Systems/Circuits

Fever During Localized Inflammation in Mice Is Elicited by a Humoral Pathway and Depends on Brain Endothelial Interleukin-1 and Interleukin-6 Signaling and Central EP$_3$ Receptors

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We examined the signaling route for fever during localized inflammation in male and female mice, elicited by casein injection into a preformed air pouch. The localized inflammation gave rise to high concentrations of prostaglandins of the E species (PGE$_2$) and cytokines in the air pouch and elevated levels of these inflammatory mediators in plasma. There were also elevated levels of PGE$_2$ in the cerebrospinal fluid, although there was little evidence for PGE$_2$ synthesis in the brain. Global deletion of the PGE$_2$ prostaglandin E receptor 3 (EP$_3$) abolished the febrile response as did deletion of the EP$_3$ receptor in neural cells, whereas its deletion on peripheral nerves had no effect, implying that PGE$_2$ action on this receptor in the CNS elicited the fever. Global deletion of the interleukin-1 receptor type 1 (IL-1R1) also abolished the febrile response, whereas its deletion on neural cells or peripheral nerves had no effect. However, deletion of the IL-1R1 on brain endothelial cells, as well as deletion of the interleukin-6 receptor α on these cells, attenuated the febrile response. In contrast, deletion of the PGE$_2$ synthesizing enzymes cyclooxygenase-2 and microsomal prostaglandin synthase-1 in brain endothelial cells, known to attenuate fever evoked by systemic inflammation, had no effect. We conclude that fever during localized inflammation is not mediated by neural signaling from the inflamed site, as previously suggested, but is dependent on humoral signaling that involves interleukin actions on brain endothelial cells, probably facilitating PGE$_2$ entry into the brain from the circulation and hence representing a mechanism distinct from that at work during systemic inflammation.

Key words: blood-brain barrier; cytokines; fever; inflammation; mouse; PGE2

Significance Statement

Fever elicited by a localized inflammation has long been suggested to depend on the activation of and signaling by peripheral nerves innervating the inflamed tissue and not on humoral messengers, in contrast to what now is clearly established for systemic inflammation. We here show that fever evoked by the injection of pyrogen into a preformed air pouch, providing an enclosed space lined by a synovial-like membrane, is dependent not on peripheral neural signals but on the activation of CNS PGE$_2$ EP$_3$ receptors, similar to what is the case during systemic inflammation. The mechanisms are nevertheless distinct because fever during localized inflammation, although it depends on cytokine signaling in the brain endothelium, does not require brain PGE$_2$ production.

Introduction

Prostaglandins (PGs) have been known as the critical mediator of fever since the 1970s, when their production by cyclooxygenase (Cox) enzymes was shown to be the target of antipyretic and anti-inflammatory drugs (Vane, 1971; Flower et al., 1972; Flower and Vane, 1972). Prostaglandins of the E species (PGE$_2$), which are synthesized from cyclooxygenase-produced PGH$_2$ by terminal isomerases such as microsomal prostaglandin E synthase-1 (mPGES-1; Jakobsson et al., 1999), were later identified as the pyrogen (Stitt, 1986). The demonstration of the expression of inducible Cox, Cox-2 (Kujubu et al., 1991; Sirois et al., 1992), and

mPGES-1 in brain vessels (Cao et al., 1996; Elmquist et al., 1997; Ek et al., 2001; Yamagata et al., 2001) gave rise to the idea that PGE_2 was produced in these vessels upon peripheral immune challenge through the action of circulating cytokines and that this centrally produced PGE_2 elicited the febrile response. Functional studies have subsequently confirmed this idea by showing that deletion of Cox-2 or mPGES-1 in brain endothelial cells attenuates fever evoked by systemic immune challenge (Wilhelms et al., 2014; Eskilsson et al., 2017).

However, in addition to this blood-borne pathway for the generation of fever, it has been suggested that fever in response to a localized inflammation may be mediated by the activation of peripheral nerves, hence not requiring the activation of central PGE_2 synthesis by circulating cytokines. Although the initial observations that surgical transection of the vagus nerve attenuated fever (Watkins et al., 1995; Sehic and Blatteis, 1996; Hansen and Krueger, 1997) later were refuted (Blomqvist and Engblom, 2018), it has been maintained that somatic afferent nerves may mediate the febrile response to a localized inflammation. Dorsal root ganglion (DRG) cells express IL-1 receptors as well as prostaglandin E receptors 3 (EP_3; Nakamura et al., 2000; Binshtok et al., 2008), the PGE_2 receptor subtype critical for fever (Ushikubi et al., 1998), and nociceptors can be sensitized by IL-1β and PGE_2 (Binshtok et al., 2008). The putative role for afferent nerve signaling to the brain in fever has been studied in models of localized inflammation using a subcutaneous air pouch or a subcutaneous Teflon chamber (Miller et al., 1997; Ross et al., 2000; Rummel et al., 2005; Zhang et al., 2008). Although in these models there seems to be leakage of IL-6 into the circulation (Cartmell et al., 2000), no Cox-2 induction in the brain was found, leading the investigators to suggest the presence of a neural rather than humoral route of signaling. Further support for a neuronal signaling pathway for fever comes from studies in which the injection of a local anesthetic at the site of local inflammation or transection of the peripheral nerve innervating the inflamed tissue were reported to attenuate fever (for a discussion of these findings, see Blomqvist and Engblom, 2018).

A conceptual problem with the idea of a neural instead of humoral signaling for fever from the site of a peripheral inflammation is that such a route would either have to feed into the central thermoregulatory system without involving PGE_2 release in the preoptic hypothalamus, which seems inconsistent with the antipyresis achieved by the largely centrally acting Cox-inhibitor paracetamol during localized inflammation, such as experimentally induced monoarthritis in animals and otitis media in humans (Kanashiro et al., 2009; van den Anker, 2013), or evoke central prostaglandin release in the hypothalamus by cells other than brain vascular cells. Here, we reexamined the mechanisms of fever during localized inflammation, using injection of the milk protein casein into a preformed air pouch (Zhang et al., 2008). We used a variety of genetically modified mice, with global or tissue-specific gene deletions of inflammatory mediators, to examine the putative pathways by which the localized inflammation gave rise to fever. We show that fever in this model is dependent on humoral signaling that involves interleukin actions on brain endothelial cells, albeit in a way that seems distinct from that at work during systemic inflammation.

Material and Methods

Animals. All experimental procedures were approved by the Animal Ethics Committee in Linköping and followed international guidelines. Adult, age, and sex-matched mice were used.

Mice with global gene deletions [EP_1 (Stock et al., 2001), EP_2 (Tilley et al., 1999), EP_3 (Fleming et al., 1998), mPGES-1 (Trebino et al., 2003), or interleukin-1 receptor type 1 (IL-1R1; Glaccum et al., 1997)], as well as mice with conditional deletions [EP_3, possessing loxP sites flanking exon 1 of the *Ptger3* gene (Lazarus et al., 2007); IL-6 receptor α, possessing loxP sites flanking exons 4–6 of the *Il6ra* gene (McFarland-Mancini et al., 2010); IL-1R1, possessing loxP sites flanking exon 5 of the *Il1r1* gene (Abdulaal et al., 2016); Cox-2, possessing loxP sites flanking exons 4–5 of the *Ptgs2* gene (Ishikawa and Herschman, 2006); and mPGES-1, possesing loxP-sites flanking exon 2 in the *Ptges1* gene (Wilhelms et al., 2014)] were used. The mice with conditional deletions were crossed with mice expressing Cre recombinase under specific promoters. These included (1) mice expressing Cre recombinase in neuronal and glia cell precursors under the control of the nestin promoter and enhancer, a widely used line for selective deletion in neural cells (Tronche et al., 1999); (2) mice expressing Cre recombinase under the TRPV1 promotor, which, with the exception of some cell groups in the hypothalamus, is expressed specifically by nociceptors in primary sensory ganglia (Cavanaugh et al., 2011). This Cre-line has previously been shown to efficaciously delete genes in these latter structures (Spencer et al., 2018); (3) the human tissue plasminogen activator (HTPA), expressed by neural crest derivates. The HTPA-Cre line is reported to be a pan neural crest cell marker that recombines almost exclusively in neural crest derivates, the exception being a minor component of limb periosteum and tendons. When combined with a reporter line, it produced homogenous staining in spinal dorsal root ganglia, indicating recombination of both glial and neuronal precursors in these structures (Pietri et al., 2003); (4) LysM, expressed by myeloid cells. The LysM-Cre line has demonstrated a deletion efficiency of 83–98% in mature macrophages and nearly 100% in granulocytes, and partial deletion in CD11c splenic dendritic cells but no significant deletion in tail DNA or purified T and B cells (Clausen et al., 1999); and (5) a tamoxifen-inducible CreERT2 from the Slco1c1 promoter, expressed by brain endothelial cells (Ridder et al., 2011). The specificity of this Cre-line has been demonstrated using reporter mice, combined with staining for cell-specific markers (Eskilsson et al., 2014; Fritz et al., 2016, 2018). It preferentially produces recombination in small- to medium-size brain blood vessels (Eskilsson et al., 2017).

