Lipopolysaccharide-Induced Fever Depends on Prostaglandin E2 Production Specifically in Brain Endothelial Cells

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Immune-induced prostaglandin E2 (PGE2) synthesis is critical for fever and other centrally elicited disease symptoms. The production of PGE2 depends on cyclooxygenase-2 and microsomal prostaglandin E synthase-1 (mPGES-1), but the identity of the cells involved has been a matter of controversy. We generated mice expressing mPGES-1 either in cells of hematopoietic or nonhematopoietic origin. Mice lacking mPGES-1 in hematopoietic cells displayed an intact febrile response to lipopolysaccharide, associated with elevated levels of PGE2 in the cerebrospinal fluid. In contrast, mice that expressed mPGES-1 only in hematopoietic cells, although displaying elevated PGE2 levels in plasma but not in the cerebrospinal fluid, showed no febrile response to lipopolysaccharide, thus pointing to the critical role of brain-derived PGE2 for fever. Immunohistochemical stainings showed that induced cyclooxygenase-2 expression in the brain exclusively occurred in endothelial cells, and quantitative PCR analysis on brain cells isolated by flow cytometry demonstrated that mPGES-1 is induced in endothelial cells and not in vascular wall macrophages. Similar analysis on liver cells showed induced expression in macrophages and not in endothelial cells, pointing at the distinct role for brain endothelial cells in PGE2 synthesis. These results identify the brain endothelial cells as the PGE2-producing cells critical for immune-induced fever. (Endocrinology 153: 4849–4861, 2012)

The febrile response, a hallmark of infection and inflammation, has been shown to depend on induced prostaglandin (PG) E2 synthesis by the sequential action of cyclooxygenase-2 (Cox-2) and microsomal prostaglandin E synthase-1 (mPGES-1) (1–5). It has also been demonstrated that the febrile response is dependent on PGE2-binding to its EP3 receptors in the median preoptic nucleus in the hypothalamus (6), demonstrating the critical role of intracerebral PGE2 for fever.

Although the role of Cox-2, mPGES-1, and PGE2 for fever thus is well established, there have been considerable arguments as to in which cells the critical PGE2 synthesis takes place. Several observations point to the brain vasculature, in which Cox-2 and mPGES-1 are abundantly expressed upon immune challenge (7–9). The brain vasculature also expresses receptors for the cytokine IL-1β (7, 8, 10), making it a dedicated structure for the transduction of peripheral immune signals to the brain through induced PGE2 synthesis (11). Studies in which the different cellular elements in the vascular wall have been characterized with specific markers (8, 12, 13) have identified the endothelial cells as the component of the blood vessel that expresses...
mPGES-1 and that hence is critical for the induced PGE2-synthesis and the PGE2-evoked and centrally elicited re-
response. However, other studies, among them work that has focused on the expression of Cox-2, have advocated
that cells of hematopoietic origin, in particular the perivas-
cular macrophages, immune cells that are located in the
vascular wall just beneath the endothelial cells, as well as
immune cells located in peripheral tissues such as the liver
and lung, may be equally important and under certain
circumstances the sole source of centrally acting PGE2
(14–17; but cf. Refs. 9, 18, and 19). However, for both
views the evidence is circumstantial, and functional ex-
periments that pinpoint the role of the different cellular
compartments have been lacking.

Here we addressed the issue of in which cells the for
the febrile reaction critical PGE2 synthesis takes place by ex-
amining central and peripheral PGE2 production and the
accompanying temperature response in mice chimeric for
mPGES-1, implying that they expressed mPGES-1, and
hence were capable of induced PGE2 synthesis, either in
their cells of hematopoietic or nonhematopoietic origin.
This enabled us to differentiate between the response me-
diated by, e.g., the nonhematopoietic endothelial cells and
that mediated by hematopoietically derived cells, includ-
ing perivascular macrophages as well as peripheral im-
une cells. Our results show that nonhematopoietic cells
are both necessary and sufficient for elevating central
PGE2 and eliciting fever after a peripheral immune chal-
lenge with lipopolysaccharide, whereas hematopoietically
derived cells, although implied in peripheral PGE2 pro-
duction, contributes little to central PGE2 levels and are
unable by themselves to elicit a febrile response. Com-
plementary immunohistochemical stainings on brain tissue
and quantitative PCR on dissociated brain vascular cells
further identified the endothelial cells as the source of in-
duced PGE2 synthesis. These findings demonstrate that
the critical site for the immune-induced PGE2-mediated
febrile response is the brain vascular endothelial cells.

