level of MDR1a, but abolished its increase in response to LPS. (B) MDR1b mRNA was increased only in the wildtype mice after LPS challenge. PKO did not affect the basal level of MDR1b, but abolished the increase in response to LPS ($n = 6/\text{group}$). *: $p < 0.05$; ***: $p < 0.005$.

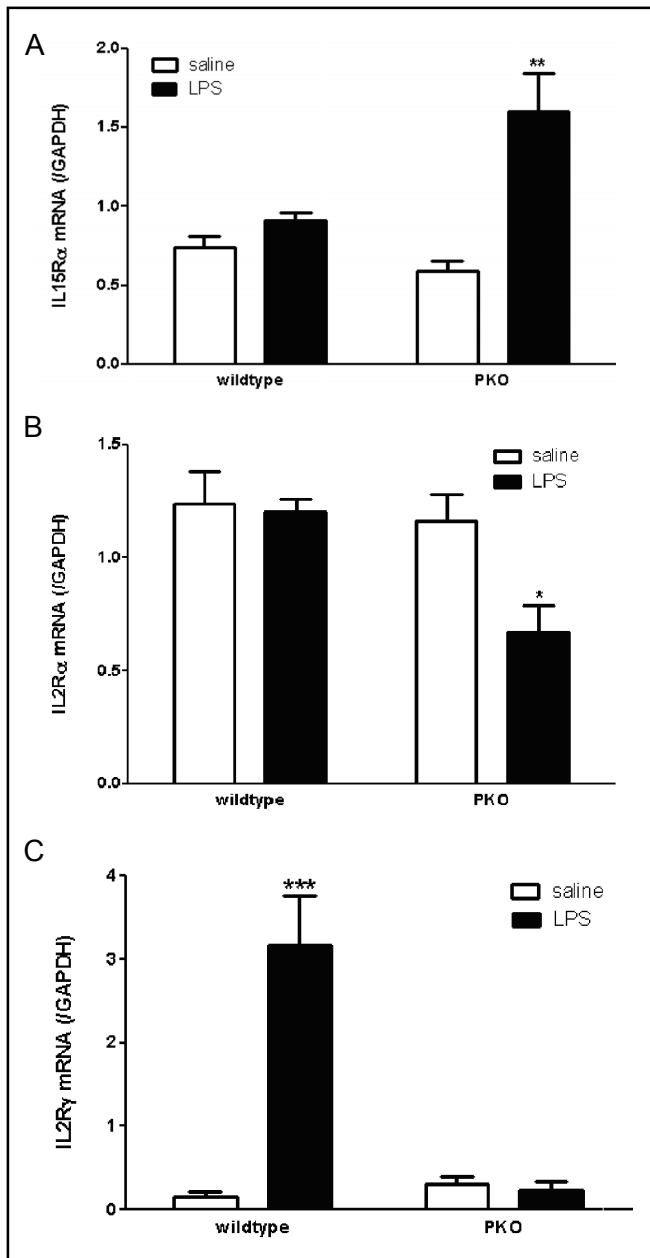


Fig. 2. The effects of LPS and PKO on receptors in the IL15/IL2 system. (A) IL15Rα mRNA was unchanged either by LPS treatment in the wildtype mice or by PKO. However, it showed an increase in the PKO mice in response to LPS. (B) IL2Rα mRNA was unchanged by either LPS or PKO, but decreased in the PKO mice after LPS challenge. (C) IL2Rγ mRNA showed a 20-fold increase in response to LPS, but the change was only seen in the wildtype mice (n = 6/group). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.005$.

mice responded to LPS by a significant elevation of IL2Rγ mRNA ($p < 0.001$; Fig. 2C). This indicates that LPS can only upregulate IL2Rγ in the presence of an intact NFκB complex.