The mice were obtained from The Jackson Laboratory or directly from the respective investigator. They were all maintained on a C57BL/6 background and housed under constant ambient temperature (21°C), on a 12 h/12 h light/dark cycle (lights on at 7 A.M.) with food and water *ad libitum*. Gene deletion in mice with the Slco1c1 CreERT2 construct was induced by intraperitoneal injection of tamoxifen (1 mg tamoxifen diluted in a mixture of 10% ethanol and 90% sunflower seed oil twice a day for 5 d) at least 5 weeks before additional experiments.

Creation of chimeric mice. Mice chimeric for mPGES-1 were created by irradiating mPGES-1 KO and WT mice to an absorbed dose of 9 Gy in two fractions. About 24 h after irradiation, the mice were injected intravenously with 2 million freshly prepared CD45+ bone marrow cells of the same or opposite genotype. They were allowed to survive for ~5 months before they were used for additional experiments (Engström et al., 2012). This procedure results in extensive replacement of hematopoietically derived cells, both in the periphery and in the brain, as reported previously (Engström et al., 2012).

Surgery. The mice were anesthetized with isoflurane and implanted intraperitoneally with a transponder that records core body temperature (E-Mitter Telemetry System, STARR Life Sciences) after which 3 ml air sterilized through a PVC filter (Filtropur S plus, 0.2 μm, Sarstedt) was injected subcutaneously at the back of the mouse to create an air pouch. The mice were then transferred to a room in which the ambient temperature was set to 29°C, providing near-thermoneutral conditions (Rudaya et al., 2005). Three d after the first air injection, the pouch was again filled with ~3 ml sterilized air, followed after another 4 d of injection into the pouch of 0.5–0.8 ml (depending on the weight of the animal) of a casein solution (10% casein, Hammarsten grade; MP Biomedicals) in 50 mM sodium bicarbonate buffer, conducted at ~7 A.M. The milk protein casein is known to elicit inflammation and fever in animals and humans, although its mode of action is not clear (Kitamura et al., 1986; Moissidis et al., 2005). The air pouch is a closed space covered by a membrane resembling the synovial membrane of a joint that creates a mechanical barrier that retains the inflammatory stimulus and products of the inflammatory response (Edwards et al., 1981; Sedgwick et al., 1983). In the initial experiments, mice were given a brief anesthesia with isoflurane to facilitate injections; however, as this procedure resulted in a drop in body temperature, it was subsequently abandoned. Control animals were injected with buffer solution only. Mice used for real-time PCR analyses, immunoassays, and immunohistochemical stainings were killed 5 h after injection, corresponding to a time point when fever is established and rising in this model (Fig. 1).

Real-time PCR. The mice were killed by asphyxiation with CO_2. The wall of the air pouch was dissected, and the brain and in some experiments dorsal root ganglia from the cervical and lumbar segments were collected. The hypothalamus was cut out as described previously (Reyes et al., 2003). Tissue was placed in RNAlater stabilization reagent (Qiagen) and kept at −70°C until further processing. RNA was extracted with RNeasy Plus Universal Kit (Qiagen), and reverse transcription was done with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems); qPCR was performed using Gene Expression Master Mix (Applied Biosystems) on a 96-well plate (7900HT Fast Real-Time PCR
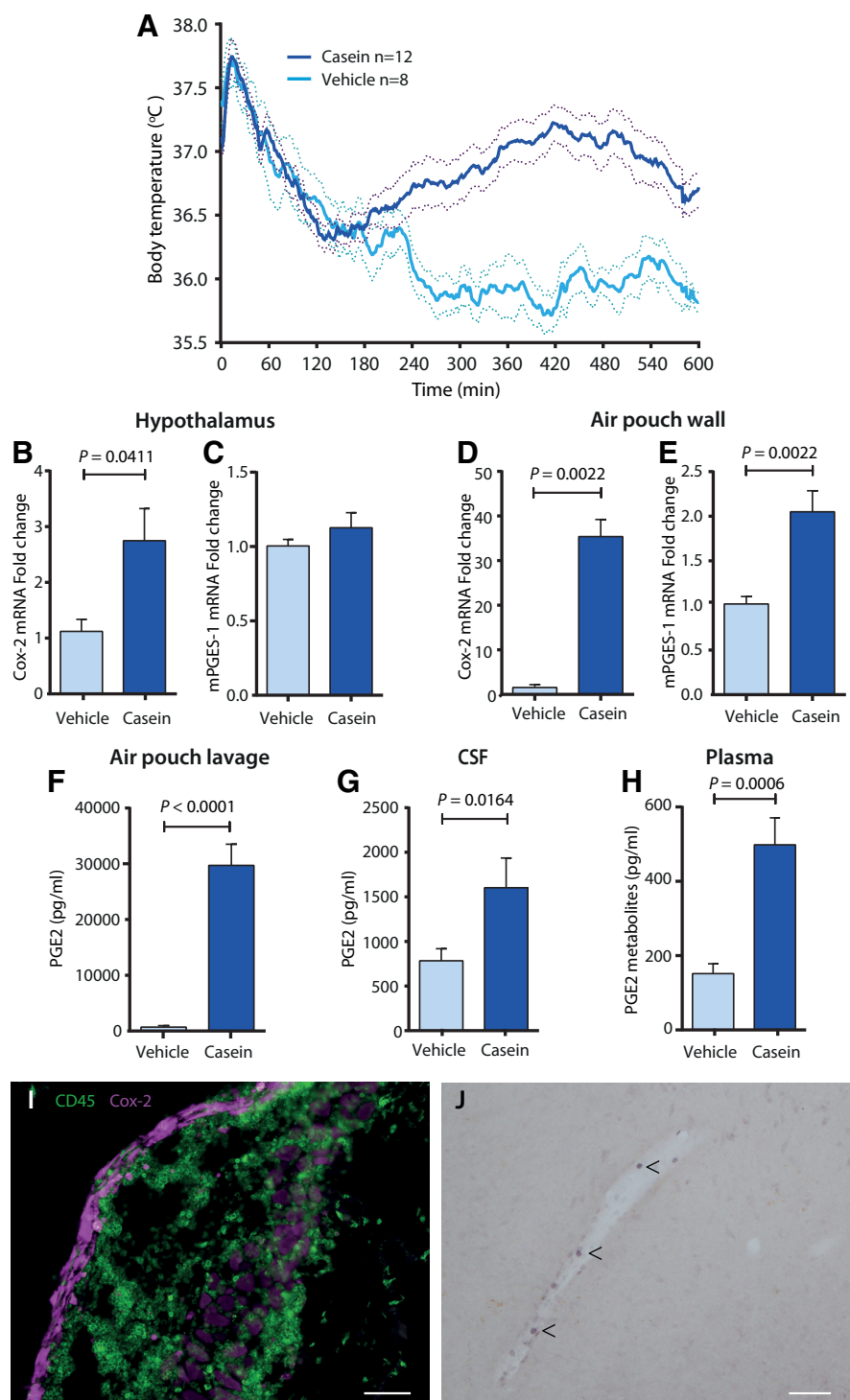

Figure 1. Fever response, and prostaglandin synthesis and PGE$_2$ levels in the air pouch, plasma, and brain elicited by casein injection into the pouch. ***A***, Temperature recordings in WT mice injected at time point 0 with casein or vehicle. The temperature peak seen in both genotypes immediately after injection is because of the handling stress during the injection procedure. Solid lines represent mean, and dotted lines represent SEM. Data are taken from WT mice in the experiment examining the role of brain endothelial IL-1 receptors (Fig. 6E). ***B–E***, qPCR Analysis of Cox-2 and mPGES-1 mRNA in the hypothalamus (***B***, ***C***) and the air pouch wall (***D***, ***E***) 5 h after injection of casein or vehicle into the air pouch; $n = 6$. ***F–H***, Concentration of PGE$_2$ or PGE$_2$ metabolites in the air pouch lavage (***F***), cerebrospinal fluid (***G***), and plasma (***H***) 5 h after casein or vehicle injection; $n = 7$–10. Error bars represent SEM. ***I***, Microphotograph of the air pouch wall stained for Cox-2 and CD45. Note the strong Cox-2 labeling in the lining of the wall, and the infiltration in the wall of CD45-positive but Cox-2 negative immune cells. Scale bar, 200 μm. ***J***, Cox-2 expressing brain vascular cells (arrowheads) 5 h after casein injection into the air pouch. Scale bar, 200 μm.

