Immune-Induced Fever Is Mediated by IL-6 Receptors on Brain Endothelial Cells Coupled to STAT3-Dependent Induction of Brain Endothelial Prostaglandin Synthesis

Anna Eskilsson, Elahe Mirrasekhian, Sylvie Dufour, Markus Schwaninger, David Engblom and Anders Blomqvist

Original Publication:

Anna Eskilsson, Elahe Mirrasekhian, Sylvie Dufour, Markus Schwaninger, David Engblom and Anders Blomqvist, Immune-Induced Fever Is Mediated by IL-6 Receptors on Brain Endothelial Cells Coupled to STAT3-Dependent Induction of Brain Endothelial Prostaglandin Synthesis, 2014, Journal of Neuroscience, (34), 48, 15957-15961.
http://dx.doi.org/10.1523/JNEUROSCI.3520-14.2014

Brief Communication

Immune-induced fever is mediated by IL-6 receptors on brain endothelial cells coupled to STAT3 dependent induction of brain endothelial prostaglandin synthesis

Anna Eskilsson[1], Elahe Mirrasekhian[1], Sylvie Dufour[2], Markus Schwaninger[3], David Engblom[1], Anders Blomqvist[1]

[1]Division of Cell Biology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, S-581 85 Linköping, Sweden, [2]Institut Curie/CNRS UMR144, Paris, France; [3]Institute of Experimental and Clinical Pharmacology and Toxicology, University of Lübeck, 23538 Lübeck, Germany

Abbreviated title: Endothelial IL-6 signaling mediates fever

18 pages, 3 figures, 198 words in Abstract, 401 words in Introduction, 1106 words in Discussion. Total number of words (Abstract, Introduction, Materials & Methods, Results, Discussion, References and Figure Legends): 4492

Conflict of interest: The authors declare no competing financial interests.

Acknowledgments: This study was supported by the Swedish Medical Research Council (A.B., D.E.), The Swedish Cancer Foundation (A.B.), The European Research Council (starting grant to D.E.), The Knut and Alice Wallenberg Foundation (D.E.), The Swedish Brain foundation (A.B., D.E.), and the County Council Östergötland (A.B., D.E).

Correspondence: Dr. Anders Blomqvist, Division of Cell Biology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, S-581 85 Linköping, Sweden. E-mail: anders.blomqvist@liu.se

The cytokine IL-6, which is released upon peripheral immune challenge, is critical for the febrile response, but the mechanism by which IL-6 is pyrogenic has remained obscure. Here we generated mice with deletion of the membrane bound IL-6 receptor α (IL-6Rα) on neural cells, on peripheral nerves, on fine sensory afferent fibers, and on brain endothelial cells, respectively, and examined its role for the febrile response to peripherally injected lipopolysaccharide. We show that IL-6Rα on neural cells, peripheral nerves and fine sensory afferents are dispensable for the lipopolysaccharide-induced fever, whereas IL-6Rα in the brain endothelium plays an important role. Hence deletion of IL-6Rα on brain endothelial cells strongly attenuated the febrile response, and also led to reduced induction of the prostaglandin synthesizing enzyme Cox-2 in the hypothalamus, the temperature regulating center in the brain, as well as reduced expression of SOCS3, suggesting involvement of the STAT signaling pathway. Furthermore, deletion of STAT3 in the brain endothelium also resulted in attenuated fever. These data show that IL-6, when endogenously released during systemic inflammation, is pyrogenic by binding to IL-6Rα on brain endothelial cells to induce prostaglandin synthesis in these cells, probably in concerted action with other peripherally released cytokines.

