Immune-induced expression of lipocalin-2 in brain endothelial cells: relationship to interleukin-6, cyclooxygenase-2 and the febrile response

Namik Hamzic, Anders Blomqvist, Camilla Nilsberth*

Linköping University, Faculty of Health Sciences, Department of Clinical and Experimental Medicine**,** Division of Cell Biology, SE-581 85 Linköping, Sweden.

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*Corresponding author. Address: Linköping University, Faculty of Health Sciences, Department of Clinical and Experimental Medicine**,** Division of Cell Biology, SE-581 85 Linköping, Sweden. Phone: +46 10 103 7469; E-mail: camilla.nilsberth@liu.se

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Abstract

Interleukin-6 (IL-6) is critical for the febrile response to peripheral immune challenge. However, the mechanism by which IL-6 enables fever is still unknown. To characterize the IL-6 dependent fever generating pathway, we used microarray analysis to identify differentially expressed genes in the brain of lipopolysaccharide (LPS)-treated IL-6 wild type and knock-out mice. Mice lacking IL-6 displayed two-times lower expression of the lipocalin-2 gene (*lcn2*), and this difference was confirmed by real-time RT-PCR. Conversely, induction of lipocalin-2 protein was observed in brain vascular cells following i.p. administration of recombinant IL-6, suggesting a direct relationship between IL-6 and lipocalin-2. Immunohistochemical analysis also revealed that LPS-induced lipocalin-2 is expressed by brain endothelial cells and is partly co-localized with cyclooxygenase-2 (Cox-2), the rate limiting enzyme for the production of inflammatory induced prostaglandin E_2 (PGE_2), the key mediator of fever. The direct role of lipocalin-2 in fever was examined in LPS-challenged lipocalin-2 knock-out mice. In both male and female mice, normal fever responses were observed at near-thermoneutral conditions (29-30°C), but when recorded at normal room temperature (19-20°C), the body temperature of lipocalin-2 knock-out female mice displayed an attenuated and delayed fever response compared with their wild type littermates. This difference was reflected in significantly attenuated mRNA expression of Cox-2 in the brain of lipocalin-2 knock-out female mice, but not of male mice, following challenge with peripheral LPS. Our findings suggest that IL-6 influence the expression of lipocalin-2, which in turn may be involved in the control of the formation of Cox-2, and hence the central PGE_2-production. We have thus identified lipocalin-2 as a new factor in the pathway of inflammatory IL-6 signaling. The effect of lipocalin-2 in LPS-induced fever is gender-dependent and ambient temperature-specific, however the effects of lipocalin-2 on fever are discrete and thus

lipocalin-2 cannot be considered as a major mediator of the IL-6-dependent fever generating pathway.

Introduction

Fever, which is a clinical hallmark of inflammation, is an adaptive brain-regulated host defense response that is elicited by infectious pathogens and other immune stimuli. It is generally accepted that prostaglandin E_2 (PGE_2) has a central role within the brain for the initiation of the neuronally regulated fever signaling (1, 2). After an immune challenge, PGE_2 is synthesized through the induction of the enzymes cyclooxygenase-2 (Cox-2) and microsomal prostaglandin E synthase-1 (mPGES-1) and acts on neurons expressing EP_3 receptors in the thermoregulatory region of hypothalamus (2).

Proinflammatory cytokines, like interleukin-1β (IL-1β) and interleukin-6 (IL-6), are produced and released into the bloodstream upon immune challenge and are involved in eliciting the febrile response. In particular, IL-6 is critical, because neither IL-6 knock-out (IL-6 KO) mice, nor animals treated with IL-6 antiserum develop fever upon peripheral immune stimulation (3-6). However, peripherally administrated IL-6 is by itself not or only weakly pyrogenic, at least in mice and rats (3-5, 7). The latter observations are supported by studies showing that systemically administered IL-6, unlike IL-1β, does not trigger PGE_2 production in the brain as measured by the induction of Cox-2 (8, 9) or by the monitoring of PGE_2 in the cerebrospinal fluid (CSF) (10), although conflicting data exist (5).

Recent work in this laboratory has demonstrated that IL-6 KO mice, despite being afebrile upon LPS challenge, had an intact central and peripheral induction of the PGE_2 synthesizing enzymes Cox-2 and mPGES-1, and displayed similar increased concentrations of PGE_2 in the CSF as fever-mounting wild-type mice. However, when PGE_2 was administered directly into the CSF, IL-6 KO mice showed a similar dose-dependent febrile response as

wild-type (WT) mice (10, 11). We therefore suggested that IL-6 may control some factor(s) in the inflammatory cascade, which render(s) IL-6 KO mice refractory to the pyrogenic action of PGE_2, or that IL-6 is involved in the mechanisms that govern release of synthesized PGE_2 onto its target neurons (10).

In the present paper, we show that a protein previously not identified to be involved in the fever generating pathway, lipocalin-2, is controlled by IL-6. We show that lipocalin-2 is induced in the brain endothelial cells upon immune stimulation, and that this induction is attenuated in the absence of IL-6, and that, conversely, IL-6 induces the expression of lipocalin-2. Furthermore, we show that, during certain conditions, absence of lipocalin-2 attenuates Cox-2 induction to lipopolysaccharide (LPS) and the concomitant febrile response.

Materials and Methods

Animals

Adult IL-6 KO mice (The Jackson Laboratory, Bar Harbor, ME), and lipocalin-2 KO mice, and their WT littermates, all on a C57B6 background, were used. Lipocalin-2 heterozygous mice were kindly provided by Dr. Jack B. Cowland, Granulocyte Research Laboratory, Copenhagen, Denmark and originally generated by Dr. Shizuo Akira, Research Institute for Microbial Diseases, Osaka University, Japan (12). They had been backcrossed onto the C57B6 background for 10 generations before being used for the present experiments.

All animals were 9-12 weeks old at the time of experiment. They were housed one per cage in a pathogen-free facility at ambient temperature of 19-20°C, unless otherwise stated, with food and water available *ad libitum*, and on a 12-h light, 12-h dark cycle (lights on at 07:00). All experimental procedures were approved by the Animal Care and Use Committee at Linköping University.