System Software; Applied Biosystems). The following TaqMan assays (all from Applied Biosystems) were used: Mm00434228_m1 (IL-1β), Mm00446190_m1 (IL-6), Mm00443258_m1 (TNF), Mm00478374_m1 (Cox-2), Mm00452105_m1 (mPGES-1), and Mm99999915_g1 (GAPDH).

Immunoassays. The mice were asphyxiated and blood was drawn from the right atrium and transferred to EDTA-coated tubes (Sarstedt) to which indomethacin (10 μM; Sigma-Aldrich) was added and centrifuged at 7000 $\times$ g for 7 min at 4°C. The plasma was immediately frozen on dry ice and kept at −70°C. Air pouch lavage was collected by injecting into the air pouch 1 ml sterile saline, which was subsequently withdrawn. The lavage was centrifuged at 15,000 $\times$ g for 10 min at 4°C. The supernatant was collected and frozen on dry ice and kept at −70°C. After blood and lavage had been collected, the carcass was placed in a stereotaxic frame, the atlanto-occipital membrane was exposed, and cerebrospinal fluid was withdrawn from the cisterna magna using a Hamilton syringe mounted on a micromanipulator and immediately frozen. Samples that contained traces of blood were discarded. The whole procedure from when the animals were killed until cerebrospinal fluid was withdrawn and frozen took <10 min.

The concentration of PGE$_2$ in the cerebrospinal fluid (diluted 1:100) and air pouch lavage (diluted 1:1000 after casein injection and 1:10 after vehicle injection) was determined using a High Sensitivity Prostaglandin E2 Enzyme Immunoassay Kit (Assay Designs). The values were calculated using a standard curve ranging from 7.81 to 1000 pg/ml ($r^2 = 0.998$). The kit antiserum showed the following cross reactivity, according to the manufacturer: PGE$_2$ 100%, PGE$_1$ 70%, PGE$_3$ 16.3%, PGF$_{1\alpha}$ 1.4%, PGF$_{2\alpha}$ 0.7%, 6-keto-PGF$_{1\alpha}$ 0.6%, PGA$_2$ 0.1%, PGB$_1$ 0.1%, and <0.1% for 13, 14-dihydro-15-ketoPGF$_{2\alpha}$, 6, 15-keto, 13, 14-dihydro-PGF$_{1\alpha}$, thromboxane B$_2$, 2-arachidonoylglyerol, anandamide, PGD$_2$ and arachidonic acid. The concentration of PGE$_2$ metabolites in plasma (diluted 1:50) was determined with a Prostaglandin E Metabolite EIA Kit (Cayman Chemical). The values were calculated using a standard curve ranging from 0.2 to 50 pg/ml ($r^2 = 0.9835$-0.9981). The kit antiserum recognizes derivatized 13,14-dihydro-15-ketoPGE$_1$ and 13,14-dihydro-15-ketoPGE$_2$ and bicycloPGE$_1$, but has <0.01% cross reactivity with arachidonic acid, leukotriene B$_4$, tetranor-PGEM, tetranor-PGFM, PGD$_2$, PGE$_1$, 6-keto PGE$_1$, PGE$_2$, PGF$_{1\alpha}$, 6-keto PGF$_{1\alpha}$, PGF$_{2\alpha}$, and thromboxane B$_2$.

Levels of IL-1β were analyzed with a mouse IL-1b/IL-1F2 immunoassay (catalog #MLB00C, R&D Systems) according to the manufacturer's instructions. The lavage from casein-injected mice was diluted 1:2 but left undiluted for mice injected with vehicle and for plasma. The values were calculated using a standard curve ranging from 12.5 to 800 pg/ml ($r^2 = 1.0$). The sensitivity of the assay was 2.31 pg/ml, and according to the manufacturer, it exhibited no cross reactivity with mouse recombinant IL-1; IL-1F10/IL-1HY2; IL-1ra; IL-1 RAPL2/IL-1 R9; IL-1 RI; IL-1 RII; IL-1 Rrp2/IL-1 R6; IL-2–7, 9, 10–13, 15, 20, 23; IL-10ra,; IL-11ra; IL-12p40; IL-23R; or ST2/IL-1 R4.

Levels of TNF and IL-6 were analyzed using a Milliplex Mouse Cytokine/Chemokine Magnetic Panel MCYTOMAG-70K (Merck Millipore), using a dilution of 1:2 for plasma and 1:200 for lavage. According to the manufacturer, assay sensitivity was 1.1 pg/ml for IL-6 and 2.3 pg/ml for TNF, and intra-assay precision was 2.3% and 2.6%, respectively. There was no or negligible cross-reactivity between IL-6 and TNF and other cytokines.

Immunohistochemistry. After asphyxiation with CO$_2$, the animals were perfused transcardially with a phosphate-buffered (0.1 M) paraformaldehyde solution (4%). Tissue of interest was post-fixed for 3 h in the same fixative, then cryoprotected with 25% sucrose in PBS and cut at 30 μm on a freezing microtome. The immunohistochemical procedures were performed according to standardized protocols (Engström et al., 2012). The primary antibodies were rabbit anti-Cox-2 (1:500–1:1000; catalog #sc-1747 M-17, Santa Cruz Biotechnology), which was detected with Alexa Fluor 555 donkey anti-rabbit (1:500; Invitrogen) or an avidin-biotin-HRP system (Vector Laboratories) with 3,3′-diaminobenzidine as chromogen, and rat anti-CD45 (1:5000; Serotec), which was detected with Alexa Fluor 488 goat anti-rat (1:500; Invitrogen).

Experimental design and statistical analyses. In all comparisons, littermates were used. Groups were designed to be age and sex matched, but within these limitations, animals were randomly assigned to the respective group. Statistical analyses were done in Prism version 9 (GraphPad). Significant differences were determined using a one-way or two-way ANOVA, both followed by a *post hoc* test with correction for multiple comparisons controlling the false discovery rate according to the linear step-up multiple-testing procedure (Benjamini et al., 2006) or, for pairwise comparisons, a nonparametric test (Mann–Whitney). Results were considered statistically significant when $p < 0.05$. Group size in the temperature recording experiments was considered as follows: With an estimated SD ~0.5°C, 12 mice in each group are required to permit detection of a difference of 0.6°C with 80% reliability at a significance level of 5%. We strived to obtain that group size; however if there was no or minimal difference in mean temperature between groups after the first rounds of an experiment (encompassing ~4–8 mice in each group), or if a very clear effect was seen, we did not further pursue that experiment, considering it was not justified from an animal ethics perspective.

Results

Casein injection into a preformed air pouch results in fever and induced PGE$_2$ synthesis

Following casein injection into the air pouch, the mice displayed fever starting ~3–4 h after the injection and lasting ~7–8 h (Fig. 1A). Because fever induced by systemic immune challenge has been shown to be elicited by PGE$_2$ (Blomqvist and Engblom, 2018), we first examined if there was increased PGE$_2$ synthesis in this model. We found a significant but weak induction of Cox-2 mRNA but not of mPGES-1 mRNA in the hypothalamus (Fig. 1B,C), and strong induction of Cox-2 as well as significant induction of mPGES-1 mRNA in the air pouch wall (Fig. 1D,E). There was a huge increase in PGE$_2$ levels in the air pouch lavage in the casein-injected mice (Fig. 1F), but increased levels of PGE$_2$ and of PGE$_2$ metabolites were also seen in the cerebrospinal fluid (Fig. 1G) and plasma (Fig. 1H), respectively. Immunohistochemical staining for Cox-2 protein showed strong, induced labeling in the lining toward the lumen of the air pouch (Fig. 1I), previously identified as consisting of fibroblasts and collagen fibers (Sedgwick et al., 1983), as well as induced expression in some brain blood vessels with immunolabeling seen in scattered cells (Fig. 1J). In the air pouch wall, many CD45-positive immune cells were seen, but these were not stained for Cox-2 (Fig. 1I).