Fever is a hallmark of peripheral inflammation and an adaptive response to the presence of pathogenic agents (Kluger, 1991). Prostaglandin E_2 (PGE_2), acting on prostaglandin E receptor type 3 (EP_3) expressing neurons in the preoptic area of the hypothalamus, is the principal mediator of fever (Engblom et al., 2003; Lazarus et al., 2007), and is synthesized in the presence of peripherally released cytokines with thermogenic properties, such as interleukin (IL)-1 and IL-6 (Conti et al., 2004). The latter has been shown to be critical for the febrile response, because mice with global deletion of the *Il6* gene do not develop fever upon peripheral immune stimulation (Chai et al., 1996; Kozak et al., 1998; Nilsberth et al., 2009). However, the mechanism by which IL-6 mediates fever is not clear. Whereas studies using genetically modified animals have shown that IL-1 elicits fever by inducing PGE_2 synthesis in brain endothelial cells (Ching et al., 2007; Ridder et al., 2011; Wilhelms et al., 2014) similar functional studies on the role of IL-6 for the febrile response have been lacking. Receptors for IL-6 exist in two forms, a soluble receptor, sIL-6R and a membrane bound receptor, IL-6Rα. The latter has been shown to be expressed in the brain parenchyma, as well as in the brain microvasculature (Vallieres and Rivest, 1997), but also on peripheral nerves (Gadient and Otten, 1996), and at several of these locations its expression is induced by immune challenge (Vallieres and Rivest, 1997), implying that immune-induced IL-6 could act at several different sites to elicit the variety of brain responses that are associated with this cytokine.

Here, we examined the role of IL-6Rα on different cell types for the generation of inflammatory fever. Using mice with floxed IL-6Rα and tissue specific Cre-lines we generated mice with deletion of the IL-6Rα in sensory nerves, neural cells, and brain endothelial cells, and studied their febrile response to peripheral immune challenge. Based on the obtained results we further examined the relationship between IL-6Rα on brain endothelial cells and the induction of

the prostaglandin synthesizing enzyme cyclooxygenase (Cox)-2 and the inhibitor of the STAT3 transcription factor SOCS3 in these cells, and we examined the febrile response in mice with endothelial specific deletion of STAT3. Our data show that IL-6, when endogenously released during systemic inflammation, is pyrogenic by binding to IL-6Rα on brain endothelial cells to induce prostaglandin synthesis in these cells via the STAT3 signaling pathway.

Material and Methods

Animals

Mice of either sex with tissue specific deletions of IL-6Rα were created by crossing mice possessing *loxP* sites flanking exons 4-6 of the interleukin-6 receptor alpha chain (*Il6ra*) (Jackson Laboratory, Bar Harbor, ME) with mice expressing Cre recombinase from the *Nestin* promoter (expressed in the central and peripheral nervous system), the endogenous *Trpv1* locus (expressed by nociceptors in primary sensory ganglia, as well as by some cell groups in the hypothalamus; Cavanaugh et al., 2011) (both from Jackson), the *human tissue plasminogen activator* (*HtPa*) (expressed by neural crest structures; Pietri et al., 2003), or a tamoxifen inducible $CreER^{T2}$ from the *Slco1c1* promoter (expressed in the cerebrovascular endothelium; Ridder et al., 2011). Mice with endothelial specific deletion of the STAT3 were generated by crossing *Slco1c1 Cre ERT2* mice with mice with *loxP* on the 5'-side of exon 18 and on the 3'-side of exon 20 of the *Stat3* gene (Jackson). The *Slco1c1 Cre ERT2* line was also crossed with Cre reporter line expressing a *Gt(ROSA)26Sor* locus with a *loxP*-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant (tdTomato) (Jackson).

The mice strains were all held on a C57/Bl6 background. Gene deletion in mice with the *Slco1c1 Cre ERT2* promoter was induced by ip injection of Tamoxifen (1 mg Tamoxifen diluted

in a mixture of 10% ethanol and 90% sunflower seed oil twice a day for five days) at least 5 weeks prior to further experiments. In each set of experiments, the mice in different subgroups were matched for sex and age. All animal experiments were approved by the Linköping Animal Care and Use Committee and followed international guidelines.