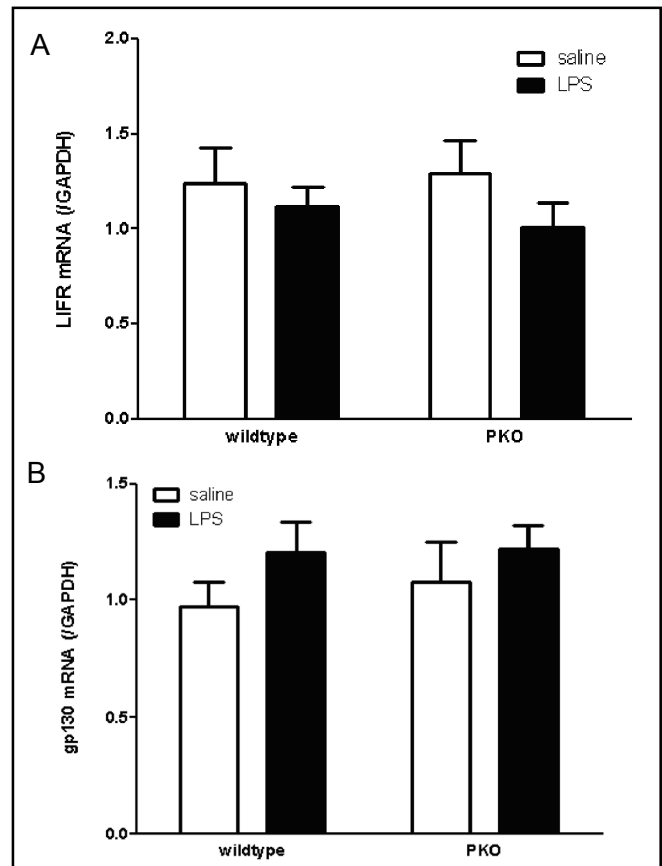


Fig. 3. Lack of changes of cerebral microvascular mRNA for LIFR (A) and gp130 (B) mRNA in response to LPS (n = 6/group).

In contrast to the robust effect of LPS on IL15/IL2 receptors and the mediatory role of NFκB, the gp190 specific receptor (LIFR, Fig. 3A) and the co-receptor gp130 for LIF (Fig. 3B) did not show a significant change either by LPS treatment or PKO at the time point studied. This serves as an important negative control.

Both IL15 and LIF receptors can be regulated by TNF. To determine whether TNF receptors play mediatory roles on the effects of LPS, we measured the mRNA levels for TNFR1 (p55, or p60) and TNFR2 (p75, or p80). For TNFR1 mRNA, there was a significant overall effect of LPS treatment ($p < 0.005$) and PKO ($p < 0.01$). There was no significant interaction between the variables of treatment and strain. LPS induced a significant increase of TNFR1 mRNA only in the PKO mice ($p < 0.01$) but not in the wildtype mice (Fig. 4A).

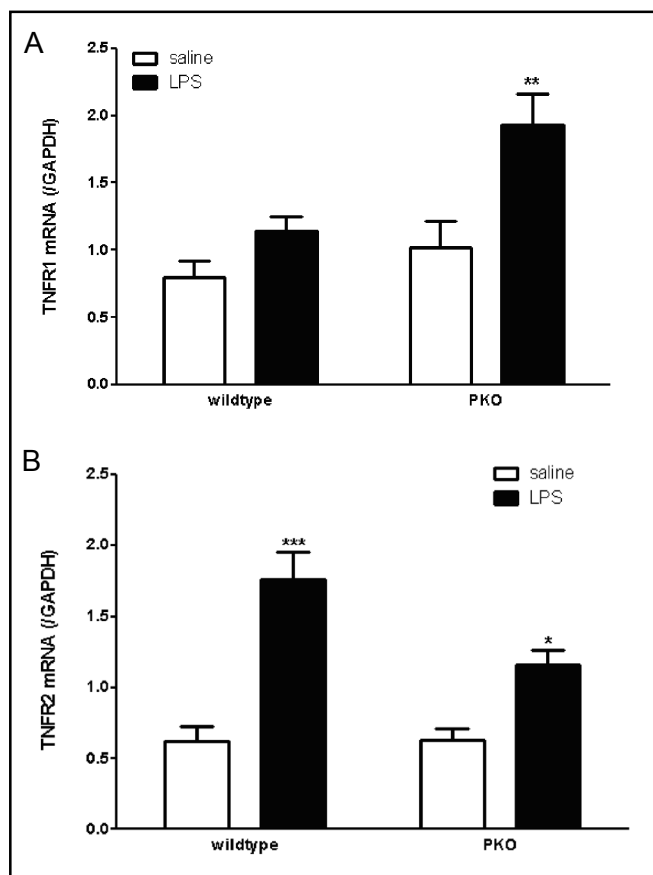


Fig. 4. The effects of LPS and PKO on TNFR1 (A) and TNFR2 (B). Significant increases were seen for TNFR2 in the wildtype mice, and for both TNFR1 and TNFR2 in the PKO mice (n = 6/group). *: p < 0.05; **: p < 0.01; ***: p < 0.005.