Materials and Methods

Animals

Wild-type C57BL/6 mice (Scanbur, Sollentuna, Sweden), and
Ptges+/− and Ptges−/− mice (20), backcrossed onto the C57BL/6
background, were used. For transplantation experiments (donor
bone marrow) Ptges−/− mice were crossed with a green fluores-
cent protein (GFP)-expressing strain [C57BL/6-Tg(CAG-
EGFP)C14-Y01-FM131Osb; kindly provided by Dr. Masaru
Okabe, Osaka university, Osaka, Japan (21)]. The resulting
heterozygous offspring were then crossed to generate
GFP+/Ptges−/− and GFP+Ptges−/− mice. Ptgs2 [Cox-2 knock-
out (KO)] mice (22) were from Taconic (Ry, Denmark). The
animals were housed one to five per cage on a 12-h light, 12-h
dark cycle (lights on at 0800 h). All experimental procedures
were approved by the Animal Care and Use Committee at
Linköping University.

Irradiation and bone marrow transplantation

The mice (3–6 months old) were irradiated to an absorbed
dose of 9 Gy in two fractions. Approximately 24 h after irra-
diation, they were injected iv with 2 × 10⁶ freshly prepared
GFP+CD45+ bone marrow cells and allowed to survive for
about 5 months. For details, see Supplemental Materials and
Methods, published on The Endocrine Society’s Journals Online

Temperature recordings

For surgical procedures, see Supplemental Materials and
Methods.

Intraperitoneal injection of lipopolysaccharide (LPS)

LPS from Escherichia coli (Sigma-Aldrich, St. Louis, MO;
O111:B4; 120 μg/kg body weight) diluted in 100 μl saline was
injected ip at around 1000 h. Temperature data were obtained
using ip transmitters (Data Science International, St. Paul, MN)
implemented 1 wk before the injection. After a washout period
of 1–2 wk, animals that had been given LPS were injected with
saline, and vice versa, and body temperature recorded as de-
scribed above.

Intravenous injection of lipopolysaccharide or IL-1β

Mice were injected iv with LPS (30 or 1 μg/kg) or recombinant
murine IL-1β (30 μg/kg; Preprotech, Rocky Hill, NJ) through an
indwelling jugular catheter implanted 3 d before injection.
The catheter was exteriorized at the back of the neck and connected
to a swivel system (CMA Microdialysis, Solna, Sweden) on the
top of the cage, permitting injection without handling the mice.

Immuunoassays for PGE2 and PGE2 metabolites in
plasma and cerebrospinal fluid (CSF)

Following asphyxiation of the mice with CO₂, blood was
drawn from the right atrium, transferred to EDTA-coated tubes
(Sarstedt, Landskrona, Sweden) to which were added indometh-
acin (10 μm), and centrifuged at 7000 × g for 7 min at 4 C.
The plasma was immediately frozen on dry ice and kept at −70 C.
CSF was then collected from the cisterna magna using a Ham-
ilton syringe, and immediately frozen. Samples that contained
traces of blood were discarded. The whole procedure from when
the animals were killed until CSF was withdrawn took less than
10 min. The concentration of PGE2 (CSF) and PGE2 metabolites
(plasma) was determined using high sensitivity PGE2 enzyme
immunoassay kit (Assay Designs, Ann Arbor, MI) and PGE me-
tabolite enzyme immunoassay kit (Cayman, Ann Arbor, MI),
respectively. For details, see Supplemental Materials and
Methods.

Immunohistochemistry

Immunohistochemistry was performed according to stan-
dard protocols in this laboratory (23). For details, see Supple-
mental Materials and Methods.
Flow cytometry and quantitative PCR (qPCR)

Blood

Venous blood was collected into ice-cold PBS containing 2.5 mM EDTA. After lysis of erythrocytes, cells were resuspended in PBS and analyzed for GFP expression by flow cytometry.

