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Endogenous interleukin-10 is required for the defervescence of fever evoked by local lipopolysaccharide-induced and *Staphylococcus aureus*-induced inflammation in rats

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We tested the hypothesis that endogenous interleukin (IL)-10 limits the fever induced by a Gram-negative bacterial toxin (*Escherichia coli* lipopolysaccharide, LPS) and a Gram-positive bacterial toxin (*Staphylococcus aureus*), when these toxins are injected into a subcutaneous air pouch (I.PO.) in rats. Injection of LPS or *S. aureus* caused fevers that were reduced in amplitude and duration by simultaneous administration of rat recombinant IL-10. The inhibition of fever by IL-10 was accompanied by a significant reduction in the toxin-evoked increases in concentrations of immunoreactive IL-6 at the site of inflammation and of IL-6 and IL-1 receptor antagonist in the circulation. Conversely, neutralisation of endogenous IL-10 in the pouch increased the amplitude and dramatically increased the duration of toxin-evoked fever, and augmented toxin-induced increases in pouch tumour necrosis factor-α, IL-1β, and especially IL-6. Our data support a crucial regulatory role for endogenous IL-10 in limiting the fever responses during both Gram-negative and Gram-positive infections.

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The pro-inflammatory cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 are believed to be key mediators underlying the febrile response (see for reviews, Kluger, 1991; Zeisberger, 1999). Although much is known about their role in the mechanisms initiating fever (for reviews, see Kluger, 1991; Elmquist *et al.* 1997; Dinarello *et al.* 1999; Zeisberger, 1999; Roth & de Souza, 2001), the mechanisms by which the fevers are maintained, limited and reduced (defervescence) have been little studied, although centrally acting endogenous antipyretic peptides (e.g. arginine vasopressin, α-melanocyte stimulating hormone and adrenocorticotrophic hormone) have been shown to limit febrile responses (see Tatro, 2000, for review).

The internalisation of lipopolysaccharide (LPS)–'LPS receptor' complexes, and (pyrogenic) cytokine–cytokine receptor complexes (Vuk-Pavlovic & Kovach, 1989; Sacht *et al.* 1999), and the downregulation of receptors for pro-inflammatory, pyrogenic cytokines (Neta *et al.* 1992; Tsujimoto & Oku, 1992; van der Poll *et al.* 1997*a*; Barrera *et al.* 2001) are possible ways in which fever responses are limited. Other means include the toxin-stimulated release of a number of endogenous ligands, including IL-1 receptor antagonist (IL-1ra), the endogenous antagonist of IL-1 (Arend 1990; Eisenberg *et al.* 1990; Hannum *et al.* 1990), soluble receptors for IL-1 and TNF (Ulich *et al.* 1994; Fernandez-Botran, 2000) and the so-called anti-inflammatory cytokines. An increasing number of cytokines have been shown to have activities that oppose or downregulate inflammatory processes. Among these are IL-4, IL-10, IL-13 and transforming growth factor-β. These so-called 'anti-inflammatory cytokines' are believed to modulate immune and inflammatory events, allowing resolution of the inflammation and a return of the damaged tissue to its normal state (Feghali & Wright, 1997; Opal & DePalo, 2000). Indeed, IL-6, although generally regarded as a 'pro-inflammatory' cytokine, has both pro-inflammatory and anti-inflammatory actions (see Dinarello, 1997).

IL-10 is an anti-inflammatory cytokine produced by Th2 lymphocytes and monocytes (Fiorentino *et al.* 1989) that potently inhibits the production of the pro-inflammatory cytokines TNF, IL-1, IL-6 and IL-8, while upregulating the expression of IL-1ra (Howard & O'Garra, 1992, Jenkins *et al.* 1994). Elevated plasma IL-10 concentrations have been reported in patients with sepsis (Marchant *et al.* 1994*b*; van der Poll *et al.* 1997*b*) and after the injection of LPS into experimental animals (Durez *et al.* 1993; Wang *et al.* 2001). In addition, exogenous administration of recombinant IL-10 protects mice from lethal endotoxaemia by reducing TNF release (Gérard *et al.* 1993; Howard *et al.* 1993; Marchant *et al.* 1994*a*), and neutralisation of endogenously produced IL-10 in endotoxaemic mice results in an increased production of several pro-

inflammatory cytokines, and enhanced mortality (Bermudez & Champsi, 1993; Standiford *et al.* 1995). In addition, it was shown that IL-10-knockout mice have an increased likelihood of inflammatory bowel disease (Rennick *et al.* 1997), higher mortality rates after experimentally induced sepsis (Berg *et al.* 1995), and develop an exacerbated and prolonged fever in response to I.P. injection of LPS, but not to localised turpentine injection (Leon *et al.* 1999). Collectively, these data imply that IL-10 may function as an endogenous antipyretic in response to systemic LPS, and may have therapeutic potential in acute and chronic inflammatory diseases.