By contrast, TNFR2 mRNA showed a significant overall increase in response to LPS (p < 0.0001), a strain effect (p < 0.05), and an interaction between treatment and strain (p < 0.05). LPS induced a significant increase of TNFR2 mRNA in both wildtype (p < 0.001) and PKO (p < 0.05) groups (Fig. 4B).

Effects of LPS treatment and NFκB KO on protein expression of MDR1 and cytokine receptors

In the wildtype mice, LPS treatment appeared to induce an increase of Pgp, LIFR, IL15Rα, and IL2Rγ in the western blotting images (Fig. 5A). The increase was significant for LIFR and IL2Rγ based on densitometric analysis (Fig. 5B). Pgp showed a trend toward an increase (p = 0.09). In the PKO mice, there was a persistent, though non-significant increase of Pgp, but reduction of

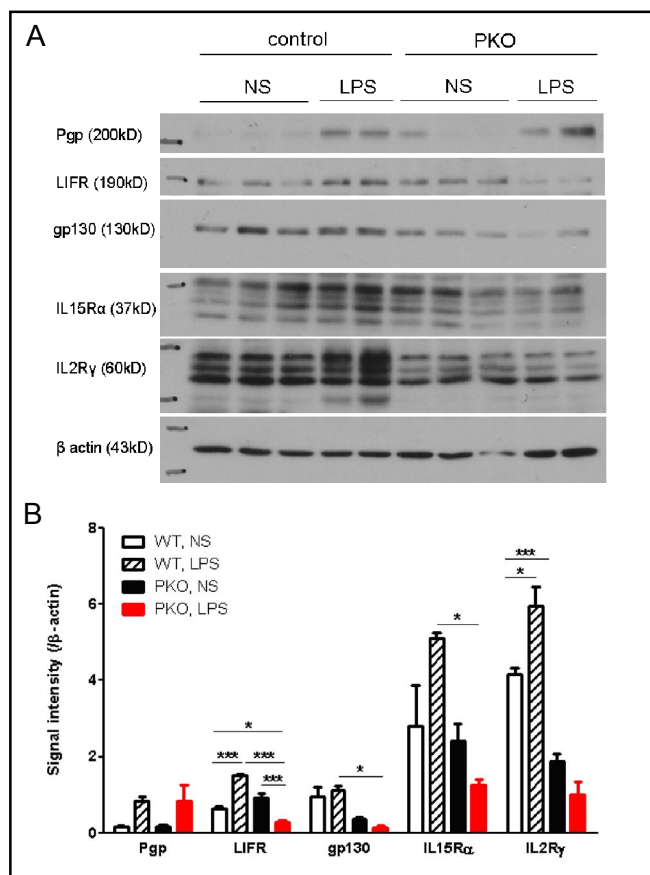


Fig. 5. The effects of LPS and PKO on protein expression of Pgp and cytokine receptors. (A) The western blotting gel image shows that in the wildtype mice LPS treatment increased Pgp, LIFR, IL15Rα, and IL2Rγ with persistent induction of Pgp. In the PKO mice LPS treatment decreased LIFR, gp130, and IL15Rα. PKO alone also induced a reduction of the IL2Rγ signal. (B) Densitometric analysis confirmed significant changes for LIFR and IL2Rγ. *: p < 0.05; ***: p < 0.005.

LIFR (p < 0.005) and non-significant decrease of gp130 and IL15Rα after LPS treatment. PKO alone (without LPS challenge) caused a decrease of IL2Rγ (p < 0.005). In the PKO mice, there was also a reduction of LIFR, gp130, and IL15Rα in response to LPS treatment.