Telemetric temperature recordings

Mice were anesthetized with isoflurane (Abbot Scandinavia, Solna, Sweden), implanted ip with a transponder that records core body temperature (Mini Mitter, Bend, OR), and then housed in a room in which the ambient temperature was set to 29°C, providing near-thermoneutral conditions (Rudaya et al., 2005), and on a 12h light/dark cycle (light on at 7 a.m.). At least one week after the implantation of the transponder the animals were injected ip with bacterial wall lipopolysaccharide (LPS) from Escherichia coli (Sigma-Aldrich, St. Louis, MO; O111:B4; 120 µg/kg body weight) diluted in 100 µl saline, or with saline only. After a washout period of 7-10 days saline injected animals were injected with LPS as described above, and LPS injected animals were injected with saline. One group of mice were implanted with an indwelling peritoneal catheter that was exteriorized at the back of the neck and connected to a swivel system, as described previously for iv catheters (Engström et al., 2012). Three days after the implantation the animals were injected with LPS or saline, as above. LPS/saline injections were done at around 9 a.m. and the recordings performed during the light-on period.

Quantitative Real Time PCR

Mice were injected ip with 120 µg/kg LPS (or vehicle) and killed 3 h later by asphyxiation with CO_2. The hypothalamus was cut out as described previously (Reyes et al., 2003), placed in RNA later stabilization reagent (Qiagen, Hilden, Germany), and kept at -70ºC until further processing.

RNA was extracted with RNeasy Universal Plus kit (Qiagen) and reverse transcription was done with High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Foster City, CA). qPCR was performed using Gene Expression Master Mix (Applied Biosystems) on a 96-well plate (7900HT Fast RT-PCR system; Applied Biosystems). TaqMan assays used were for *IL6ra*: Mm00439653_m1; for *Ptgs2* (Cox-2): Mm00478374_m1; for *Nfkbia* (Iκb): Mm00477800_g1; for *Socs3:* Mm00545913_s1; and for *GAPDH*: Mm99999915_g1 (Applied Biosystems).

Immunohistochemistry

The immunohistochemical procedures were carried out according to standardized protocols (Engström et al., 2012).The primary antibody was rabbit anti-Cox-2 (1:500; sc-1747 M-17; Santa Cruz Biotechnologies, Santa Cruz, CA), which was detected with Alexa Fluor 488 donkey anti-rabbit (1:500, Life Technologies, Carlsbad, CA).

Statistics

Statistical analyses were done in Graph Pad Prism (GraphPad Software, La Jolla, CA), using a two-way ANOVA followed by Bonferroni's *post-hoc* test for multiple comparisons. Results were considered significant when $P < 0.05$.

Results

IL-6Rα on neural cells, including sensory nerves, is dispensable for the febrile response to LPS

Both mice with deletion of IL-6Rα in neural cells (IL-6Rα$^{\Delta Nestin}$) and their wild-type (WT) littermates (IL-6Rα$^{fl/fl}$) showed a prominent rise of their body temperature after ip injection of LPS (120 µg/kg body weight) that peaked around 5-6 h post injection (Figure 1a). A similar temperature response was seen in mice with deletion of IL-6Rα in peripheral nerves (IL-

6Rα$^{\Delta HtPa}$; Figure 1b), as well as in mice with deletion of the IL-6Rα in fine sensory nerves (IL-6Rα$^{\Delta TRPV1}$; Figure 1c). Although there were no statistically significant differences in the temperature responses to LPS between the genetically modified animals and their WT littermates in any of the three mouse lines examined, there was in all of them a tendency to a reduced temperature response to LPS in the genetically modified mice during the first 3 h post-injection (Figures 1a-c), corresponding to the second phase of fever (Rudaya et al., 2005). Because these early responses are difficult to properly examine in models in which the handling stress causes a prominent initial stress induced hyperthermia (the rapid initial temperature peak in all traces in Figures 1a-c), we also examined the febrile response to LPS after injection into an indwelling peritoneal catheter, whose outer end was accessible outside the animals cage. Using this approach, in which no handling stress interfered with the body temperature recordings, no attenuation of the febrile response was seen in the genetically modified mice (Figure 1d).