The fever response to casein injection is dependent on the synthesis of PGE$_2$ by nonhematopoietic cells

Given the above findings, we examined whether deletion of PGE$_2$ synthesis would inhibit fever induced by casein injection
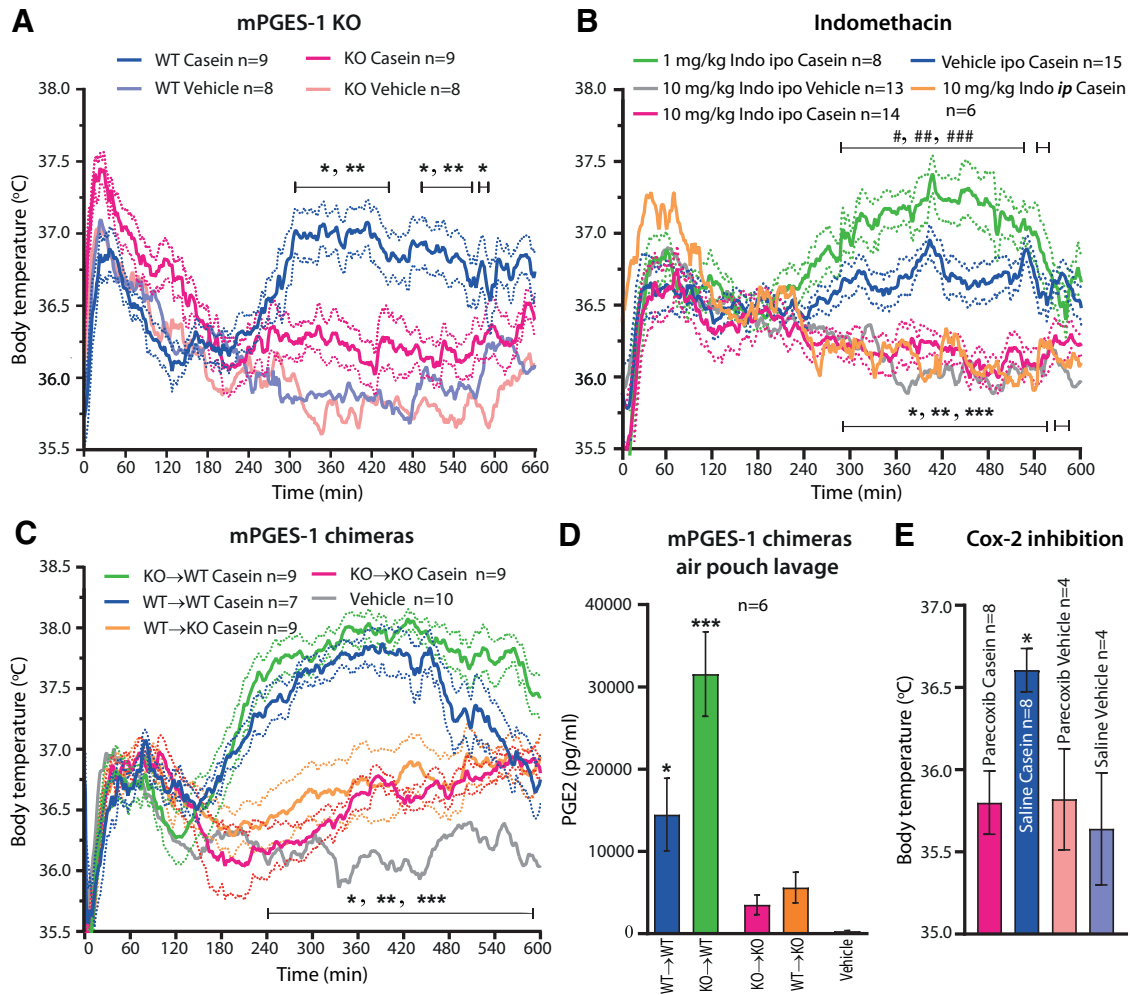
Figure 2. Temperature response and PGE$_2$ production after casein injection into a preformed air pouch in mPGES-1 KO and chimeric mice, and treatment with cyclooxygenase inhibitor. **A**, Attenuated fever in mPGES-1 KO mice. *$p < 0.05$ and **$p < 0.01$ for WT mice versus mPGES-1 KO mice injected with casein (two-way ANOVA for the period of 4–10 h: $F_{(3,30)} = 17.71$, $p < 0.0001$). **B**, Effect of indomethacin (Indo) injected into the air pouch (ipo) or intraperitoneally (ip). Note that both routes of administration of 10 mg/kg indomethacin abolished casein-induced fever. Injection of 1 mg/kg indomethacin into the air pouch resulted in augmented fever to casein. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ for Vehicle ipo Casein versus 10 mg/kg Indo ipo Casein, and #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ for Vehicle ipo Casein versus 1 mg/kg Indo ipo Casein (two-way ANOVA for the period of 4–10 h: $F_{(4,51)} = 17.26$, $p < 0.0001$). **C**, mPGES-1 chimeric mice. WT→WT, WT mice transplanted with WT bone marrow; KO→WT, WT mice transplanted with KO bone marrow; KO→KO, KO mice transplanted with KO bone marrow; and WT→KO, KO mice transplanted with WT bone marrow. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ for KO→WT mice versus WT→KO mice injected with casein (two-way ANOVA for the period of 4–10 h: $F_{(4,39)} = 32.02$, $p < 0.0001$). Solid lines represent mean, and dotted lines represent SEM. **D**, PGE$_2$ concentration in air pouch lavage in mPGES-1 chimeric mice 5 h after casein injection into the pouch. Vehicle, mixed chimeras. Error bars represent SEM. *$p = 0.0155$ for WT→WT versus KO→KO and *$p = 0.0358$ for WT→WT versus WT→KO. ***$p < 0.0001$ for KO→WT versus KO→KO and WT→KO (one-way ANOVA: $F_{(4,25)} = 15.66$, $p < 0.0001$). **E**, Effect of parecoxib on casein-induced fever. Parecoxib, 10 mg/kg, or saline was injected intraperitoneally 3 h after casein or vehicle injection into the air pouch (i.e., immediately before the appearance of the casein-induced fever); the temperature recordings were done 2.5 h later, close to the peak of the casein-induced fever and before the effect of parecoxib disappears (Nilsberth et al., 2009a). *$p = 0.0173$ for parecoxib casein versus saline casein (one-way ANOVA: $F_{(3,20)} = 4.625$, $p = 0.0129$).

into the air pouch. We found that global deletion of the PGE$_2$ synthesizing enzyme mPGES-1 almost completely abolished the febrile response (Fig. 2A). Similarly, the nonselective Cox inhibitor indomethacin (10 mg/kg body weight; Alpharma) injected either intraperitoneally or into the air pouch, as well as the selective Cox-2 inhibitor parecoxib (10 mg/kg; Dynastat, Pfizer), injected intraperitoneally, also abolished the fever response (Fig. 2B,E). We then examined the role of hematopoietic versus nonhematopoietic cells for the casein-induced fever. We generated mPGES-1 chimeric mice by transplantation of bone marrow of the opposite genotype to mPGES-1 knock-out mice and wild-type mice, and used animals receiving bone marrow of their own genotype as controls (Engström et al., 2012). Wild-type mice transplanted with knock-out bone marrow (KO→WT) displayed fever similar to wild-type mice with wild-type bone marrow (WT→WT). Knock-out mice transplanted with wild-type bone marrow

(WT→KO) showed attenuated fever, like knock-out mice with knock-out bone marrow (KO→KO; Fig. 2C). Furthermore, the PGE$_2$ levels in the air pouch lavage were much higher in WT→WT and KO→WT animals than in KO→KO and WT→KO animals (Fig. 2D). These data suggest that nonhematopoietic cells are synthesizing the PGE$_2$ that is critical for the fever response to casein injected into the preformed air pouch. This idea is consistent with our observation that Cox-2 was densely expressed by cells in the lining of the air pouch wall but not by infiltrating immune cells (Fig. 1I).

EP$_3$ receptors in the CNS are critical for the febrile response to casein injection