Efflux transport of vinblastine in mice after LPS treatment and/or NFκB mutation

The increased expression of MDR1a and 1b mRNA and elevation of Pgp in the wildtype mice in response to LPS suggests an accelerated efflux transport. Thus, we expected that the wildtype mice would show decreased brain uptake of vinblastine after LPS treatment. However, there was no significant change in the wildtype mice. Since the PKO mice also showed a tendency toward higher Pgp expression in cerebral

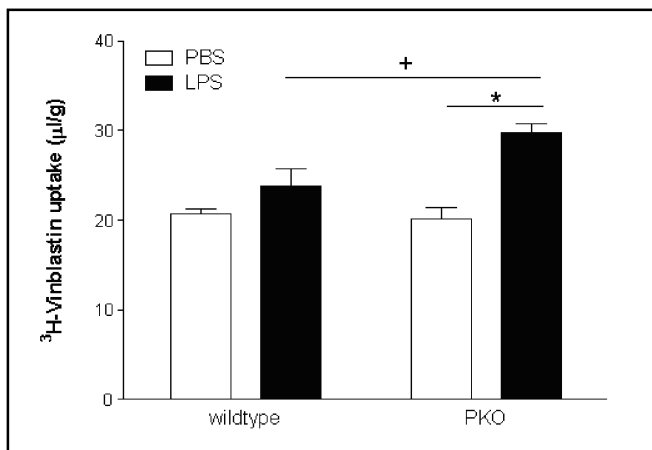


Fig. 6. The uptake of ³H-vinblastine after 1 min of in-situ brain perfusion was unchanged by LPS treatment alone or PKO alone. However, the PKO mice showed a significant (*: $p < 0.05$) increase of brain uptake after LPS treatment. There was also a trend (+: $p = 0.08$) toward an increase in the PKO mice in comparison with the wildtype mice after LPS treatment ($n = 3/\text{group}$).

microvessels, a lower vinblastine uptake after LPS treatment would also be expected from the PKO mice. Nonetheless, the results were opposite from the predictions. Vinblastine uptake was increased in the PKO mice after LPS treatment in comparison with the vehicle-treated group ($p < 0.05$). PKO mice also showed a trend ($p = 0.08$) toward a greater response to LPS than the wildtype mice with higher elevation of vinblastine uptake (Fig. 6).

Discussion

NF κ B is a ubiquitous transcriptional factor that plays an essential role in many biological functions, including reactions to neuroinflammation. It is well known that LPS induces NF κ B activation after binding to its receptors such as CD14 and toll-like receptor (TLR)-4 [22, 23]. LPS also affects the cytokine network and efflux drug transporters [24–26]. However, the role of NF κ B in mediating the effects of LPS on cytokine receptors and Pgp at the BBB level has not been fully elucidated. Here, by use of PKO mice deficient in the p50 subunit of NF κ B, we showed that LPS increased the expression of Pgp and selective cytokine receptors in cerebral microvessels composing the BBB, a complex process with regulation at both mRNA and protein levels. We also showed

differential involvement of NF κ B that can induce either upregulation or downregulation of cytokine receptors and modulate the response to LPS. Furthermore, despite the higher level of expression, the efflux transport function of Pgp was depressed in LPS-treated PKO mice. These findings broaden our understanding of the active role of the BBB in response to LPS-induced neuroinflammation.

In the non-activated state, the two main subunits of NF κ B - p50 and p65 - exist as a homodimer or heterodimer. The p50/p65, p50/p50, or p65/p65 subunits are located in the cytoplasm and bound by the inhibitory subunit I κ B. Once activated, such as by LPS or cytokines, there is rapid phosphorylation, ubiquitination, and trafficking of I κ B to proteasomes for degradation. Concurrently, the activated dimer is translocated to the nucleus where it binds to gene promoters and modulates transcriptional activation [27, 28]. Since the p65 subunit KO is lethal with intrauterine death of mice [29], the commonly used embryonic PKO is the p50 subunit KO surviving to adulthood [4]. These PKO mice have an altered basal metabolic rate, with lower body weight, higher food intake (/body weight), lower body temperature, more sleep time (including an increase of slow wave and rapid eye movement sleep), and less locomotor activity [30]. Since a low dose of LPS causes a greater sickness response in the PKO mice than the wildtype mice, it can be concluded that NF κ B protects mice against the detrimental consequences of LPS exposure [31], although compensatory changes after p50 deletion are also present [30].