Brain and liver

Single cells were prepared as described in Supplemental Materials and Methods. The live cells [propidium iodide negative (PI)] from each sample were first sorted using yield sorting mode and then resuspended in PBS with 10% fetal bovine serum and incubated with rat antimonster FC-Block (CD32/16), CD45 PE-Cy5.5, CD206-Alexa 647 (brain) or F4/80 PE (liver), and CD31 PE-Cy7 for 10 and 15–20 min, respectively, on ice. Dead cells were again excluded by PI staining. CD31^{+}CD45^{-}, CD45^{+}CD31^{-}, and CD45^{+}CD206^{+} or CD45^{+}F4/80^{+} cells were gated based on fluorescence minus one controls and isotype staining controls for each antigen expression (for antibody suppliers, see Supplemental Materials and Methods). The cell populations were then sorted directly into RNasey lysis buffer (QIAGEN, Hilden, Germany) with β-mercaptoethanol (Sigma; 143 mM) after purity analysis and stored at −80°C until use. For qPCR, total RNA was extracted, reversely transcribed, and the cDNA preamplified. For details, see Supplemental Materials and Methods.

Statistics

Temperature data were analyzed by a two-way ANOVA, followed by Bonferroni post hoc test. Data on PGE2 or PGE2 metabolites were analyzed by a one-way ANOVA followed by Newman-Keuls multiple comparisons test, or, for comparisons between treatments within the same group of chimera (plasma at 40 min), with a Student’s t test.

Results

Creation of chimeric mice by whole body γ-irradiation and bone marrow transplantation

Immune-induced PGE2 is produced by mPGES-1, the PGE2 synthase encoded by the Ptges gene. We have previously demonstrated that mPGES-1 is critical for fever (2–4). Mice chimeric for mPGES-1 were created by subjecting littermates of wild-type (WT) and Ptges^{−/−} (KO) mice to potentially lethal whole body γ-irradiation (9 Gray) followed by transplantation of CD45^{+} enriched GFP^{+} bone marrow cells from mice of the opposite genotype (KO and WT, respectively). Hence, two different chimeras were created: WT mice with Ptges^{−/−} hematopoietic cells and Ptges^{−/−} mice with WT hematopoietic cells. In addition, WT mice transplanted with WT bone marrow, and Ptges^{−/−} mice transplanted with Ptges^{−/−} bone marrow were generated and included as controls.

To allow as complete reconstitution of hematopoietic cells as possible, while at the same time assuring that mice did not become senescent, mice were allowed to survive for 5 months after irradiation and transplantation. Examination at this time point of LPS-induced IL-1β in plasma of chimeric mice (see Supplemental Materials and Methods) showed a normal inflammatory response (Supplemental Fig. 1).

The degree of reconstitution was examined in peripheral blood, brain, liver, and lung from tissue harvested after animals had been immune challenged for recordings of the temperature response. Overall, the data showed extensive replacement of native hematopoietic cells with transplanted cells.

Analysis of reconstitution in peripheral blood, as determined by flow cytometry of the proportion of GFP^{+} cells among the blood leukocytes, revealed that approximately 90% of the white blood cells originated from transplanted bone marrow cells (Supplemental Fig. 2).

Examination of tissue sections through the brain revealed GFP^{+} cells (i.e. cells derived from transplanted cells) in the walls of blood vessels, with only few GFP^{+} cells being seen in the brain parenchyma (Fig. 1, A–C). The morphology of the cells in the two compartments differed; whereas the cells in the walls of the blood vessel had an amoeboid appearance characteristic of perivascular macrophages (Fig. 1B), those found in the parenchyma displayed characteristics of microglial cells (Fig. 1C). Brain sections from six mice, encompassing all four groups of hybrid mice, which had been subjected to an LPS challenge to monitor their temperature response, were stained for both GFP and the mannose receptor (CD206) (Fig. 1D), which identifies perivascular macrophages (24). Quantitative analysis on six randomly selected fields from each of the six mice showed that 87.5% (SEM 8.7) of the CD206^{+} population also expressed GFP and that 91.5% (SEM 5.4) of the GFP^{+} population also expressed CD206. Similar results were obtained in four transplanted mice that had not been given LPS, verifying that the reconstitution had not taken place as a consequence of the immune challenge but was present before that. Thus, the data show that approximately nine of 10 GFP^{+} cells in the examined brains were perivascular macrophages, and nine of 10 of the perivascular macrophages were derived from transplanted hematopoietic cells.

In additional hybrid mice, the proportion of GFP^{+} cells among the CD206^{+} population in the brain was determined by flow cytometry, providing corroborative evidence for the high degree of reconstitution of the perivascular cells from transplanted GFP expressing hematopoietic cells (Supplemental Fig. 3). Accordingly, the irradiation and transplantation procedure generated a highly selective and efficacious replacement of native perivascular cells with transplanted cells. The specificity of the reconstitution was further supported by labeling for GFP and the endothelial cell marker von Willebrand factor. In no case did GFP^{+} cells also express the von Willebrand factor (Fig. 1E) and were in fact located...
on the parenchymal side of the endothelial cells, consistent with their perivascular identity.