LPS and IL-6 administration

All injections were given to awake animals as a single dose dissolved in 100 µl saline or sterile water, administered intraperitoneally 2 h into the lights-on period (9 a.m.). LPS was from *Escherichia coli* (Sigma Chemical Co., St. Louis, MO; 0111:B4) and given at a dose of 120 µg/kg body weight. Murine recombinant IL-6 (Peprotech, Rocky Hill, NJ; catalog #216-16) was given at a dose of 900 ng/mouse.

RNA extraction and hybridization

The mice were asphyxiated with CO_2 3 h or 4 h after i.p. injection of LPS and rapidly perfused transcardially with saline (NaCl, 0.9 %), before samples of the hypothalamus, liver, lung, and spleen were collected. The harvested tissues were stored in RNAlater stabilizing agent (Qiagen, Hilden, Germany) at 4°C overnight and then at -20°C until further processed. Total RNA from the hypothalamus was extracted using RNeasy Lipid Tissue Kit (Qiagen) according to the manufacturer's instructions, including deoxyribonuclease treatment with ribonuclease-free deoxyribonuclease set (Qiagen). The integrity of extracted RNA was checked by using an Agilent RNA 6000 Nano Kit and Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA). The concentrations and purity of extracted RNA were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE).

Array protocol

A mouse GeneChip 1.0 ST array (Affymetrix, Santa Clara, CA), expressing the whole mouse genome (28,853 genes) was used. Each gene was represented by approximately 27 probes spread across the full length of the gene. In brief, double-stranded cDNA was synthesized with random hexamers tagged with a T7 promoter sequence and subsequently used as a template and amplified to produce antisense cRNA. Single stranded DNA (ssDNA) was

produced in the second cycle of cDNA synthesis, using prime reverse transcription of the cRNA. Following fragmentation and labeling, ssDNA was hybridized to the GeneChip which then was stained, washed and scanned using a Fluidics 450 station and GeneChip operating software (Affymetrix). The hybridization, washing, and scanning was performed by Bioinformatics and Expression Analysis core facility at the Karolinska Institute in Huddinge, Stockholm. The raw expression values from the array represented as CEL files were uploaded and normalized using robust multi array (RMA) as a normalization method. The differentially expressed genes satisfying the conditions of fold change cutoff 1.5 and a *P*-value of < 0.05 from all of the genes probed in the GeneChip were searched out with the GeneSpring GX Software (Agilent).

Real Time RT-PCR

RNA was reversely transcribed to cDNA by random hexamer priming using PrimeScript first-strand cDNA synthesis kit (TaKaRa Bio, Shiga, Japan). Real-time RT-PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR System using TaqMan Fast Universal PCR Master Mix according to the manufacturer's instructions. The following TaqMan Gene Expression assays were used: Mm01324470_m1 (*lcn2*), Mm00478375_g1 (*ptgs2*), and Mm00452105 (*ptges*) (Applied Biosystems, Foster City, CA). As endogenous control, the glucose-3 phosphate dehydrogenase gene (*gapdh*) TaqMan Gene Expression assay Mm99999915_g1 was used (Applied Biosystems). Gene expression was calculated using the $\Delta\Delta$Ct method (Ct = threshold cycle). Each gene was normalized with the corresponding *gapdh* expression in the same sample and expressed as the fold difference in relation to vehicle-injected WT mice.

Western Blot

Mice were asphyxiated with CO_2 3 h after i.p. injection with LPS and briefly perfused transcardially with saline (NaCl, 0.9 %). The hypothalamus was quickly dissected, snap frozen and stored at -80°C until analysis. The dissected hypothalami were homogenized in lysis buffer (0.1 M PBS, 1 % nonidet P40, 0.5 % deoxycholate and 0.1 % sodium dodecyl sulfate). Before homogenization, protease inhibitor (Complete Mini; Roche, Basel, Switzerland) was added and the homogenates were centrifuged at 14,000 *g* for 20 min.

The quantitation of the total protein was determined by using BCA protein assay reagent (Pierce Biotechnology, Rockford, IL). Proteins (20 µg) were fractionated on 12 % mini-Protean TGX gels (Bio-Rad, Hercules, CA) and transferred to a Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Amersham, UK). Non-specific binding was blocked by immersing the membrane in blocking solution (TBS, pH 7.6; 0.1 % Tween-20, 2 % enhanced chemiluminescence advance blocking agent) (GE Healthcare, Little Chalfont, UK) for 1 h at room temperature. The membrane was incubated overnight at 4°C with goat anti-lipocalin-2 antibody (1:2000, 0.2 µg/ml; R&D Systems, Minneapolis, MN) followed by chicken anti-goat HRP-conjugated secondary antibody (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. The bands were visualized using an enhanced chemiluminescence advance western blotting detection kit (GE Healthcare). To restore and remove primary and secondary antibodies, the membrane was immersed in stripping buffer (100 Mm 2-mercaptoethanol, 2 % sodium dodecyl sulfate, 62.5 Mm Tris-Hcl; pH 6.7) for 30 min at 50°C, washed with buffer and then blocked for 30 min at room temperature. The membrane was then incubated with rabbit anti-GAPDH antibody (1:10,000; 200 µg/ml; Santa Cruz), overnight at 4°C, used here as a loading control, followed by donkey anti-rabbit HRP-conjugated secondary antibody (1:50,000; 200

µg/0.5 ml; Santa Cruz) for 1 h at room temperature after which proteins were visualized as described above.