Deletion of IL-6Rα on brain endothelial cells attenuates the febrile response to LPS

Next we examined the febrile response in mice with deletion of the IL-6Rα in brain endothelial cells (IL-6Rα$^{\Delta Slco1c1}$). As shown in Figure 2a the genetically modified mice displayed an attenuated temperature response to ip injected LPS compared to their WT littermates. Statistical analysis of the temperature differences during 3 to 8 h post injection (the 8 h time point was chosen as cut off because at later time points body temperature start to rise because of increased activity of the mice in anticipation of the dark period as seen in the temperature recording from the saline injected control mice; Figure 2a) showed significant genotype ($F_{1,60}$ = 6.14; P = 0.0160), treatment ($F_{1,60}$ = 35.51; P < 0.0001), and interaction ($F_{1,60}$ = 45.31; P = 0.0247) effects.

Post hoc test showed significant differences between LPS treated IL-6Rα$^{\Delta Slco1c1}$ and WT mice (P = 0.0025).

Mice with deletion of IL-6Rα on brain endothelial cells show reduced induction of Cox-2 in the hypothalamus after immune challenge

We next examined if the attenuated fever in IL-6Rα$^{\Delta Slco1c1}$ mice was associated with reduced induction in the hypothalamus of Cox-2, the rate limiting enzyme for the synthesis of PGE_2 (Li et al., 1999; Engström Ruud et al., 2013). qPCR analysis showed that the immune induced expression of *Ptgs2* (encoding Cox-2) in the hypothalamus of IL-6Rα$^{\Delta Slco1c1}$ mice was much reduced, compared to that seen in WT (IL-6Rα$^{fl/fl}$) littermates (Figure 2b; genotype: $F_{1,31}$ = 14.47, P = 0.006; treatment: $F_{1,31}$ = 96.75, P = 0.0001; interaction: $F_{1,31}$ = 1.52, P = 0.2275; LPS IL-6Rα$^{\Delta Slco1c1}$ vs LPS WT: P = 0.0011). The IL-6Rα$^{\Delta Slco1c1}$ mice also showed reduced induction of *Il6rα*, as expected (Figure 2c; genotype: $F_{1,31}$ = 9.16, P = 0.0049; treatment: $F_{1,31}$ = 6.13, P = 0.0189; interaction: $F_{1,31}$ = 1,28, P = 0.2657; LPS IL-6Rα$^{\Delta Slco1c1}$ vs LPS WT: P = 0.0066). Cox-2 is expressed by endothelial cells upon immune challenge (Cao et al., 1996; Engström et al., 2012). Cox-2 immunohistochemistry on hypothalamic sections from mice that expressed red fluorescent protein (tdTomato) in cells recombined by *Slco1c1-CreER^{T2}* verified that Cre-induced recombination and deletion of the IL-6Rα occurred in Cox-2 expressing endothelial cells, because most of the Cox-2 expressing cells also expressed tdTomato (Figure 2d). Taken together, these data imply that the reduced Cox-2 expression seen in the IL-6Rα$^{\Delta Slco1c1}$ mice, is directly related to impaired IL-6Rα activity in endothelial cells.

Induction of Cox-2 by IL-6/IL-6Rα involves the STAT3 system

To further analyze the intracellular signaling pathway by which IL-6/IL-6Rα induces the expression of Cox-2 we examined to what extent deletion of the IL-6Rα in endothelial cells affected the immune-induced expression of *Nfkbia*, encoding IκB and an index of NFκB activation (Cheng et al., 1994), or *Socs3*, encoding a protein in the STAT inhibitor family (Starr et al., 1997). Whereas both IL-6Rα$^{\Delta Slco1c1}$ mice and their WT littermates showed similar LPS-induced expression of *Nfkbia* in the hypothalamus (Figure 3a), there was attenuated expression of *Socs3* in the mutant mice (Figure 3b; genotype: $F_{1,31} = 4.71$, $P = 0.0379$; treatment: $F_{1,31} = 344.69$, $P < 0.0001$; interaction: $F_{1,31} = 13.41$, P = 0.0009; LPS IL-6Rα$^{\Delta Slco1c1}$ vs LPS WT: $P = 0.0002$). Because the data above suggested that deletion of IL-6Rα in brain endothelial cells affects signaling in the STAT3 pathway, we finally generated mice with deletion of *Stat3* in brain endothelial cells (by crossing mice with floxed *Stat3* alleles with *Slco1c1-CreER*^{T2} mice) and examined their febrile response to peripheral immune challenge. We found that such mice showed an attenuated temperature elevation 3-8 h after ip injection of LPS, similar to that seen in IL-6Rα$^{\Delta Slco1c1}$ mice (Figure 3c; genotype: $F_{1,41} = 5.94$, $P = 0.0192$; treatment: $F_{1,41} = 45.62$, $P < 0.0001$; interaction: $F_{1,41} = 3.37$, $P = 0.0738$; LPS STAT3$^{\Delta Slco1c1}$ vs LPS WT: $P = 0.0111$).