Reconstitution in the liver was examined using staining for GFP and the pan-macrophage marker F4/80 (25) (Fig. 1F). Quantitative analysis on confocal micrographs from 6 transplanted mice showed that 67.7% (SEM 4.1) of the liver F4/80+ population expressed GFP, and that 84.6% (SEM 1.9) of the total GFP+ population was F4/80+. In the lung (Fig. 1G), staining for GFP and the lung macrophage marker CD68 (26, 27) showed that 78.3% (SEM 2.3) of the CD68+ cells expressed GFP and that 64.7% (SEM 3.4) of the GFP+ population expressed CD68. Thus, in both liver and lung, two thirds or more of the immune cells were replaced by transplanted cells. Although it was slightly lower than in blood and brain, this degree of reconstitution should, when KO bone marrow cells were transplanted to WT mice, imply a very large reduction in any PGE2 synthesis of hematopoietic cells, and, most importantly, it should largely restore the PGE2 synthesis in hematopoietic cells when WT cells were transplanted to mice of KO background.

**mPGES-1 in nonhematopoietic cells is critical for the febrile response to ip LPS**

The temperature response of the four different groups of transplanted mice 5 months after irradiation and transplantation is shown in Fig. 2A. Mice were given an ip injection of LPS (120 μg/kg body weight), and core body temperature was recorded continuously by telemetry. As seen, WT mice transplanted with WT bone marrow (WT→WT) displayed a characteristic fever with two clearly discernible phases, the first initial phase being partly obscured by the hyperthermia elicited by the handling during the injection (28). This temperature curve is similar to that observed in naïve WT mice using the same immune challenge paradigm (4). As also expected, similar to what is seen in mice with a global mPGES-1 gene deletion (2, 4), KO mice transplanted with KO bone marrow (KO→KO) displayed a rapid temperature fall immediately after the initial temperature peak elicited by the handling stress during the injection and then remained at a
body temperature below or around that of control mice injected with saline. The temperature response of the two chimeras resembled that of mice carrying the same background genotype. Thus, WT mice transplanted with KO bone marrow (KO<sub>3</sub>WT) showed a febrile response that was similar to that of the WT<sub>3</sub>WT mice, and although the body temperature was slightly lower throughout most of the observation period, the difference was not statistically significant. In contrast, KO mice transplanted with WT bone marrow (WT<sub>3</sub>KO) were unable to mount a febrile response, similar to the KO<sub>3</sub>KO mice. Like the latter they displayed a rapid temperature fall after the initial stress-induced hyperthermia, and although their body temperature thereafter for most of the observation period was slightly higher than that of the KO<sub>3</sub>KO mice, this difference was not statistically significant.

The initial phase of fever in response to iv LPS is dependent on mPGES-1 in nonhematopoietic cells

Because the handling-induced hyperthermia associated with the ip injection may obscure rapid LPS elicited temperature responses (28, 29), we also examined the febrile response in chimeric mice injected with LPS through an indwelling jugular catheter, a procedure that permitted injections during low stress condition for the mice. The dose (0.75 μg; ∼30 μg/kg body weight) was selected to yield a clearly discernible first phase of fever (29). As shown in Fig. 2B, WT mice, both when transplanted with WT or KO bone marrow, displayed with a latency of 10–15 min after the LPS injection a rapid and transient temperature elevation, which peaked at 25–40 min after the injection. In contrast, KO mice, irrespective of whether they were transplanted with KO or WT bone marrow, showed no febrile response but instead a temperature fall that started 10–20 min after injection (Fig. 2B).

The febrile response to LPS in chimeric mice follows PGE2 levels in the cerebrospinal fluid