Immunohistochemistry

Mice were asphyxiated by CO_2 3 h after i.p. injection with LPS or recombinant IL-6 and perfused with 0.9 % saline, followed by 4 % ice-cold paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). The brains were post-fixed for 3 h in the same fixative at 4°C and subsequently transferred to ice-cold PBS containing 30 % sucrose overnight. Sections were cut transversely at 30 µm on a freezing microtome, collected in sterile bins containing cold cryoprotectant (0.05 M sodium phosphate buffer, 30 % ethylene glycol, 20 % glycerol) and stored at -20°C until use. For single-labeling lipocalin-2 immunoreactivity, sections were incubated in goat anti-lipocalin-2 antibody (1:1000; R&D Systems) overnight at room temperature, followed by biotinylated horse anti-goat antibody (1:1000; Vector Laboratories, Peterborough, UK) and avidin-biotin complexes (1:1000; Vector). Color was developed using 3.3´-diaminobenzidine tetrahydrochloride (Sigma) containing 0.01 % H_2O_2 and 2.25 % nickel ammonium sulfate in 0.1 M sodium acetate buffer (pH 6.0) for 3 min. For dual-labeling, sections were incubated at room temperature overnight in a mixture of goat anti-lipocalin-2 antibody (1:500; R&D Systems) and either of rabbit anti-von Willebrand Factor (vWF) (1:500; Abcam, Cambridge, UK), used here as marker for endothelial cells (13,14), rat anti-mouse CD206 (1.500; Serotec, Raleigh, North Carolina), a marker for perivascular macrophages (15), or rabbit anti-Cox-2 (1.500; Santa Cruz). Secondary antibodies were Alexa Fluor 568 donkey anti-goat IgG (1:500; Invitrogen, Carlsbad, CA) and either Alexa Fluor 488 donkey anti-rabbit IgG (1:500; Invitrogen), or Alexa Fluor 488 donkey anti-rat IgG (1:500; Invitrogen). Sections were analyzed on a Nikon Eclipse E600 microscope connected to a Nikon C1 confocal unit with argon 488 nm, and HeNe 543 nm and 633 nm lasers. Control

experiments included incubation of tissue sections from control and immune-challenged animals with each antiserum alone or with secondary antisera only.

Telemetric Recordings

Mice were briefly anesthetized with 1 % isoflurane (Abbot Scandinavia, Solna, Sweden), implanted i.p. with a transmitter that records core temperature (Data Science International, St. Paul, MN), and allowed one week to recover. Body temperature recordings were started at least 1 day before immune challenge, collecting the basal temperature of each mouse to assure that it displayed normal body temperature with normal circadian variation. Baseline body temperature was calculated over an 8 hours period during the light period of the day. Following i.p. injection with the inflammatory stimulus, data were sampled during 10 s every 2 min throughout the entire observation period.

Statistics

Microarray data as well as data on lipocalin-2 protein levels were analyzed using unpaired, two-tailed Students`*t*-test. Temperature data was analyzed with two-way ANOVA followed by *post hoc* t-test, $P<0.05$ was considered statistically significant. The analysis of the fever curves was divided into the different fever phases before analysis: 0-60 min (first phase) excluded due to stress response after injection; 60-220 min second phase of fever; 220-360 min third fever phase; 360-480 min recovery phase. qPCR data for each transcript and tissue were analyzed with one-way ANOVA, followed by Bonferroni *post hoc* test with correction for multiple comparisons. A P-value < 0.05 was considered as statistically significant. Data are expressed as mean ± SEM.

Results

Microarray Data Analysis

The thermoregulatory center in the brain is found in the anterior part of the hypothalamus (16, 17). In order to examine whether there was any difference in the gene expression profile after inflammatory stimuli between WT mice and IL-6 KO mice, we conducted a genome wide microarray study on mRNA extracted from the hypothalamus. Male WT mice injected intraperitoneally with LPS (120 µg/kg body weight) displayed when compared with those injected with vehicle a dramatic alteration of the expression of several genes, as reported previously (18, 19). A total of 164 genes were altered 1.5-fold or more ($P < 0.05$). The majority of the genes were up regulated, and included a number of immune and acute-phase response genes and some chemokines, such as *ifit1, mpa2, gbp4 and cxcl10*. The top-scored up regulated and down regulated genes are listed in Table 1A.

Thus, the LPS treatment generated a robust inflammatory response as detected by the microarray system. However, when we compared the gene expression between WT and IL-6 KO mice that had been given LPS, surprisingly few genes were differentially expressed between these two genotypes (Table 1B), despite the fact that IL-6 KO mice in contrast to the WT mice do not display a febrile response to the immune challenge (10). The gene that displayed the largest difference in expression following LPS injection between the two genotypes was *lcn2* (encoding for an acute phase protein, lipocalin-2), which was twice as abundantly expressed in WT than in IL-6 KO mice.

The other two genes that displayed significant expression differences between the two genotypes were E030010A14Rik (RIKEN cDNA E030010A14 gene), with so far no established biological function, and *adamts9* (a disintegrin-like and metalloprotease domain with thrombospondin type 1 motifs 9), described to be implicated in melanoblast and limb

development (20, 21), but that also has been suggested to function as a tumor suppressor gene in some cancers (22, 23).

Induced expression of lipocalin-2 in brain and viscera after LPS administration in WT and IL-6 KO mice

Because of the known association of lipocalin-2 to inflammatory processes (12, 24, 25) as well as its involvement in thermogenesis (26), we next verified the results from the microarray analysis on the *lcn2* gene by examining the expression of *lcn2* in the hypothalamus with real-time RT-PCR 3 h after LPS injection, using the same experimental paradigm as above. The expression of *lcn2* was strongly induced in WT mice upon LPS-treatment, displaying over 200 times higher expression ($P < 0.001$) than in saline-injected mice, with no difference between sexes (Fig. 1A-B). Notably, this difference was far larger than that seen in the microarray analysis, implying differences in the sensitivity of the two methods. While there was as strong LPS elicited induction of *lcn2* also in the brains of IL-6 KO mice, it was significantly lower than in the WT mice, the difference between the genotypes being close to 3 times ($P < 0.001$) (Fig. 1A-B). This difference corresponded well with the data from the microarray study that showed a fold-change of 2.06 in *lcn2* expression between LPS treated WT and IL-6 KO mice (Table 1B).

Lipocalin-2 mRNA expression levels were also examined in the liver, lung and spleen of male WT and IL-6 KO after i.p. injection of LPS. The expression of *lcn2* in the liver was barely detectable in saline-injected mice, whereas it was strongly up regulated following treatment with LPS (close to 1000 times higher in LPS-treated WT mice than in saline-treated mice**;** $P < 0.001$) (Fig. 1C). As in the brain, the induction of *lcn2* in the liver was less pronounced in the LPS-treated IL-6 KO mice, the difference between the genotypes being close to two times ($P < 0.05$) (Fig. 1C). In the lung and spleen, *lcn2* displayed a weak

constitutive expression, which in both genotypes was moderately up regulated following treatment with LPS (Fig. 1D-E). Thus, in the lung, *lcn2* displayed close to 10 times higher expression in LPS-treated WT mice than in saline-injected WT mice ($P < 0.001$), and while the LPS induced change in expression was somewhat less pronounced in the IL-6 KO mice (around 7 times), there was no statistically significant difference between the two genotypes (Fig. 1D). In the spleen, *lcn2* displayed 20 times higher expression in WT mice treated with LPS than in saline-injected WT mice ($P < 0.001$), and while again the up regulation by LPS was slightly lower in the IL-6 KO mice, there was again no statistically significant difference between the two genotypes (Fig. 1E).