We next examined where the PGE$_2$ induced by the casein injection into the air pouch exerted its pyrogenic effect. We first confirmed that deletion of the EP$_3$ receptor, which has been shown
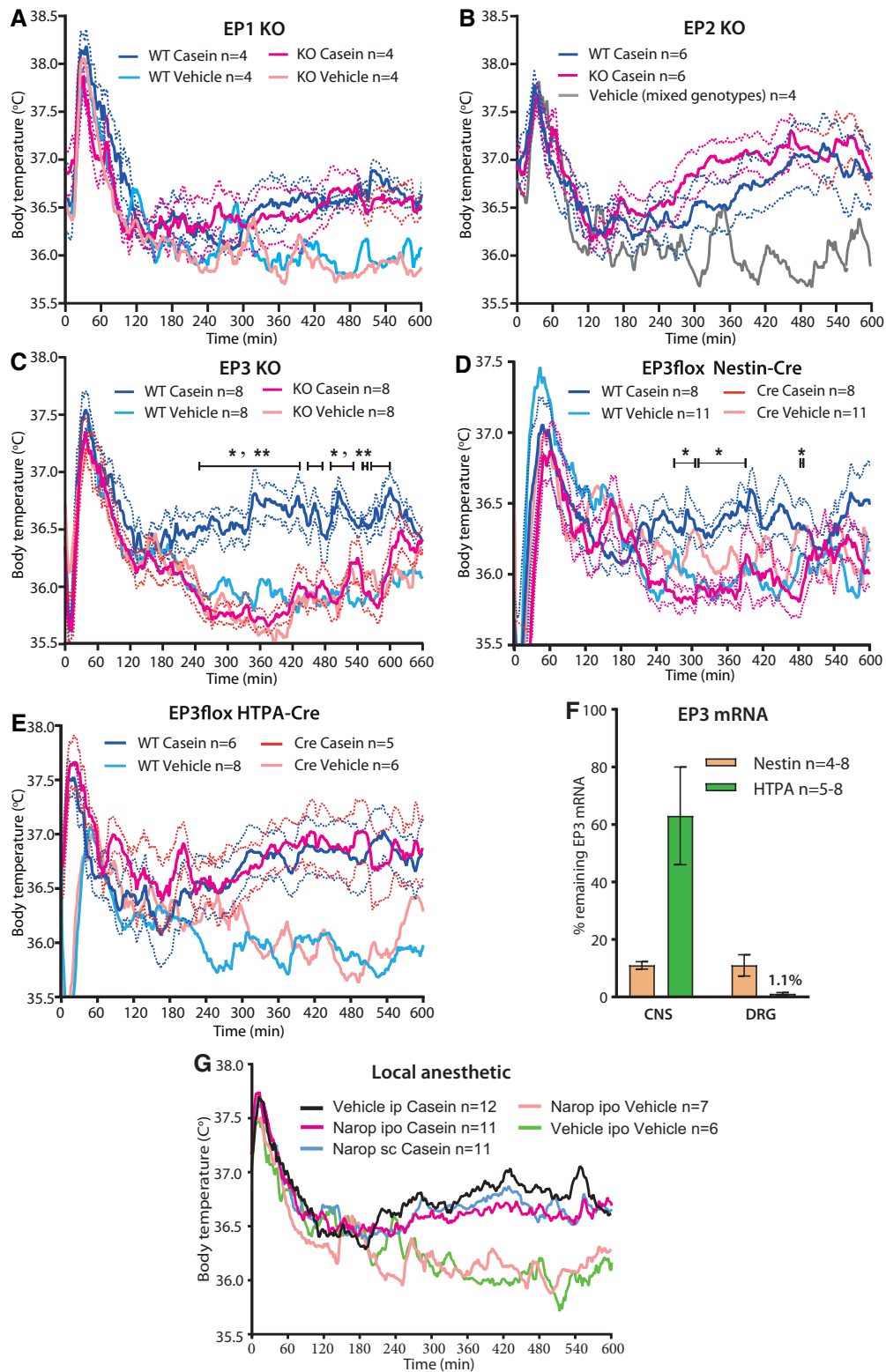
Figure 3. Effect of EP receptor deletion and nerve blockade on casein-induced fever. *A*, *B*, No difference in the febrile response between WT and EP$_1$ (*A*) and EP$_2$ (*B*) KO mice. *C*, *D*, Absence of fever in mice with global deletion of EP$_3$ (*C*); *p < 0.05 and **p < 0.01 for WT mice versus EP$_3$ KO mice injected with casein (two-way ANOVA for the period of 4–10 h: $F_{(3,28)} = 20.29$, $p < 0.0001$) or EP$_3$ deletion in neural cells (*D*); *p < 0.05 for WT mice versus EP$_3$flox Nestin-Cre mice injected with casein (two-way ANOVA for the period of 4–10 h: $F_{(3,34)} = 5.545$, $p = 0.0033$). *E*, Normal febrile response in mice with deletion of the EP$_3$ receptor in neural crest derivates. Solid lines represent mean, and dotted lines represent SEM. *F*, qPCR Analysis of the amount of remaining EP$_3$ receptor mRNA in the CNS and DRG after deletion in EP$_3$ floxed mice with Nestin-Cre and HTPA-Cre, respectively. Error bars show SEM. *G*, No effect of nerve blockade by injection of a local anesthetic (ropivacaine, Narop) into the air pouch (ipo), immediately before the injection of casein into the pouch. To account for any systemic effects of the local anesthetic, the same dose was also injected subcutaneously (sc), on the belly. Solid lines represent mean.
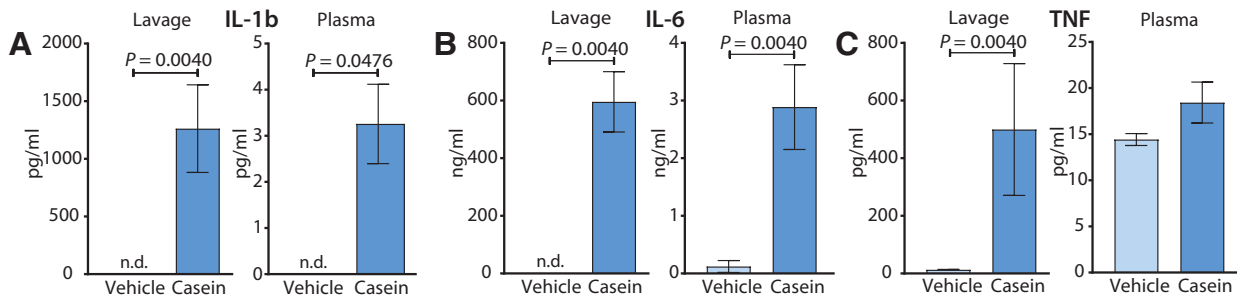
Figure 4. *A–C*, Levels of IL-1β (*A*), IL-6 (*B*), and TNF (*C*) in air pouch lavage and plasma 5 h after casein or vehicle injection into the air pouch; $n = 3–4$ in the vehicle groups and $n = 6–8$ in the casein groups. Error bars represent SEM. n.d., Not detectable.

to be critical for PGE$_2$-induced fever in other models (Ushikubi et al., 1998; Engblom et al., 2003), abolished the febrile response to casein injection (Fig. 3*C*). There was no effect of deletion of the EP$_1$ or EP$_2$ receptor (Fig. 3*A,B*). We then generated mice with deletion of EP$_3$ receptors on peripheral nerves, or on neural cells, by crossing mice with a conditional deletion of the EP$_3$ receptor (Lazarus et al., 2007) with mice expressing Cre recombinase under the HTPA promotor (expressed by neural crest derivates; Pietri et al., 2003) or mice expressing Cre recombinase under the Nestin promotor (expressed by neuronal and glia cell precursors; Tronche et al., 1999). Mice expressing Cre recombinase under the Nestin promotor showed no febrile response to the casein injection (Fig. 3*D*), whereas mice expressing Cre recombinase under the HTPA promotor displayed the same febrile response as control (wild type) mice (Fig. 3*E*). We further explored the possibility that nerves innervating the region of the air pouch could be involved by injecting the air pouch with a local anesthetic (8 mg/kg ropivacaine; Narop, Aspen Nordic) 5 min before the injection of casein to block any inflammation-elicited neural signaling from this structure. The dose chosen was the highest that could be administered without risking systemic effects. However, mice injected with the local anesthetic into the air pouch showed the same febrile response to casein as did mice injected with vehicle (Fig. 3*G*).