Discussion

The present findings show that the pyrogenic effect of IL-6 is exerted by its binding to IL-6 receptors on brain endothelial cells, and that the ligand binding in turn leads to induced expression of the prostaglandin synthesizing enzyme Cox-2 via intracellular signaling that involves the STAT3 pathway. In contrast, IL-6 receptors on neural cells, including peripheral nerves are not involved.

The role of IL-6 for the generation of fever has long been obscure. On the one hand, IL-6 is critical for the febrile response, since genetic deletion of IL-6 or its neutralization blocks fever (Chai et al., 1996; Kozak et al., 1998; Cartmell et al., 2000; Nilsberth et al., 2009), but, on the other hand, IL-6 by itself is not or only weakly pyrogenic, at least in rodents (Wang et al., 1997; Cartmell et al., 2000; Rummel et al., 2006; Nilsberth et al., 2009). Furthermore, the relation between IL-6 and pyrogenic Cox-2 induction has not been clear. IL-6 signaling has been suggested to be upstream, downstream or parallel to the prostaglandin production (Kagiwada et al., 2004; Rummel et al., 2006; Nilsberth et al., 2009). Neutralizing antibodies directed against IL-6 have been shown to attenuate cerebral Cox-2 induction in response to localized peripheral inflammation (Rummel et al., 2006) but mice lacking IL-6 show Cox-2 induction in the brain endothelium (but no fever) in response to ip injection of LPS (Nilsberth et al., 2009) and intravenous IL-1 injections (Kagiwada et al., 2004). The present study unequivocally shows that IL-6 is upstream of the pyrogenic Cox-2 induction and indicates that previous results from mice with global knockout of IL-6 might be confounded by mechanisms compensating for the mutation.

The previous findings that ip injection of IL-6 failed to induce Cox-2 expression in the brain, which is consistent with the absence of a pyrogenic response (Wang et al., 1997; Cartmell et al., 2000; Rummel et al., 2006; Nilsberth et al., 2009), may seem contradictory to the present findings that reduced IL-6 receptor expression in the brain endothelium resulted in attenuated Cox-2 expression and attenuated fever. However, it has been suggested that for IL-6 to exert a pyrogenic effect, there has to be a concomitant action of other cytokines such as IL-1. Thus, when a relatively high dose of IL-6 was injected into rats, it generated no fever; however, when injected together with a low, by itself non-pyrogenic dose of IL-1β, fever arose (Cartmell et al.,