To confirm that the induced gene expression of *lcn2* seen in the hypothalamus was reflected in differences in protein levels, the hypothalami from IL-6 KO and WT male mice, harvested 3 h after i.p. injection of LPS, were analyzed by Western blot (WB). As shown in Fig. 1F, the protein levels of lipocalin-2 were significantly higher (about 2 times; $P = 0.02$) in WT mice compared with IL-6 KO mice, which hence was in line with the results from qPCR analysis.

Peripherally administrated IL-6 induces the expression of *lcn2* in brain endothelial cells

In light of the above findings, we examined whether peripherally administrated IL-6 had the capacity to induce lipocalin-2 in the brain. Male WT mice were injected i.p. either with recombinant murine IL-6 (900 ng/mouse) or vehicle and the expression of lipocalin-2 was examined by immunohistochemistry 3 h after injection. In WT mice injected with IL-6 an immune-positive staining for lipocalin-2 appeared in cells associated with the brain blood

vessels, whereas no lipocalin-2 like immunoreactivity was observed in WT mice injected with the vehicle (Fig. 2).

Lipocalin-2 is expressed in brain vascular cells and is partly co-expressed with Cox-2 after LPS challenge

The cellular distribution of lipocalin-2 in the brain upon LPS-stimulation in IL-6 KO mice and their WT littermates was studied by immunohistochemistry 3 h after LPS injection. In control animals injected i.p. with saline, no lipocalin-2 like immunoreactivity was observed. However, after LPS-challenge (120 µg/kg body weight), immune-positive staining for lipocalin-2 appeared in cells associated with the brain blood vessels in both IL-6 KO and WT mice (Fig. 3A). The labeling was preferentially localized to the perinuclear regions of cells with rounded nuclei, suggesting that the labeled cells were endothelial cells. However, to positively identify the cells that expressed lipocalin-2, sections were also stained for the vWF and CD206. Dual labeling for the lipocalin-2 and vWF revealed round lipocalin-2 labeled nuclei within vWF labeled cells (Fig. 3B), whereas dual labeling for lipocalin-2 and CD206 showed that the two markers labeled distinct cell populations (Fig. 3C). These stainings hence identified the lipocalin-2 expressing cells as endothelial cells (13-15).

As a final step we examined if lipocalin-2 and Cox-2 were expressed in the same cells. As shown in Fig. 3D, the majority of brain endothelial cells stained both for lipocalin-2 and Cox-2, although single-labeled cells also were seen. Brain sections from mice that had been given saline showed only constitutive neuronal expression of Cox-2, and no labeling of either Cox-2 or lipocalin-2 in the blood vessels.

Attenuated LPS-induced fever response in lipocalin-2 deficient females

Since IL-6 is critical for LPS-induced fever, and since, as shown here, absence of IL-6 attenuates the induced expression of lipocalin-2 in brain endothelial cells, i.e. in the cells that also express the inducible PGE_2 synthesizing enzymes Cox-2 and mPGES-1 (27, 28), we next examined if lipocalin-2 was involved in fever regulation. LPS (120µg/kg b.w.) or saline was given i.p. to WT and lipocalin-2 KO mice and the temperature response was monitored either during near-thermoneutral conditions (29-30°C) or at ambient room temperature (19-20°C).

The basal body temperature of both male and female mice was recorded one day prior to injection with LPS or saline. No significant difference was observed between gender or genotype in basal body temperature neither at near-thermoneutral conditions (36.61±0.51 male KO mice; 36.13±0.24 male WT mice; 36.59±0.40 female KO mice; 36.15±0.26 female WT mice) nor at 20°C (36.56±0.56 male KO mice; 36.22±0.27 male WT mice; 36.72±0.28 female KO mice; 36.74±0.18 female WT mice).

Intraperitoneal injection of LPS resulted in a typical triphasic fever (29, 30) with the first phase being obscured by the hyperthermia induced by handling stress associated with the injection. At an ambient temperature of 29-30°C, both genotypes displayed normal febrile response to LPS (Fig. 4A, B), however lipocalin-2 deficient males displayed higher body temperature than WT mice at the end of the observation period ($P < 0.05$). When the temperature response to LPS was recorded at 19-20°C, however, a more pronounced difference between the two genotypes was seen. Thus, while lipocalin-2 KO and WT males showed almost indistinguishable temperature responses (Fig. 4C), female lipocalin-2 KO mice displayed a significantly attenuated fever response compared with their WT littermates (Fig. 4D), starting at approximately 4 h post LPS challenge ($P < 0.05$). This difference in the fever response between the genotypes was present for 70 minutes.

Cox-2 mRNA expression is lower in lipocalin-2 deficient female mice following LPS challenge

In order to examine if the observed difference in the febrile response between lipocalin-2 KO and WT mice was due to a differential expression of Cox-2 and/or mPGES-1 in the hypothalamus, the mRNA levels of the corresponding genes were determined using real-time RT-PCR. Female lipocalin-2 KO and WT mice were injected i.p. with LPS (120 µg/kg body weight) or saline, and sacrificed 4 h later (the time point corresponding to the time point when the febrile response of the two genotypes started to differ), followed by dissection of the hypothalamus with subsequent RNA isolation and real-time RT-PCR analysis. As previously shown (10), Cox-2 and mPGES-1 mRNA were induced upon LPS stimulation (Fig. 5A-B). A trend towards lower expression of both genes was seen in LPS-treated lipocalin-2 KO mice when compared with their WT littermates, however only for Cox-2 the difference was statistically significant ($P < 0.05$) (Fig. 5A). The mRNA levels of Cox-2 in the hypothalamus were also determined in male mice devoid of lipocalin-2, using the same experimental paradigm. Cox-2 was induced in both genotypes treated with LPS, although to a lesser degree than in female mice due to higher basal mRNA expression of Cox-2 in male mice. As shown in Fig. 5C, there was no difference in Cox-2 mRNA expression between LPS-treated WT and lipocalin-2 KO male mice.