To evaluate the efficacy of the EP$_3$ gene deletion, we compared the EP$_3$ mRNA expression in whole brain and dorsal root ganglia in the EP$_3$ floxed mice expressing Nestin-Cre or HTPA-Cre with that found in littermates not expressing Cre (WT), using qPCR. In offspring expressing Nestin-Cre the amount of EP$_3$ receptor mRNA was ~10% of that seen in WT littermates both in the CNS and in dorsal root ganglia. In contrast, in offspring expressing HTPA, ~60% of the EP$_3$ mRNA remained in the CNS but only ~1% in the dorsal root ganglia (Fig. 3*F*). Hence, although the absence of fever in the Nestin-Cre EP$_3$ mice could be consistent with EP$_3$ deletion either in the CNS or in afferent peripheral somatic
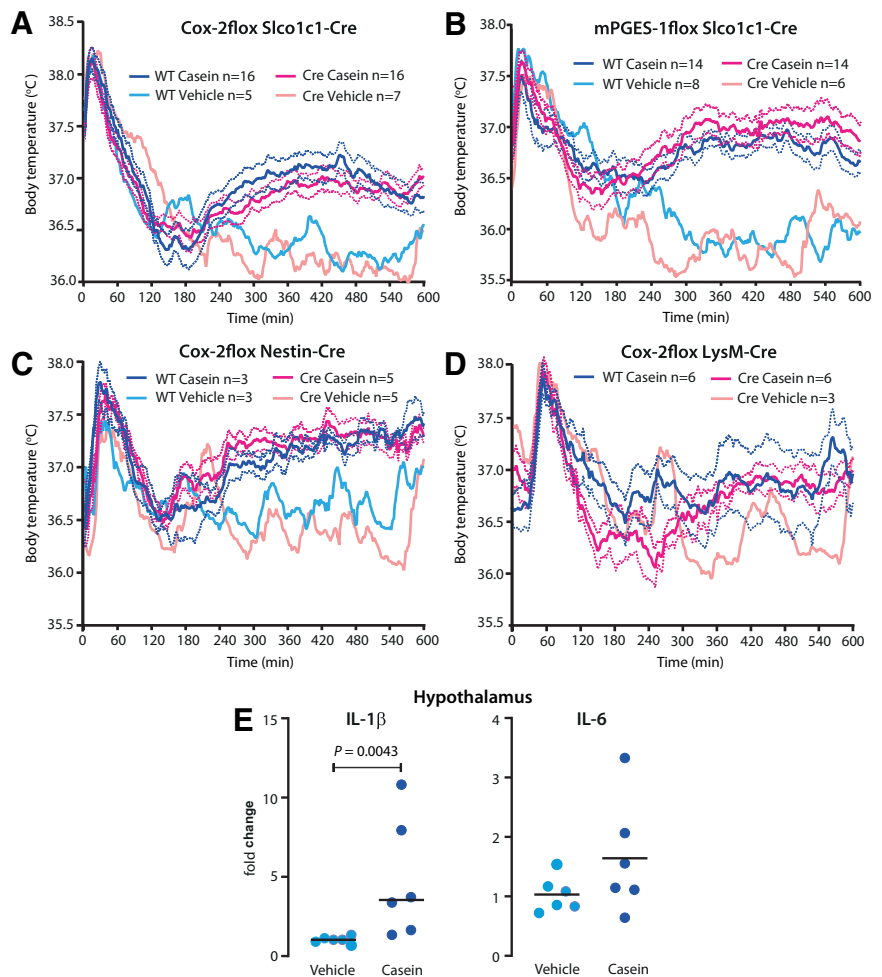

Figure 5. *A–D*, Normal fever to casein injected into a preformed air pouch in mice with tissue-specific deletion of PGE$_2$ synthesizing enzymes. *A*, Endothelial deletion of Cox-2. *B*, Endothelial deletion of mPGES-1. *C*, Cox-2 deletion in neural cells. *D*, Cox-2 deletion in myeloid cells. Solid lines represent mean, and dotted lines represent SEM. *E*, Statistically significant but minor induction of IL-1β mRNA but not of IL-6 mRNA in the hypothalamus after casein injection in WT mice. Horizontal bars represent mean.

nerves, the presence of fever in the HTPA-Cre EP$_3$ mice cannot be ascribed to the presence of EP$_3$ receptors on peripheral nerves, demonstrating that the EP$_3$ receptors in the CNS are critical.

Casein injection into an air pouch results in high concentrations of cytokines in the pouch but only low levels in the circulation

Although fever in the air pouch model was associated with high levels of PGE$_2$ in the air pouch, as well as elevated PGE$_2$ levels in
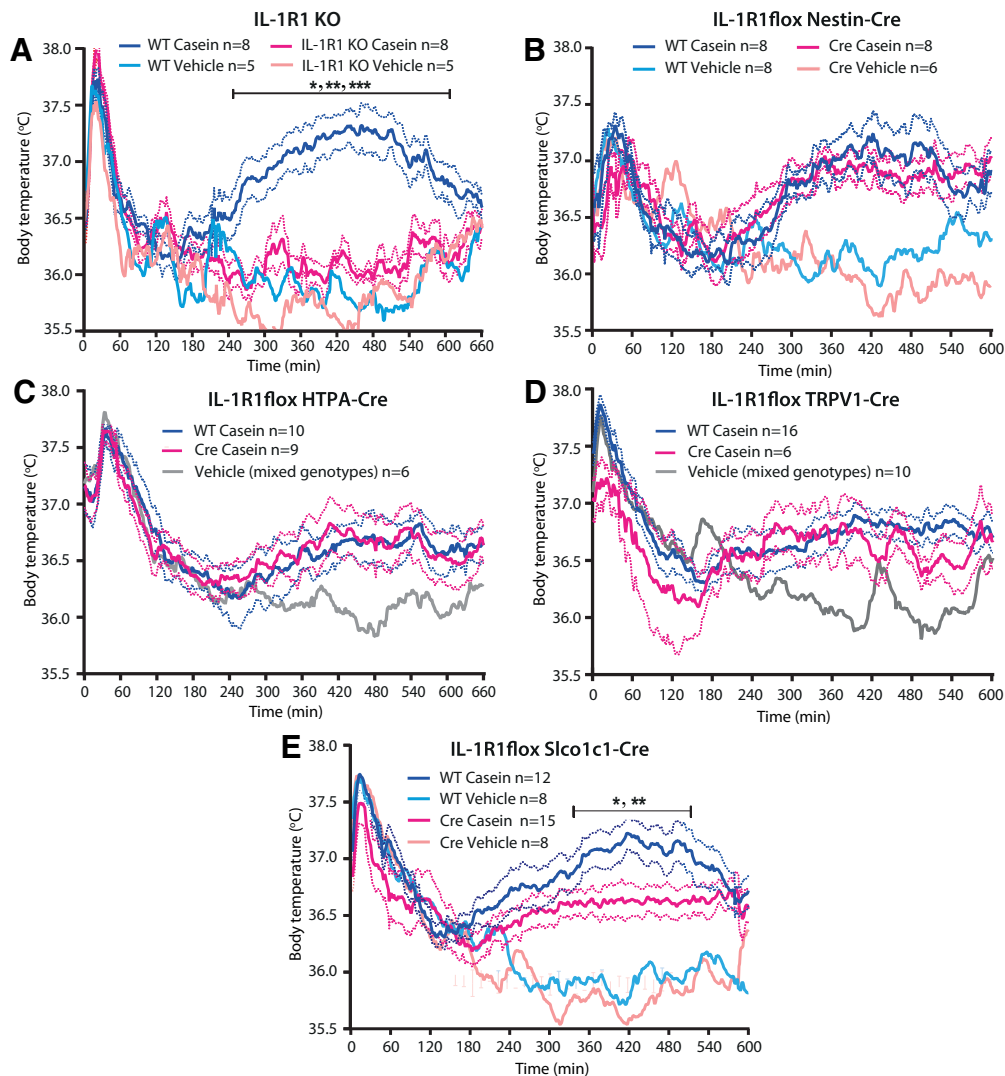
Figure 6. Fever response to casein injected into a preformed air pouch in mice with IL-1R1 deletion. **A**, Absence of fever in mice with global deletion of IL-1R1. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ for WT mice versus IL-1R1 KO mice injected with casein (two-way ANOVA for the time period of 4–10 h: $F_{(3,22)} = 27.79$, $p < 0.0001$). **B–D**, No difference in the febrile response between WT mice and mice with deletion of IL-1R1 in neural cells (**B**), neural crest derivates (**C**), or thin afferent nerves (**D**). **E**, Attenuated fever in mice with deletion of IL-1R1 in brain endothelial cells. *$p < 0.05$, and **$p < 0.01$ for WT mice versus IL-1R1flox Slco1c1-Cre mice injected with casein (two-way ANOVA for the time period of 4–10 h: $F_{(3,39)} = 14.72$, $p < 0.0001$). Solid lines represent mean, and dotted lines represent SEM.

plasma and cerebrospinal fluid (Fig. 1F–H), and deletion of PGE$_2$ synthesis attenuated fever (Fig. 2A), it was not clear if all PGE$_2$ observed derived from the air pouch or whether PGE$_2$ also was synthesized at other sites and that such synthesis elicited the fever. Because systemic administration of pyrogenic inflammatory mediators, such as lipopolysaccharide, has been shown to result in high levels of circulating proinflammatory cytokines, such as IL-1β, IL-6 and TNF (Elander et al., 2009; Matsuwaki et al., 2017), which are critical for PGE$_2$ production and fever (Leon, 2002; Eskilsson et al., 2014; Matsuwaki et al., 2017), we examined the production of these cytokines in the air pouch and the extent to which they were also found in the circulation. We found that casein injection into the air pouch resulted in high levels in the pouch of IL-1β, but there were also elevated levels in plasma (Fig. 4A), however, at a concentration several magnitudes lower than that seen after immune challenge with LPS (Elander et al., 2009; Matsuwaki et al., 2017). Casein injection resulted in high concentrations in the air pouch also of IL-6 (Fig. 4B) and TNF (Fig. 4C). IL-6 was also elevated in plasma (Fig. 4B), present in a concentration about one order of magnitude

lower than that seen after intraperitoneal injection of LPS (Elander et al., 2009; Matsuwaki et al., 2017). TNF levels in plasma were low and did not differ between mice injected with casein and those injected with vehicle (Fig. 4C).