The aforementioned data strongly suggest that PGE2 production in cells of the hematopoietic lineage plays but a minor role for the febrile response and that the nonhematopoietic cells instead are critical. To further address this issue, we examined in the different chimera the relationship between PGE2 levels in the brain and blood and the febrile response. Cerebrospinal fluid was withdrawn from the cisterna magna 40 min and 3 h after ip injection with LPS and analyzed for the PGE2 content by immunoassay. These time points were chosen because they correspond to the short first phase of fever and to the late, third, sustained phase of fever, respectively [(29) and present data]. Moreover, these phases have been suggested to be elicited by distinct mechanisms (16). We found a significant elevation of the PGE2 level at 40 min in the cerebrospinal fluid of LPS-treated WT mice, irrespective of whether they had been transplanted with WT or KO bone marrow (Fig. 3A). LPS-treated KO<sub>3</sub>KO mice displayed PGE2 levels in the cerebrospinal fluid similar to those seen in saline injected...
plasma is difficult to measure reliably (30–32) showed induced expression 40 min after ip LPS injection in all hybrids except the KO→KO group (Fig. 3B). Notably, the rescue group, i.e. animals in which WT bone marrow was transplanted to KO mice (WT→KO), showed a strong induction of PGE2 metabolites in plasma at 40 min, similar to WT→WT and KO→WT mice, indicating a significant role for hematopoietic cells for plasma PGE2 levels at this time point. However, as was shown in Fig. 2, WT→KO mice, in contrast to the WT→WT and KO→WT mice, did not display any febrile response at 40 min, and the data thus did not show any clear relationship between plasma PGE2 levels and body temperature.

At 3 h after the LPS injection, plasma concentration of PGE2 metabolites (Fig. 3B) remained high in the WT→WT group and low in the KO→KO group, with KO→WT and WT→KO mice displaying levels in between. Notably, although the concentration of PGE2 metabolites was not significantly different between KO→WT and WT→KO mice but differed significantly in both these groups from WT→WT mice, they displayed very different temperature responses, with KO→WT mice showing a febrile response similar to WT→WT mice and WT→KO mice showing no fever (see Fig. 2). Again, these data thus show that the plasma PGE2 levels have at best a minor influence on body temperature. This conclusion was also confirmed by the regression analysis of the relationship between body temperature and plasma levels of PGE2 metabolites (Supplemental Fig. 4).

### Identification of PGE2 synthesizing cells

The data from chimeric mice clearly show that fever is dependent on intact PGE2 synthesis in nonhematopoietic cells and is best associated with elevated central but not peripheral PGE2 levels, suggesting a critical role for the cerebral endothelium. As a final step, we therefore examined the expression of the inducible PGE2-synthesizing enzymes Cox-2 and mPGES-1 in the brain as well as in peripheral tissues. For the analysis of Cox-2, we used immunohistochemistry. Although mPGES-1 has been unequivocally demonstrated in rat brain tissue by both immunohistochemistry and *in situ* hybridization by us and others (7, 8, 12), neither method produced any labeling in mouse brain tissue [consistent with the sparse evidence in the literature (33)]. Therefore, we instead used qPCR on dissociated brain cells separated by flow cytometry.

### Prostaglandin-synthesizing enzymes are induced in brain endothelial cells but not in perivascular macrophages

Immunohistochemical staining for Cox-2 in the brain showed prominent induction in blood vessels 3 h after LPS injection (120 μg/kg, ip) (Fig. 4A). The labeling was seen...
in round nuclei outlining the vessel wall (Fig. 4B). Simultaneous staining for the von Willebrand factor demonstrated perinuclear Cox-2 labeling surrounded by von Willebrand positive cytoplasm (Fig. 4F). Dual labeling for Cox-2 and GFP showed that the Cox-2-positive cells were distinct from the perivascular macrophages expressing GFP (Fig. 4, B–D). This was not a phenotype change of perivascular macrophages derived from transplanted hematopoietic cells because cells labeled for the macrophage marker CD206 in nontransplanted wild-type mice neither expressed Cox-2 after LPS challenge (Fig. 4E).

Because the expression pattern of Cox-2 in the brain has been suggested to depend on the dose of LPS injected, and possibly also the route of administration, we also injected a low dose of LPS iv (1 μg/kg). This procedure previously has been reported to preferentially induce Cox-2 in perivascular cells and not in endothelial cells (15). However, the same picture was seen as after injection of 120 μg/kg LPS ip: Cox-2 labeling was observed in endothelial cells only, whereas perivascular macrophages were unlabeled (Fig. 4G). Similarly, iv injection of IL-1β (30 μg/kg body weight), another immune stimulus, which, although being induced by LPS (34), also has been suggested to elicit Cox-2 preferentially in perivascular cells (15), only labeled endothelial cells (Fig. 4H). Hence, taken together these data demonstrate that Cox-2 induction occurs exclusively in endothelial cells.