Discussion

The role of IL-6 in fever has been studied for many years, but is still elusive. While the presence of IL-6 has been shown to be necessary for the fever, IL-6 by itself seems unable to elicit a clear febrile response (3-4, 7). Here we demonstrate that IL-6 influences the expression in the brain of lipocalin-2, an acute phase protein that through binding to bacterial siderophores and hence preventing bacterial iron acquisition is an important component of the

innate immune response (12). Using a genome wide microarray analysis we show that *lcn2* is one of few genes in the brain that is differently expressed in IL-6 knock-out mice. We also show that IL-6 *in vivo* induces lipocalin-2 expression in the brain, and we demonstrate that immune challenge with LPS results in rapid and strong induction of lipocalin-2 in brain endothelial cells, being co-localized with the induced expression of the prostaglandin synthesizing enzyme Cox-2. Finally, we demonstrate that at normal ambient temperature, but not at conditions near thermoneutrality, absence of lipocalin-2 results in an attenuation of the late febrile response to LPS in female mice, and that this attenuation is associated with decreased expression of Cox-2 in brain endothelial cells. Interestingly, the late phase of the febrile response has been suggested to be elicited by mechanisms different from those that evoke the two earlier phases, because, as shown in rats, it is in contrast to the early phases attenuated by vagotomy (31), and it is accompanied by the induction of cytosolic PLA_2-α in the hypothalamus (32). Our data indicate that it also may be differentially influenced by lipocalin-2, in an ambient temperature-specific and gender-dependent manner.

The results from the microarray analysis and RT-qPCR demonstrated that IL-6 was not the only factor affecting lipocalin-2 expression. In mice devoid of IL-6, a substantial up-regulation of lipocalin-2 was still seen, although it only reached approximately one third of the expression level observed in WT mice. It can also be concluded from present results that the effect of lipocalin-2 on inflammatory induced fever is small, particularly considering the complete absence of LPS-induced fever in IL-6 KO mice (3, 10). Hence, lipocalin-2 cannot be considered as a major mediator of the IL-6-dependent fever generating pathway.

Lipocalin-2 has recently attracted considerable interest, both in relation to thermogenesis and inflammation. Thus, it was shown that lipocalin-2 deficient mice were unable to maintain their body temperature when being subjected to cold stress, hence displaying an impaired adaptive thermoregulation that was suggested to be due to the

decreased mitochondrial oxidative capacity in brown adipose tissue (BAT) and skeletal muscle (26). This is a phenotype very similar to that reported for IL-6 deficient mice, which also display significantly impaired ability to defend their core temperature during cold stress (33), and these data hence suggest that lipocalin-2 and IL-6 may be linked in the control of thermogenesis.

Furthermore, in a model with sterile abscess, lipocalin-2 was rapidly detected at high levels in plasma and was also strongly induced in the liver (34). In that study, similar to what was found in the present work, the *lcn2* induction was abrogated in the absence of IL-6. The authors also demonstrated that IL-6 induced *lcn2* in cultured hepatocytes, being in line with our *in vivo* demonstration of induced lipocalin-2 synthesis in the brain following IL-6 administration. These findings hence strongly support a direct relationship between IL-6 and lipocalin-2.

Only quite recently, the role of lipocalin-2 in the brain has been examined. In two studies, published after the present work had been undertaken, lipocalin-2 was shown to be induced in choroid plexus epithelial cells and in brain endothelial cells after immune challenge with LPS (24, 35). These findings were corroborated in the present study and taken one step further by showing that lipocalin-2 co-localized with Cox-2, the rate limiting enzyme for the production of inflammatory induced PGE_2. Lipocalin-2 also co-localized with the vWF, but not with CD206, indicating that the expression occurred exclusively in endothelial cells and not in perivascular macrophages.

The finding that the lipocalin-2 expression was influenced by IL-6, which itself is critical but not sufficient for the febrile response, and the observation that lipocalin-2 is necessary for cold induced thermogenesis, led us to examine the role of lipocalin-2 in the febrile response. While, as discussed above, lipocalin-2 KO mice displayed the same temperature elevation as WT mice in response to LPS during near-thermoneutral conditions,

female lipocalin-2 KO mice that were immune challenged at normal ambient temperature (19-20°C) displayed an attenuated febrile response which also was associated with, and hence possibly caused by, attenuated Cox-2 induction in the brain. These data indicate that lipocalin-2, and also IL-6, may influence thermogenesis at least in part by influencing Cox-2 expression. However, this issue requires further examination. While the present findings may also seem contradictory to our previous observation that Cox-2 induction (and PGE_2 levels) in the brain did not differ between IL-6 KO and WT mice (10), it should be noted that those experiments were carried out during near-thermoneutral conditions.

The mechanism of the sex-differences in temperature response can only be speculated on. It was recently reported that lipocalin-2 KO female mice had significantly reduced levels of 17β-estradiol in serum and that estrogen receptor α was down regulated in these mice (36), illustrating a connection between lipocalin-2 and gender specific hormones. A relationship between the levels of female gonadal hormones and Cox-2 expression and fever has been demonstrated, however in way that is opposite to the findings in the present study (37, 38).

The precise role of lipocalin-2 in brain endothelial cells and its relation to the expression of Cox-2 in these cells remains to be clarified. However, an induction of Cox-2 has been demonstrated in lipocalin-2 treated astrocytes (39), which hence is in line with our observation that mice lacking lipocalin-2 displayed reduced Cox-2 expression in the hypothalamus. The influence of lipocalin-2 on Cox-2 could be an autocrine effect, mediated by lipocalin receptors in endothelial cells (24, 39), because there is no evidence that lipocalin-2 influences the immune-induced mRNA expression of pyrogenic cytokines that in turn are known to induce Cox-2, such as IL-1β (24). The lipocalin-2 receptors, megalin and brain type organic cation transporter, have been shown to be expressed both constitutively (24) and in response to the inflammatory stimuli (39) in brain endothelial cells, and they have been described to mediate the uptake of positively charged molecules bound to lipocalin-2 (40, 41).