Prostaglandin synthesis in brain endothelial or neural cells or in hematopoietic/myeloid cells is not critical for fever induced by casein injection

Because circulating pyrogenic cytokines (IL-1β and IL-6) were present in the circulation, although at low levels (Fig. 4A,B), and as there was induction, although weak, of Cox-2 in the brain endothelium (Fig. 1J), we next examined whether deletion of prostaglandin synthesis in brain endothelial cells would affect the casein-induced fever as has been shown for fever induced by peripheral injection of LPS and IL-1β (Wilhelms et al., 2014; Eskilsson et al., 2017). Mice with conditional deletion of Cox-2 (Ishikawa and Herschman, 2006) or mPGES-1 (Wilhelms et al., 2014) were crossed with mice expressing Cre recombinase under the promotor for the thyroxine transporter Slco1c1 (Ridder et al.,

2011), which is almost exclusively expressed by brain endothelial cells (Ridder et al., 2011; Eskilsson et al., 2014; Wilhelms et al., 2014; Fritz et al., 2018). However, neither endothelial cell deletion of Cox-2 nor of mPGES-1 affected the fever elicited by casein injection into the air pouch, but mice with these deletions showed temperature responses almost identical to those of wild-type mice (Fig. 5A,B). Neither did deletion of Cox-2 in neural cells using a Nestin-Cre mouse line affect the febrile response (Fig. 5C). We also examined whether deletion of PGE_2 synthesis in hematopoietic/myeloid cells would affect casein-induced fever. However, deletion of Cox-2 in cells expressing the LysM domain, by using a LysM-Cre line (Clausen et al., 1999), had no effect on the febrile response to casein injection into the air pouch (Fig. 5D). Although we did not specifically delete any putative PGE_2 production by microglial cells, there was little evidence of inflammatory activation of these cells, as shown previously in this model (Zhang et al., 2008), because there was minor or no induced expression in brain tissue of the transcripts for the cytokines IL-1β and IL-6 (Fig. 5E), which are produced by the microglial cells on immune stimulation (Norden et al., 2016). In contrast, both these cytokines are strongly upregulated in models of systemic immune challenge (Nilsberth et al., 2009b).

Hence, although proinflammatory cytokines were present in the circulation, albeit in low concentrations, and a weak induction of Cox-2 occurred in brain vascular cells, there was no evidence that any PGE_2 production in the brain or in hematopoietic/myeloid cells was critical for the fever seen in the casein air pouch model.

Cytokine signaling in brain endothelial cells is critical for fever induced by casein injection

As IL-1β has been shown to be critical for the febrile response to localized inflammation, such as a turpentine abscess (Zheng et al., 1995; Horai et al., 1998), and as very high levels of this cytokine were found in the air pouch in the present model, we examined its role for the febrile response. We found that mice with global deletion of IL-1R1 were afebrile following casein injection into the air pouch (Fig. 6A), as reported previously (Zhang et al., 2008). The fever depending on IL-1R1 was not mediated by IL-1R1 on nerves innervating the air pouch because deletion of the IL-1R1 on neural cells or on neural crest derivates or thin sensory peripheral nerves obtained by crossing mice with conditional deletion of the IL-1R1 (Abdulaal et al., 2016) with mice expressing Cre under the Nestin, HTPA, and TRPV1 promoter, respectively, had no effect on the febrile response (Fig. 6B–D). However, when IL-1R1 was deleted in brain endothelial cells, the casein-induced fever was attenuated (Fig. 6E). A similar finding was obtained in mice with deletion of the membrane-bound IL-6Rα. Deletion of IL-6Rα on brain endothelial cells also attenuated the fever induced by casein injection into an air pouch (Fig. 7A), whereas deletion of this receptor on neural cells or sensory peripheral nerves had no effect (Fig. 7B,C).

Discussion

Several previous studies of localized inflammation in which pyrogen was injected into a preformed air pouch or into an artificial subcutaneously implanted Teflon chamber have suggested that the febrile response seen in such models is elicited by neural signaling from the site of inflammation (Miller et al., 1997; Ross et al., 2003; Rummel et al., 2005; Zhang et al., 2008; Quan, 2014) and not by a humoral pathway as is the case for systemic
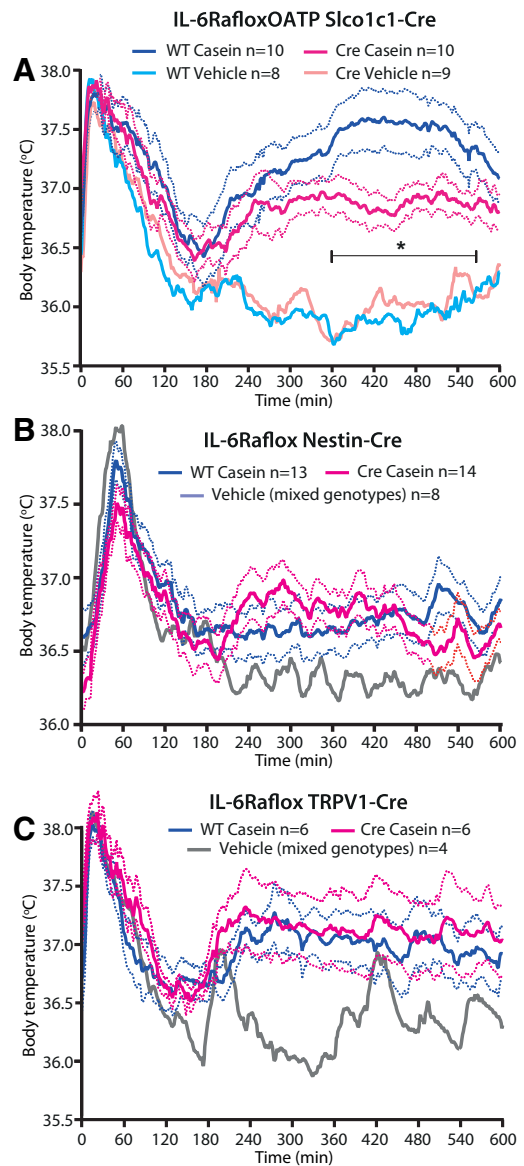

Figure 7. Fever response to casein injected into a preformed air pouch in mice with IL-6Rα deletion. ***A***, Attenuated fever in mice with deletion of IL-6Rα in brain endothelial cells. *$p < 0.05$ for WT mice versus IL-6Rαflox Slco1c1-Cre mice injected with casein (two-way ANOVA for the time-period of 4–10 h: $F_{(3,33)} = 20.39$, $p < 0.0001$). ***B***, ***C***, No difference in the febrile response between WT mice and mice with deletion of IL-6Rα in neural cells (***B***) or thin afferent nerves (***C***). Solid lines represent mean, and dotted lines represent SEM.

inflammation (Blomqvist and Engblom, 2018). This idea has been further fostered by the observations that dorsal root ganglia express IL-1R1 and EP_3 receptors and that peripheral nerves respond to IL-1β and PGE_2 (Nakamura et al., 2000; Binshtok et al., 2008), present at a high concentration at the site of inflammation (Miller et al., 1997; Rummel et al., 2005; Zhang et al., 2008; present study). Furthermore, in several studies of localized inflammation, previous investigators have failed to detect any induction in the brain of Cox-2 (Rummel et al., 2005; Zhang et al., 2008; but see Rummel et al., 2006), considered to be an obligatory step in the synthesis of pyrogenic PGE_2 (Ivanov and Romanovsky, 2004). Nevertheless, in the models of localized inflammation, there is almost invariably elevated levels of IL-6 in plasma (Cartmell et al., 2000; Ross et al., 2003; Rummel et al., 2005, 2006; Zhang et al., 2008), and in studies in which an IL-6 antiserum was given systemically, the febrile response was
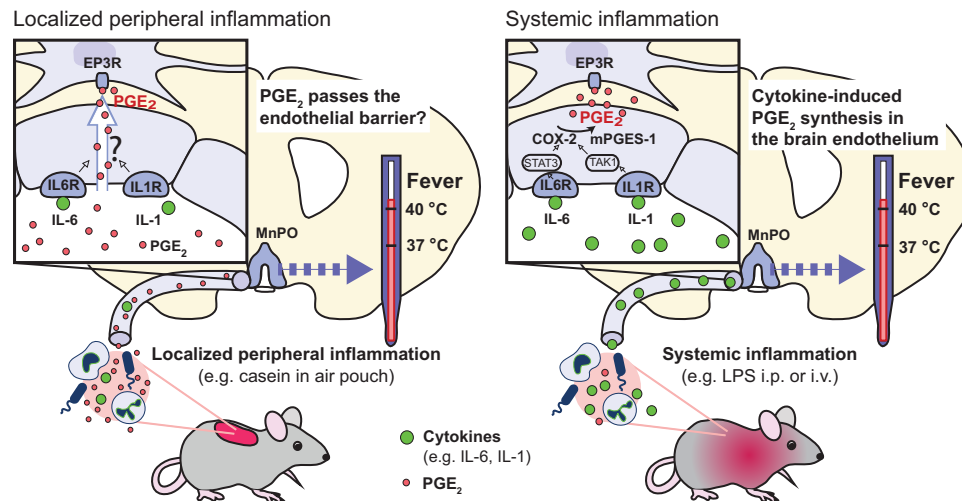
Figure 8. Suggested pathways across the blood-brain barrier for the generation of fever during localized (left) versus systemic (right) inflammation. During localized inflammation, high concentrations of PGE_2 and cytokines are generated at the inflamed site. The PGE_2, and to some extent the cytokines (in particular, IL-6), leak into the circulation to reach the brain. Through a yet unknown mechanism, partly dependent on the presence of receptors for IL-1 and IL-6 on brain endothelial cells (IL1R, IL6R), PGE_2 may enter the brain to reach PGE_2 EP_3 receptor-expressing (EP3R) thermoregulatory neurons in the median preoptic nucleus of the hypothalamus (MnPO). In contrast, systemic inflammation yields high concentrations of cytokines in the circulation. The binding of the cytokines to their receptors on brain endothelial cells results, via the STAT3 and TAK1 pathways, respectively, in induced expression of Cox-2 and mPGES-1 and the subsequent production of PGE_2 in these cells and its transport into the brain parenchyma.