qPCR analysis on cells obtained by flow cytometry (Fig. 5) showed a prominent up-regulation (9 times; P < 0.05) of mPGES-1 mRNA in endothelial cells (CD31+/CD45−) 3 h after ip LPS injection (Fig. 5D). In contrast, mPGES-1 mRNA levels in perivascular cells (CD206+/CD45−) were low to undetectable, both in LPS treated and NaCl treated mice (Fig. 5D). Notably, a similar qPCR analysis on cells obtained from the liver by flow cytometry (Fig. 6) showed strong up-regulation of mPGES-1 mRNA in macrophages (Kupffer cells) and undetectable values in endothelial cells, i.e. a pattern quite opposite to that seen in the brain, thus further supporting the distinct role of the brain endothelium in induced PGE2-synthesis. qPCR analysis of mPGES-1 mRNA in the hypothalamus of chimeric mice further supported the finding that hematopoietically derived brain cells do not take part in the PGE2 production (Supplemental Fig. 5).

Taken together with the immunohistochemical data on Cox-2 expression, being localized to endothelial cells in the mouse brain, these findings hence identify the endothelial cells as the PGE2-producing cells in the brain during an immune challenge.
were preferentially seen at the periphery of the lobule but occasionally also were more centrally located (Fig. 7A). Dual labeling revealed extensive overlap with F4/80 immunoreactivity and in transplanted mice also with GFP, demonstrating that the Cox-2 induction occurred in immune cells, such as Kupffer cells (Fig. 7, B and C). In the

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**FIG. 5.** Flow cytometry showing induced expression of mPGES-1 in endothelial cells (CD45^−CD31^+) but not in perivascular cells (CD45^−CD206^+) 3 h after ip injection of LPS. A, Representative fluorescence-activated cell sorting profiles show sorting strategy. Dead cells and debris were first excluded using forward scatter channel (FSC) area (A) vs. side scatter channel (SSC-A) and FSC height (H) vs. FSC width (W) and further excluded by PI staining (upper panels). The cell populations were then obtained by two-step sorting. First, the live cells (PI−) were enriched using a yield-sorting mode (upper panels). Then the live (PI−) CD45^−CD31^+ and CD45^−CD206^+ cells were gated based on fluorescence minus one (FMO) controls and isotype staining controls (lower panels). The CD45^−CD206^+ cells, seen in the middle column of the lower panel in A represents microglial cells, which, although mostly being CD45low, in some areas display average to high immunoreactivity for CD45 (cf. Fig. 1C). B, Purity analysis of the sorted cell populations CD45^−CD31^+ and CD45^−CD206^+ cells. Note that although the sorting for CD45^−CD31^+ cells (endothelial cells) in A did not yield a very large population (cf. also Fig. 6), the population selected for further analysis was clearly shown to be distinct. C, Quantitative PCR verification of the cell sorting. CD206 mRNA expression was normalized against that in the endothelial cells (EC) from mice injected with NaCl. Perivascular macrophages (PVC) display 1000-fold higher expression of CD206 than the endothelial cells (n = 3 in each group). D, LPS-induced mPGES-1 mRNA expression. The Δcycle threshold (CT) values, obtained by comparison with glyceraldehyde-3-phosphate dehydrogenase, were normalized against the endothelial cells from mice treated with saline (n = 3). In perivascular cells from one LPS-treated and two saline-treated mice, mPGES-1 mRNA levels were undetectable; the CT values were then set to 30, as a conservative sensitivity limit for preamplified samples. Error bars, SEM. *, P < 0.05 compared with endothelial cells from saline-treated mice.
lung, ip LPS injection induced at the 3-h time point Cox-2 expression in endothelial-like cells in the walls of vessels (Fig. 8A), suggestive of pulmonary veins (Fig. 8D). Although extensive GFP labeling was seen in the lung of bone marrow-transplanted mice, no GFP positive cells expressed Cox-2 (Fig. 8B, B1), and Cox-2 expression neither colocalized with CD68 (Fig. 8C, C1), indicating that Cox-2 expression occurred in nonhematopoietic cells. These data thus confirmed the presence of LPS-induced synthesis of PGE2 in peripheral organs in both immune cells and nonimmune cells, being consistent with elevated plasma levels of PGE2 in both WT→KO and KO→WT