2000). In the present experimental paradigm, in which LPS was given as immune stimulus, there is peripheral release of both IL-1β and IL-6 (Elander et al., 2009). Both these cytokines act on brain endothelial cells to induce Cox-2 expression and fever, but whereas the IL-1 receptor (IL-1R1) signals via the p38 MAPK and c-Jun pathway, activation of the IL-6 receptor leads to Cox-2 expression through activation of STAT3 (present study; see also Rummel et al., 2006). Taken these observations together, it is likely that for a febrile response to occur several intracellular signaling pathways have to be activated by the concerted action on the brain endothelium of several peripherally released cytokines. Thus, although the present study shows that deletion in the brain endothelium of STAT3, on which IL-6 signaling is dependent (Heinrich et al., 2003), resulted in attenuated fever, activation of this signaling pathway alone is not sufficient for eliciting Cox-2 induction and prostaglandin synthesis, but requires also the activation of other Cox-2 inducing pathways. Notably, the dependence of IL-6 for LPS-induced fever is dose-dependent. Whereas IL-6 knockout mice show no fever to a low or moderate dose of LPS such as the one administered in the present study (Chai et al., 1996; Kozak et al., 1998; Nilsberth et al., 2009), they display a febrile response when given a very high dose of LPS (Kozak et al., 1998). These data suggest that the IL-6 signaling is critical for the febrile response to a moderate systemic immune challenge, but that it can be bypassed during severe sepsis, possibly by the redundant activation of other pyrogenic cytokines that similar to IL-6 signal via the gp130 adaptor protein (Kozak et al., 1998).

In addition to cytokine release into the blood stream, peripheral injection of LPS elicits elevated levels of cytokines, including IL-6, in the CNS (e.g. Elander et al., 2009). However, with the proviso that constitutive gene deletions did not induce compensatory mechanisms in the animals, the present study demonstrates that IL-6 receptors on nerve cells do not contribute to the

febrile response. This is also of interest considering the plethora of studies showing that IL-6 injected intracerebroventricularly triggers febrile responses, which can be abolished by pretreatment with Cox inhibitors (e.g. LeMay et al., 1990; Chai et al., 1996). The present findings thus imply that the temperature response seen following icv injection of IL-6 most likely is due to ligand binding on endothelial cells.

Because the febrile response, as well as the Cox-2 induction, was not completely blocked in mice in which in the IL-6Rα was deleted from brain endothelial cells, it is possible that other non-neural cells in the brain, such as microglial cells, also could play a role. However, as we previously reported, the recombination in the brain endothelial cells elicited by the *Slco1c1 Cre ERT2* line may not be complete; when the Cox-2 gene was targeted about 15% of the Cox-2 protein induction remained (Wilhelms et al., 2014). It is conceivable that remaining IL-6Rα expression in endothelial cells accounts for the residual fever (and Cox-2 expression) seen in the present study.

It has been shown that several centrally elicited disease symptoms can be alleviated by transection of peripheral nerves, suggesting the presence of neuroimmune afferent neural pathways (Quan, 2014). For example, vagotomy was found to block LPS-induced hyperalgesia and feeding depression (Bret-Dibat et al., 1995). Furthermore, sciatic nerve transection abolished neuronal activation in a central autonomy relay nucleus, the paraventricular nucleus of the hypothalamus, that was elicited by a localized peripheral inflammation (Belevych et al., 2010). Fever has also been ascribed to the activation of peripheral nerves (Ross et al., 2003); however, in the present study there was no attenuation of the febrile response in mice with deletion of the IL-6Rα in peripheral nerves in general (IL-6Rα$^{\Delta HtPa}$) or specifically in nociceptors in primary

sensory ganglia (IL-6R$\alpha^{\Delta TRPV1}$). Thus, any febrile response elicited by primary afferent signaling is not dependent on a direct effect of IL-6 on such nerves.

In conclusion, we show here that IL-6 exerts its pyrogenic effect by binding to IL-6 receptors on brain endothelial cells, which, in the context of a systemic inflammation with release of other cytokines that also act on the brain endothelium, leads to induced prostaglandin synthesis by these cells.