abolished (Cartmell et al., 2000; Rummel et al., 2006), suggesting the presence of a humoral route for eliciting fever also in these models (Rummel et al., 2011).

Here, we examined the signaling pathway for the febrile response to a localized inflammation, casein injection into a preformed air pouch, by using genetically modified mice. We confirmed previous data that the localized inflammation gives rise to high concentrations of PGE_2 and cytokines at the site of inflammation and moderate levels of IL-6 in the circulation (Cartmell et al., 2000; Ross et al., 2003; Rummel et al., 2005; Zhang et al., 2008), but we also detected trace levels of IL-1β and TNF in plasma. We found that the PGE_2 in the air pouch was generated by nonhematopoietic cells, most likely those lining the inner surface of the pouch that displayed Cox-2 immunoreactivity, but that elevated PGE_2 levels were present also in plasma and cerebrospinal fluid. Whereas global deletion of the PGE_2 EP_3 receptor abolished fever, deletion of this receptor on neural crest derivates (and hence on peripheral nerves) had no effect. Similarly, whereas global deletion of the IL-1R1 abolished fever, its deletion in neural crest derivates as well as specifically on thin afferent nerves was without effect. These data refute the idea that binding of PGE_2 or IL-1β onto receptors on peripheral nerves is responsible for the fever seen during localized inflammation. Furthermore, blocking neural signaling from the inflamed site by the injection of a local anesthetic into the air pouch did not affect the febrile response. Hence, our findings provide no evidence that neural signaling from the site of inflammation is critically involved.

Instead, the present data show that fever evoked by a localized inflammation, at least in the present experimental paradigm, is elicited by humoral signaling but by a mechanism that appears distinct from that eliciting fever during systemic inflammation, although they have some characteristics in common (Fig. 8). It is now well established that following peripheral immune challenge with, for example, intraperitoneal or intravenous injection of LPS or IL-1β, fever is elicited by the synthesis of PGE_2 by brain endothelial cells in the preoptic region of the hypothalamus and

that this synthesis in turn is driven by binding of IL-1β and IL-6 to their receptors on the endothelial cells and the subsequent activation of the NfκB and STAT3 signaling pathways, respectively (Ek et al., 2001; Yamagata et al., 2001; Ridder et al., 2011; Engström et al., 2012; Eskilsson et al., 2014, 2017; Wilhelms et al., 2014). Here, we demonstrate that although endothelial deletion of receptors for IL-1 and IL-6 attenuated the fever, similar to what has been shown for systemic inflammation (Eskilsson et al., 2014; Matsuwaki et al., 2017), endothelial deletion of the PGE_2 synthesizing enzymes Cox-2 and mPGES-1 had no effect, which contrasts with the strong attenuation of fever seen after such deletions in models of systemic inflammation (Wilhelms et al., 2014; Eskilsson et al., 2017). Furthermore, whereas systemic immune challenge results in the release of high concentrations of proinflammatory cytokines into the circulation, blood cytokine levels were low in the present model, and although the localized inflammation elicited an induction of Cox-2 in the brain, it was feeble (and therefore likely overlooked in several previous studies). Also, in contrast to the generalized inflammatory activation of brain cytokines seen after systemic immune challenge (Nilsberth et al., 2009b), there was only a weak induced transcription of IL-1β and none of IL-6 in the brain parenchyma.

However, similar to what has been found for fever elicited by systemic immune challenge, fever elicited by casein injection into an air pouch depended on the formation of PGE_2 and its binding to EP_3 receptors in the CNS (Lazarus et al., 2007). Consistent with this finding, PGE_2 levels were elevated in the cerebrospinal fluid. This PGE_2 could originate from the brain and/or the circulation. Although there was only sparse induction of Cox-2 protein in the brain vessels and only minor upregulation of Cox-2 mRNA (and none of mPGES-1 mRNA) in the hypothalamus, and although endothelial deletion of Cox-2 or mPGES-1 did not affect the fever, it cannot be excluded that some cytokine-driven PGE_2 production occurred in cells other than brain endothelial cells. However, this idea is inconsistent with the lack of effect by deletion of prostaglandin synthesis in neural cells and myeloid cells and the feeble microglial activation.

Alternatively, the brain endothelial cells in which recombination does not occur (~15% in the hypothalamus; Wilhelms et al., 2014) could be responsible, but this idea is difficult to reconcile with the strong attenuation of fever seen in other models of peripheral immune challenge after deletion of brain endothelial Cox-2 or mPGES-1, using the same genetically modified mouse lines (Wilhelms et al., 2014) as used here. It is also difficult to reconcile with the attenuation of the febrile response observed here when receptors for IL-1 and IL-6 were deleted in endothelial cells using the same Cre lines as used for deleting the PGE$_2$ synthesizing enzymes. These findings lead to the second possibility that the elevated PGE$_2$ concentrations in the brain, and the central PGE$_2$ that elicited fever, derive from PGE$_2$ in the circulation, which also was elevated in this model. The PGE$_2$ in the circulation in turn is likely to be a result of leakage from the air pouch, which contained very high PGE$_2$ levels, similar to what has been shown for IL-6 (Cartmell et al., 2000).

The role of circulating PGE$_2$ for the generation of fever has been a matter of controversy. Circulating PGE$_2$ is rapidly metabolized, with >90% being cleared by a single passage through the lungs (Hamberg and Samuelsson, 1971), suggesting that it would be unlikely to reach the brain in any significant amount. Nevertheless, intravenous injection of PGE$_2$ has been shown to elicit fever (Steiner et al., 2006; Ootsuka et al., 2008). Some of the same investigators also demonstrated that lung macrophages rapidly produced PGE$_2$ after immune challenge and that peripheral administration of neutralizing antibodies attenuated the first phase of fever (seen during the first 60 min after intravenous injection of LPS; Romanovsky et al., 1998; Rudaya et al., 2005), suggesting that this febrile phase was elicited by circulating PGE$_2$ (Steiner et al., 2006). However, this idea has been challenged: It was shown that mice with deletion of the prostaglandin synthesizing enzyme mPGES-1 in hematopoietic cells displayed a normal first phase of fever, whereas mice that expressed mPGES-1 only in hematopoietic cells showed no fever. The latter mice nevertheless showed significantly elevated levels of PGE$_2$ metabolites in plasma but not of PGE$_2$ in the cerebrospinal fluid (Engström et al., 2012). In the same vein, in a rat tumor model, high levels of PGE$_2$ were recorded in plasma, yet there was no fever and no elevated PGE$_2$ levels in the cerebrospinal fluid (Ruud et al., 2013). However, the situation could be different when elevated PGE$_2$ levels occur during sustained inflammatory conditions that could change the permeability of the blood-brain barrier. Cytokines such as IL-6 have been shown to increase endothelial and blood-brain barrier permeability (Paul et al., 2003; Alsaffar et al., 2018), which could include the transport of PGE$_2$. Such a mechanism would be consistent with the attenuated fever in the present study in mice with endothelial deletion of IL-1R1 and IL-6Rα. Alternatively, the genetic interventions might have altered the impact of endogenous cryogens such as α melanocyte-stimulating hormone, nitrous oxide, vasopressin, or glucocorticoids (Kozak et al., 2000), for example, by facilitating the release or action of any of these substances. Both these putative mechanisms need further examination.

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