**FIG. 6.** Flow cytometry showing induced expression of mPGES-1 in liver macrophages (CD45−/F4/80+) but not in liver endothelial cells (CD45−/CD31+) 3 h after ip injection of LPS. A, Representative fluorescence-activated cell sorting profiles shows sorting strategy, which was the same as for brain samples (see Fig. 5). The pan-macrophage marker F4/80 was used instead of CD206 (used for the sorting of the brain tissue (see Fig. 5)) because it is a marker for Kupffer cells and because CD206 (that identifies brain macrophages) does not differentiate between liver macrophages and endothelial cells (50). B, Purity analysis of the sorted cell populations CD45−/CD31+ and CD45−/F4/80+ cells. Note that although the sorting for CD45−/CD31+ cells (endothelial cells) in A did not yield a very large population (cf. also Fig. 5), the population selected for further analysis was clearly shown to be distinct. C, LPS-induced mPGES-1 mRNA expression. The ΔΔCT value for liver Kupffer cells (KC) (CD45−/F4/80+), obtained by comparison with GAPDH, was normalized against the ΔΔCT value for Kupffer cells from mice treated with saline (n = 3). In endothelial cells (EC) (CD45−/CD31+), mPGES-1 mRNA levels were not detectable (nd), neither in LPS-treated nor NaCl-treated mice (n = 3). Error bars, SEM. ***, P < 0.001 compared with endothelial cells from saline-treated mice.
hybrids (cf. Fig. 3). They also showed that the transplanted cells had the capacity to produce prostaglandins at the appropriate sites, as evidenced by their induced Cox-2 expression in the liver.

No induced expression of PGE2 synthesizing enzymes is seen at the time of the first phase of fever

Examination of brain, liver and lung at 40 min after the ip LPS injection showed no clear Cox-2 induction, and qPCR on brain endothelial cells and perivascular macrophages showed low levels of mPGES-1 mRNA, irrespective of whether mice had been injected with LPS or saline (not shown).

Discussion

The present study shows that the febrile response to LPS in mice is dependent on the presence of mPGES-1, the inducible terminal enzyme in the PGE2 synthesizing cascade (35), in cells of nonhematopoietic origin. Furthermore, we demonstrate that the febrile response is associated with increased PGE2 levels in the cerebrospinal fluid, whereas there was only a weak relationship between body temperature and PGE2 metabolites in plasma, indicating that the PGE2 production that gives rise to fever takes place in the brain. Finally, we show that endothelial cells are the site of Cox-2 induction in response to LPS and that these cells also show high inducible levels of mPGES-1. Because Cox-2- and mPGES-1-dependent PGE2 synthesis is necessary for LPS induced fever (1, 2, 4), the present data hence identify the brain endothelial cells as the cells critical for the febrile response.

Although this study is the first to identify the cells that are critical for the PGE2 production that gives rise to fever, some previous observations that are consonant with the present finding should be noted. Thus, Scammell et al. (36) have demonstrated that local injection of a Cox inhibitor into the anterior preoptic region attenuates the fever elicited by systemic administration of LPS, and recently Vardeh et al. (37) showed that conditional deletion of Cox-2, the isoform critical for fever, in neural lineages did not affect the febrile response to LPS. Taken together, these two studies show that prostaglandin production in the brain is involved in the generation of fever but that this occurs in brain cells other than neurons and glia, being well in line with the present observations. Furthermore, quite recently Ridder et al. (38) showed that the MAPK kinase TGFβ-activated kinase 1 in brain endothelial cells is needed for IL-1β-induced COX-2 production and that mice lacking the Tak1 gene in brain endothelial cells displayed a blunted fever response to IL-1β. Although that study, unlike the present work, does not directly target the critical final step in the induced PGE2-synthesis, it is in strong support of an essential role of endothelial cells for immune-induced fever.

Our findings that cells of hematopoietic origin play at best a minor role for the LPS induced febrile response, not only during the second and third phases of fever but also during the first initial phase, is at odds with the prevailing hypothesis, based largely on studies in rats, not mice, that has ascribed the initial fever to hematopoietic cells and in particular those in peripheral organs. That idea, which has the inherent weakness that PGE2 in the blood is rapidly...
converted \textit{in vivo} to its 13,14-dihydro-15-keto metabolite, with more than 90\% of circulating PGE2 being cleared by a single passage through the lungs (30, 39), is based on the findings that iv injection of PGE2 in supra-physiological concentrations elicits fever (16, 40, 41), that mPGES-1 mRNA, which, however, is not the rate-limiting enzyme for the induced PGE2-synthesis (1), is induced more rapidly in peripheral organs than in the brain (42), and that LPS-induced fever can be attenuated by iv but not intracerebroventricular injection of PGE2-neutralizing antibodies (16). The latter experiment suffers from the obvious limitation that iv injected antibodies in fact may have better access to blood-brain barrier cells than those injected intracerebroventricularly. Importantly, it was never examined in the latter study (16) whether intracerebral injection of neutralizing antibody could attenuate the later phases of fever that are known to be associated with strong induction in the blood-brain barrier of PGE2-synthesizing enzymes or that the fever during these phases were unaffected when the antibody was injected iv, making the interpretation of the obtained data hazardous.