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Figures

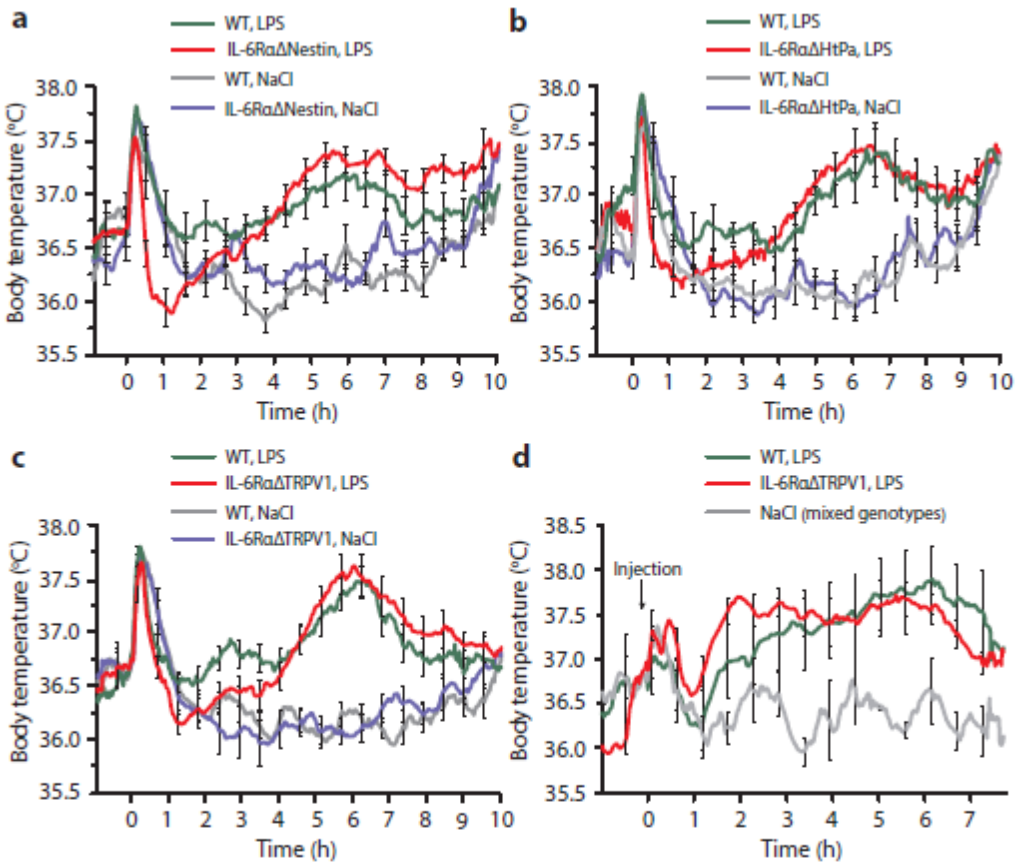

Figure 1

Figure 1. The febrile response to LPS is not dependent on IL-6Rα on nerve cells. **a-c**, IL-6Rα$^{\Delta Nestin\text{-}Cre}$ mice (***a***), IL-6Rα$^{\Delta HtPa}$ mice (***b***), and IL-6Rα$^{\Delta TRPV1}$ mice (**c**) injected ip with 120 µg/kg LPS showed a temperature response similar to their WT (IL-6Rα$^{fl/fl}$) littermates. In (***a***) *n* = 12-14, in (***b***) *n* = 5 in WT mice and 9 in L-6Rα$^{\Delta HtPa}$ mice, and in (***c***) *n* = 13-14. ***d***, IL-6Rα$^{\Delta TRPV1}$ mice and their WT littermates showed no difference in the early phases of febrile response to immune challenge with LPS via indwelling intraperitoneal catheter that permitted injecting the animals without inducing handling stress hyperthermia. *n* = 4-5.