It remains to be clarified, what role, if any, such cation transport may have for the centrally elicited inflammatory response.

In conclusion we have identified lipocalin-2 as a new factor in the pathway of inflammatory IL-6 signaling across the blood-brain barrier. Our data show that lipocalin-2 is induced in brain endothelial cells, known to express PGE_2-synthesizing enzymes upon systemic immune challenge (28, 42), and that this expression in part is under the control of IL-6. While our data also indicate that lipocalin-2 is involved in the immune-induced thermogenesis, its contribution is minor and the critical role of IL-6 for the febrile response cannot be explained by its induction of lipocalin-2.

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Figure Legends

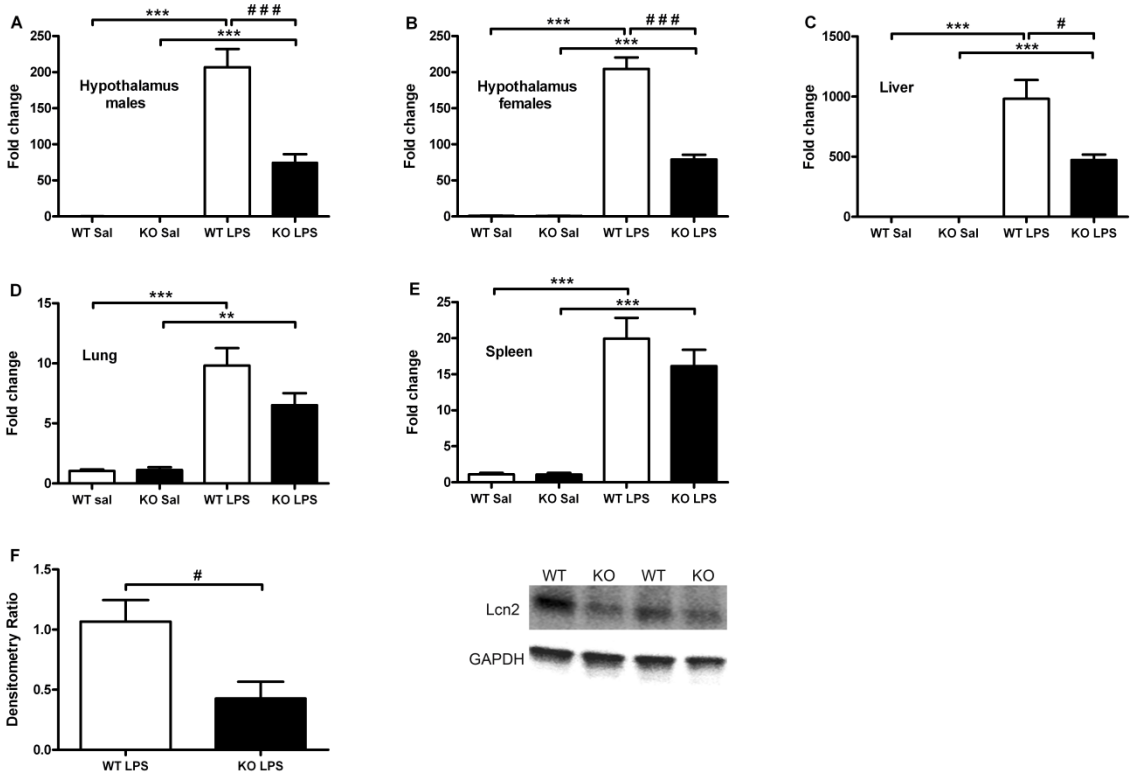

Fig. 1. LPS induces the expression of lipocalin-2 both centrally and peripherally.

(A-B) Relative expression, as determined by qPCR, of lipocalin-2 mRNA in the hypothalamus of [A] male, and [B] female WT and IL-6 deficient mice 3 h following treatment with intraperitoneal LPS (120 µg/kg b.w.) or saline. (C-E) Relative expression of lipocalin-2 mRNA in the liver [C], lung [D], and spleen [E] of male WT and IL-6 deficient mice. WT and IL-6 deficient mice are represented by white and black bars, respectively. $n = 7$ in LPS treated groups, except for lipocalin-2 mRNA in hypothalamus of female mice where $n = 3$ and $n = 6$ for WT and IL-6 deficient mice, respectively. $n =$ 3-4 in saline treated groups. Fold changes are relative to values obtained from WT mice given saline. (F) Densitometric quantitation of Western blot for lipocalin-2 in the hypothalamus of LPS-treated male WT and IL-6 deficient mice 3 h post injection. $n = 7$ in both groups. The qualitative Western blots shows lipocalin-2 protein expression in two of a total of seven LPS-treated mice of each genotype. Values are normalized against GAPDH. $^{**}P < 0.01$, and $^{***}P < 0.001$ between animals of the same genotype treated with LPS and saline, respectively. $\#P < 0.05$, and $\#\#\#P < 0.001$ between animals of the two different genotypes treated with LPS.

Fig. 2

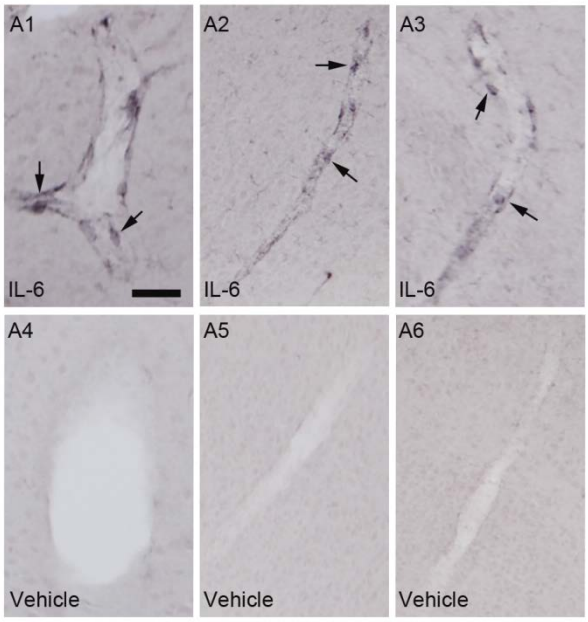

Fig. 2. Recombinant murine IL-6 induces the expression of lipocalin-2 in the brain.