Additional evidence for a role of peripheral immune cells in fever comes from a study on mice that were hybrids for the Toll-like receptor 4 (43). In that study, mice that lacked functional Toll-like receptor 4 in hematopoietic cells showed absence of the first phase of fever after iv injection of LPS (43), whereas the presence of functional receptors in hematopoietic cells in mice of mutant background rescued the initial febrile response, suggesting a critical role for hematopoietic cells for the initiation of fever. Although we here show in a similar experimental paradigm the opposite phenotypes, \textit{i.e.} intact initial fever in WT mice with a KO genotype in hematopoietic cells, and absence of initial fever in KO mice with WT hematopoietic cells, the findings in the two studies are not necessarily mutually exclusive. The obvious reconciling interpretation is that intact Toll-like receptor 4 is necessary in hematopoietic cells for the release of proinflammatory cytokines (44), which in turn act on endothelial cells in the brain to induce PGE2 release and thereby eliciting fever. The mediator acting on the endothelial cells may be IL-1 because IL-1 type 1 receptors are exclusively expressed on endothelial cells and not on perivascular cells (10) and selective genetic ablation of IL-1 type 1 receptors on brain endothelial cells yields mice refractory to the fever producing properties of IL-1 (45). It should in this context also be noted that brain macrophages are not likely to be able to respond to peripherally injected LPS because brain uptake of circulating LPS is extremely low (46).

Previous morphological studies on the expression of PG synthesizing enzymes in the brain have largely been done in rats, in which both Cox-2 and mPGES-1 have been demonstrated with immunohistochemistry and \textit{in situ} hybridization. Although the reports on which cells that express Cox-2 are contradictory, as discussed above, mPGES-1 expression has consistently been ascribed only to endothelial cells (8, 12, 13, 47). In the single previous study in the mouse in which cell specific markers were used, Cox-2 expression was shown in endothelial cells (48), and so far mPGES-1 has been demonstrated in the mouse...
brain only by in situ hybridization, without identifying the expressing cells (33). We were unable to obtain an mPGES-1 signal in the brain either by immunohistochemistry or in situ hybridization, despite previous successful stainings of rat brain tissue with both techniques (7, 11); however, our alternative approach of qPCR analysis of endothelial cells and perivascular cells isolated by flow cytometry, clearly demonstrated a strong LPS-induced induction in endothelial cells, with, in contrast, very low mRNA levels in perivascular cells, pinpointing the endothelial cells as the PGE2 producing cells in the brain vasculature.

Although the increased PGE2 levels in the brain at the peak of LPS fever, i.e. at around 3 h after the injection, are consistent with induced transcription and subsequent translation of PGE2-synthesizing enzymes, there was no evidence at the time of the initial phase of fever neither for induced Cox-2 expression in brain (or in liver or lung), nor for induced mPGES-1 transcription in brain vascular cells. This is in contrast to what has been reported in rats (16), suggesting that species differences may exist. Nevertheless, also at this time point, PGE2 levels were increased in the cerebrospinal fluid in the chimeras that displayed fever (WT→WT and KO→WT) as compared with the chimeras that were afebrile (WT→KO and KO→KO). Considering that the temperature elevation starts within less than 15 min after iv injection of LPS (Fig. 2B), the data suggest that this early PGE2 release is not dependent on newly translated prostaglandin synthesizing enzymes but occurs from a pre-existing pool. Notably, the time course of the initial phase of fever follows the dynamic of the temperature response when PGE2 is injected intracerebroventricularly (49): a rapid rise after iv injection of LPS (Fig. 2B), the data suggest that this early PGE2 release is not dependent on newly translated prostaglandin synthesizing enzymes but occurs from a pre-existing pool. Notably, the time course of the initial phase of fever follows the dynamic of the temperature response when PGE2 is injected intracerebroventricularly (49): a rapid rise followed by a somewhat slower decrease and is hence consistent with a momentary release of PGE2 and its subsequent removal from the brain. The present data are therefore consonant with the interpretation that this release, similar to that which results in the later phases of fever, occurs from central mPGES-1-expressing nonhematopoietic cells, i.e. brain vascular endothelial cells.

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