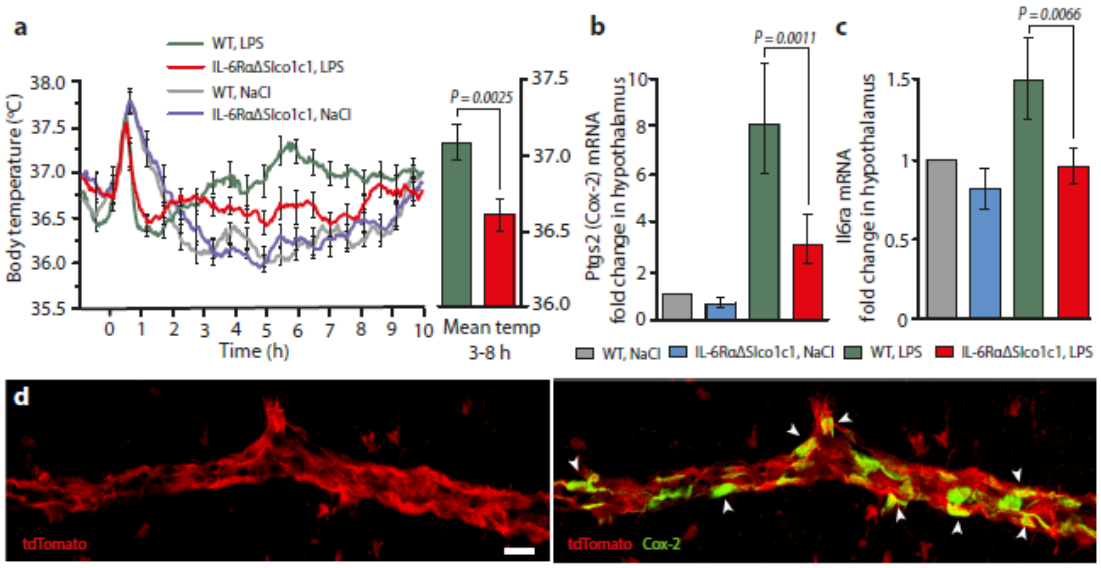

Figure 2

Figure 2. Deletion of IL-6Rα on brain endothelial cells results in attenuated fever and hypothalamic Cox-2 expression. ***a***, Blunted febrile response in IL-6Rα$^{\Delta Slco1c1}$ mice to 120 µg/kg LPS ip. *n* = 16. ***b***, ***c***, Attenuated induction of *Ptgs2* (Cox-2) (***b***) and of *Il6rα* (***c***) mRNA in the hypothalamus of IL-6Rα$^{\Delta Slco1c1}$ mice. *n* = 7-10. ***d***, Co-localization (arrowheads) between red fluorescent reporter protein (tdTomato) and Cox-2 in the hypothalamus of Gt(ROSA)26Sor$^{\Delta Slco1c1}$ mice. Scale bar = 50 µm.

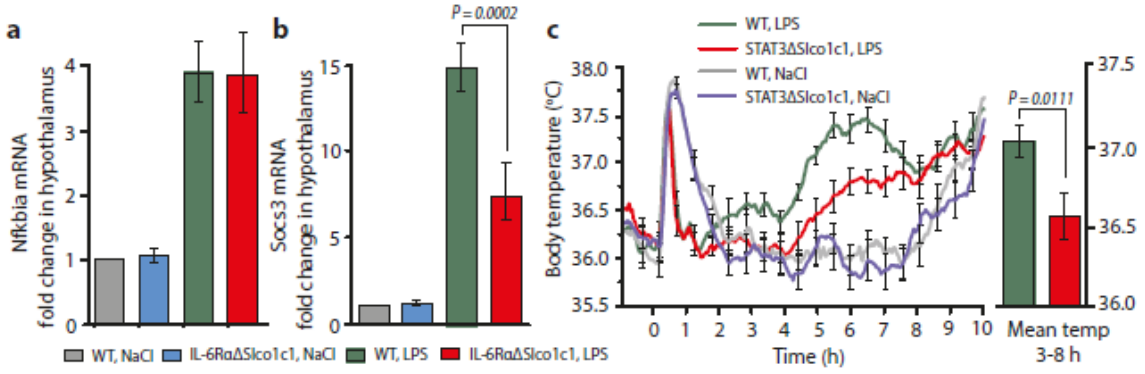

Figure 3

Figure 3. Induction of Cox-2 by IL-6/IL-6Rα involves the STAT3 system. ***a***, ***b***, Induction of *Nfkbia* (***a***) and *Socs3* (***b***) mRNA in the hypothalamus of IL-6Rα$^{\Delta Slco1c1}$ and WT (IL-6Rα$^{fl/fl}$) mice. *n* = 7-10. ***c***, Attenuated fever to 120 μg/kg LPS ip in STAT3$^{\Delta Slco1c1}$ mice. *n* = 10-12.