Immunohistochemical detection of lipocalin-2 in brain vascular cells in WT mice 3 h following intraperitoneal injection of recombinant murine IL-6 (900 ng/mouse) [A1-A3] or vehicle [A4-A6]. Arrows indicate cells that are immune-positive for lipocalin-2. Scale bar = 100 μm.

Fig. 3

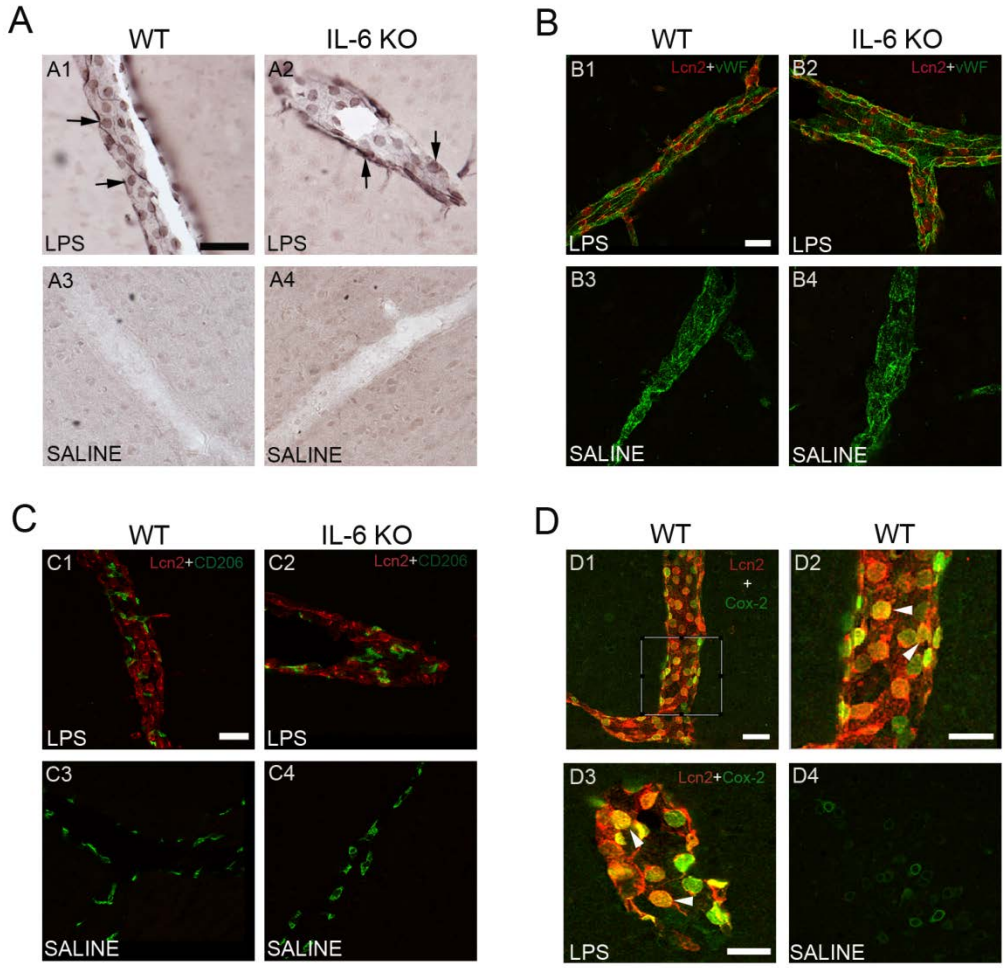

Fig. 3. Lipocalin-2 is expressed in brain endothelial cells and co-localized with Cox-2.

(A) Immunohistochemical demonstration of lipocalin-2 in brain vascular cells in WT and IL-6 KO mice 3 h following intraperitoneal injection of LPS (120 µg/kg b.w.) [A1 and A2] or saline [A3 and A4]. Black arrows point at cells immunoreactive for lipocalin-2. (B-D) Confocal images of brain vascular cells showing lipocalin-2 (Lcn; red) and [B] the von Willebrand factor (vWF; green), [C] an endothelial cell marker, CD206 (green), a marker for perivascular macrophages, and [D] cyclooxygenase-2 (Cox-2; green). B1 and B2, C1 and C2, D1 and D3 are from mice injected intraperitoneally with LPS (120 µg/kg b.w.), and B3 and B4, C3 and C4, and D4 are from mice injected with saline. Croped image of D1 is shown in D2.White arrows in D point at endothelial cells that express both Cox-2 and lipocalin-2. Scale bar = 20 µm in [A], 50 µm in [B, C, D1 and D4], and 30 µm in [D2 and D3].