We have shown that the proinflammatory cytokine TNF increases the expression of Pgp in cerebral endothelia [2] and activates MDR1b promoter activity exclusively by NF κ B binding to the promoter region [3]. To determine whether LPS acts through NF κ B signaling at the BBB level, we treated wildtype and PKO mice with LPS before obtaining enriched cerebral microvessels 48 h later. It is evident that LPS increased both MDR1a and MDR1b mRNA, with a corresponding increase of Pgp protein in the wildtype mice. Although the activation of transcription of the efflux transporter was no longer present in the PKO mice, Pgp protein remained elevated in response to LPS. This suggests compensatory changes at the BBB in the absence of the p50 subunit, and is consistent with previous observations in sleep studies on these mice [30]. Despite the elevation of mRNA and protein after LPS, the wildtype mice showed no major change in the efflux transport of vinblastine. The PKO mice even had an increase of vinblastine uptake by the brain, indicating depressed transport function regardless of the higher level

of protein expression. This is supported by a recent study with a 3 mg/kg dose of LPS in CD1 mice by use of verapamil, another substrate for Pgp. The effect of LPS on increasing the uptake of ^3H -verapamil was time-dependent, and not associated with disruption of the BBB shown by co-administered ^{14}C -sucrose [26]. The results also show differences of in-vivo BBB assays with cellular uptake measurements [2]. This is an important finding; contrary to conventional beliefs, the efflux transport function can be depressed in severe neuroinflammation.

LPS increases the expression of TNF and IL15, among many other cytokines [24, 32, 33]. Therefore, we also determined the levels of their respective receptors in enriched cerebral microvessels. The changes revealed the expected complexity. For the IL15 system in the wildtype mice, LPS failed to increase IL15R α and its cognate receptor IL2R α , but induced the co-receptor subunit IL2R γ . This was more evident at the mRNA level than the protein level. In the PKO mice, LPS caused a major elevation of IL15R α , decreased IL2R α , and failed to affect the low basal level of IL2R γ . This is also more apparent at the mRNA level. For the TNF system, LPS increased the mRNA of TNFR2 without affecting that of TNFR1 in the wildtype mice, but caused a significant increase in both receptor subtypes in the PKO mice. For all of these receptors, PKO did not significantly affect the basal level of expression. However, the changes resulting from p50 deletion were accentuated after LPS challenge. By contrast, the levels of LIFR and gp130, which showed distinctive changes in response to TNF [8, 9], were unchanged in either wildtype or PKO mice. The differential role of NF κ B in the expression of these receptors reflects complex cellular mechanisms of regulation of different genes in the cytokine network.

Although the Pgp system is one of the major ATP-binding cassette transporters at the BBB [34], many other cytokines and receptors also undergo regulatory changes during inflammation. For instance, NF κ B is essential in transcriptional activation of IL15 [35], but there was no change in IL15 mRNA at the BBB level in our preliminary study even though the BBB is activated during neuroinflammation, as shown by elevated γ -glutamyl transpeptidase activity after stimulation by LPS or TNF [20]. Upregulation of TNF transport has also been shown in mice with experimental autoimmune encephalomyelitis [36]. The upregulation of IL15 and TNF receptor subtypes that may mediate the blood-to-brain transport of these cytokines correlates with the increase of efflux transporter function despite the reduction of efflux transport of vinblastine. The cytokine cascades, secondary mediators, and altered transport of many substrates as well as cytokine themselves [37-39] all contribute to the sickness behavior and eventual functional outcome [40].

In summary, we showed that NF κ B signaling at the BBB mediates the effect of LPS in upregulating Pgp efflux transporter expression and decreasing its transport function. We also showed that NF κ B participates in the selective effects of LPS in regulating several receptor subtypes for IL15 and TNF without affecting those for LIF. This novel information provides further insight into the complexity of regulation at the BBB during neuroinflammation.

Acknowledgements

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