Fig. 4

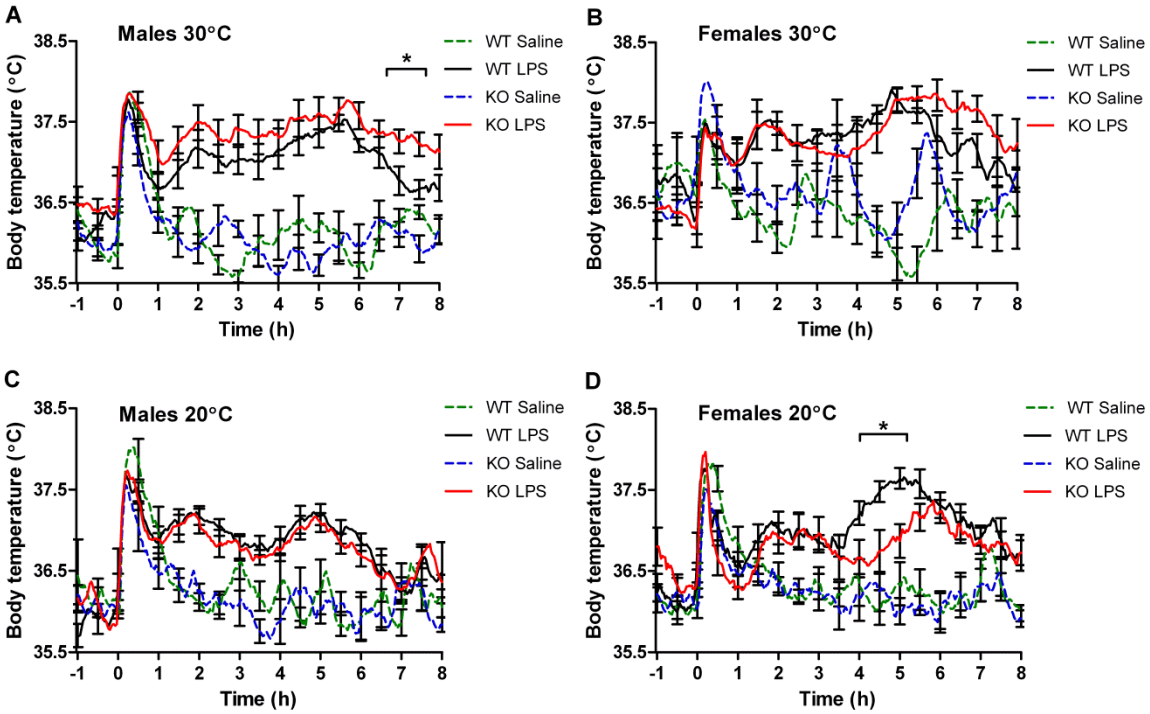

Fig. 4. Lipocalin-2 deficient female mice display an attenuated febrile response upon peripheral LPS challenge.

(A-B) Telemetric recordings of body temperature in [A] lipocalin-2 KO male mice (n = 8), and [B] lipocalin-2 KO female mice (n = 6), and their WT littermates (n = 8 and 4) at conditions near thermoneutrality (29-30°C) following injection of LPS (120 µg/kg b.w.). Control mice were given saline (n = 4-7). (C-D) Telemetric recordings of body temperature in [C] lipocalin-2 KO male mice (n = 9), and [D] lipocalin-2 KO female mice (n = 7), and their WT littermates (n = 8 and 7 respectively) at normal room temperature (19-20 °C) following intraperitoneal injection of LPS (120 µg/kg b.w). Control mice were given saline (n = 4-8). Error bars indicate SEM. *P < 0.05 refers to a comparison between WT and lipocalin-2 KO mice treated with LPS.

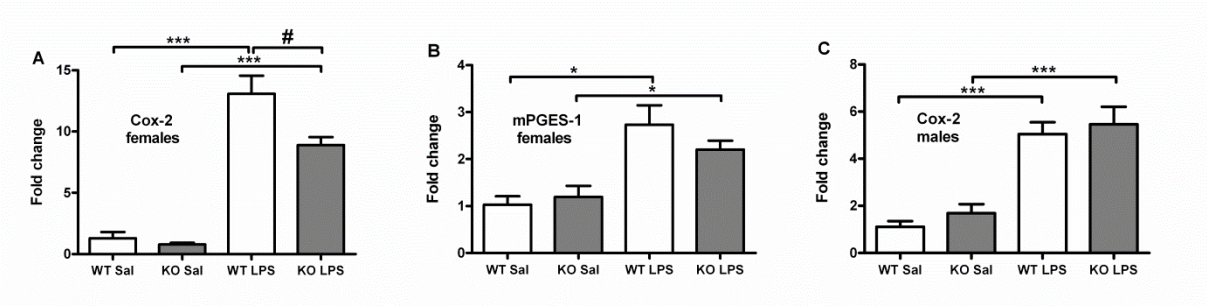

Fig. 5. Cox-2 but not mPGES-1 is differently expressed in female mice that lack lipocalin-2.

(A-B) Relative expression of [A] Cox-2 mRNA and [B] mPGES-1 mRNA in the hypothalamus of WT and lipocalin-2 KO female mice 4 h after intraperitoneal injection of LPS (120 µg/kg b.w) or saline. (C) Relative expression of Cox-2 mRNA in the hypothalamus of WT and lipocalin-2 KO male mice 4 h following treatment with intraperitoneal LPS (120 µg/kg b.w.) or saline. WT and lipocalin-2 deficient mice are represented by white and grey bars, respectively. $n = 8$ in all LPS groups except for Cox-2 mRNA in the hypothalamus of male WT and lipocalin-2 KO mice where $n = 6$. $n = 4$-6 in saline treated groups. Fold changes are relative to values obtained from WT mice given saline. *$P <$ 0.05, and ***$P < 0.001$ between animals of the same genotype treated with LPS and saline, respectively. #$P < 0.05$ between animals of the two different genotypes treated with LPS.

Table 1

Differentially expressed genes (> 1.5-fold, $P < 0.05$) in the hypothalamus of LPS-treated WT mice (n = 7) compared with vehicle injected controls (n = 4). Genes was classified as up regulated if the expression was higher in LPS-treated WT mice than in the vehicle injected controls, and as down regulated if the expression in WT mice treated with LPS was lower than in mice that received vehicle only. Additional LPS regulated genes are listed in Table S1.

Gene	**Fold Change**	***P*-value**	**Regulation**
cxcl10	29.5	5.3×10^{-10}	Up
rsad2	10.6	6.6×10^{-10}	Up
lcn2	10.5	8.4×10^{-9}	Up
gbp5	8.8	3.9×10^{-10}	Up
gbp2	8.0	1.0×10^{-8}	Up
gbp4	7.5	2.3×10^{-6}	Up
ifit1	6.5	7.5×10^{-8}	Up
tgtp	5.5	1.8×10^{-7}	Up
mpa2	5.4	9.3×10^{-9}	Up
slco1a4	2.7	1.9×10^{-6}	Down
cldn5	2.2	7.1×10^{-6}	Down
slc40a1	1.9	2.0×10^{-4}	Down
ranbp3l	1.9	4.7×10^{-3}	Down

Table 2

Differentially expressed genes (> 1.5-fold, $P < 0.05$) in the hypothalamus of LPS-treated WT mice (n =7) compared with IL-6 KO mice (n = 7). Data from mice 3 h after LPS treatment are shown. Genes was classified as down regulated if the expression is lower in LPS treated IL-6 KO mice than in LPS-treated WT mice.

Gene	**Fold Change**	***P*-value**	**Regulation**
lcn2	2.1	7.5×10^{-4}	Down
E030010A14Rik	1.6	5.2×10^{-4}	Down
adamts9	1.5	2.4×10^{-3}	Down