Toxins from both Gram-negative (*Escherichia coli* LPS) and Gram-positive (*Staphylococcus aureus*) bacteria initiate the release of a cascade of pro-inflammatory cytokines following their administration to experimental animals (see Kluger, 1991; Plata-Salamán *et al.* 1998; Turrin *et al.* 2001). Although Gram-negative and Gram-positive pyrogens elicit similar fevers, similar acute-phase reactions and apparently similar sickness behaviours, differences in the processes underlying the responses to the two types of toxin were suggested some years ago (Goelst & Laburn, 1991; Mitchell & Laburn, 1997). It was reported that *S. aureus* may be capable of causing fever independently of the cytokine cascade (Goelst & Laburn, 1991). Consequently, responses to *S. aureus* might be expected to be less susceptible to IL-10-induced inhibition of the production of endogenous pyrogenic cytokines, if that indeed is the mechanism of action of IL-10. More recently, evidence has accumulated to indicate that LPS and *S. aureus* toxin activate different cell-surface Toll-like receptors (TLR), TLR4 and TLR2, respectively (see Akira, 2003), and that the stimulation of these different receptors can lead to different profiles of cytokine production, and potentially, different sensitivities to the actions of anti-inflammatory cytokines such as IL-10 (see O'Neill, 2002).

Using a model of localised inflammation in rats, we have examined the potential antipyretic role of IL-10 in fevers evoked by Gram-negative and Gram-positive toxins. In this particular model, in which an exogenous pyrogen, such as LPS, is injected into a subcutaneous air pouch (Miller *et al.* 1997), the exogenous pyrogen remains at the site of its administration (Cartmell *et al.* 2001) and the fever appears to be dependent upon LPS-induced IL-6, which finds its way into the blood from the pouch, in contrast to LPS and LPS-induced TNF and IL-1, which are produced but remain in the pouch (Cartmell *et al.* 2000). The sequence of events in response to intrapouch (I.PO.) injection of *S. aureus* has yet to be elucidated. Given the capacity of IL-10 to inhibit the production of the pro-inflammatory cytokines TNF, IL-1 and IL-6, and to increase IL-1ra production, our experiments were designed to investigate the role of exogenous and endogenous IL-10 on experimentally induced fever and cytokine production, both at the site of inflammation and in the circulation.

METHODS

Male and female Sprague-Dawley rats (250–300 g) were used in all experiments. Animals were housed in a controlled environment at an ambient temperature of 24 ± 2 °C (mean ± S.E.M.) on a 12:12 h light:dark cycle (lights on from 07.30 to 19.30 h). Food (pelleted rat chow, Epol, Johannesburg, South Africa) and water were provided *ad libitum*. Unless specified otherwise, at the end of the experimental period, animals were killed by an overdose of a rising concentration of carbon dioxide. All procedures were approved by the Animal Ethics Committee of the University of the Witwatersrand (South Africa) under clearance 2001/73/4.

Measurement of body temperature

The core body temperature of unrestrained rats was measured by remote biotelemetry, using temperature-sensitive radiotelemeters (TA10TA-F40, Data Sciences) implanted intraperitoneally whilst animals were under ketamine (80 mg kg^{-1} I.M.) and xylazine (4 mg kg^{-1} I.M.) anaesthesia. Animals were housed individually after surgery and allowed at least 1 week to recover from surgery before experimentation. Transmitter output frequency (Hz) was monitored at 10 min intervals by an antenna mounted in a receiver board, which was situated beneath the cage of each animal, and the data was logged into a peripheral processor (VitalView, Minimitter, Sunriver, OR, USA) connected to a personal computer. The telemeters and receiver were calibrated against a quartz thermometer (Quat, Heraeus) to an accuracy of 0.1 °C.

Air pouch

A subcutaneous air pouch was formed, as described by Edwards *et al.* (1981), immediately after implantation of the radiotelemeter while animals were still under ketamine and xylazine anaesthesia (see above). Briefly, 20 ml of sterile air (filtered through 0.2 μm Acrodisc, Gelman Sciences, USA) was injected into the subcutaneous tissue of the dorsal midline, caudal to the scapulae. Three days after the initial pouch formation, animals were briefly re-anaesthetised (3 % halothane (Fluothane, Zeneca) in oxygen) and the air pouches were reinflated with a further 10 ml of sterile air, to maintain open cavities. On day 6, rat recombinant (rr) IL-10, sheep anti-rat IL-10 serum (anti-IL-10), pre-immune sheep serum (PIS), pyrogen, or vehicle (saline) was injected directly into the air pouches of lightly restrained (hand held), conscious animals.

Materials

The following reagents were employed: rrIL-10, in the form of recDNA IL-10^{tyr149}, a stable analogue with full biological activity (Ball *et al.* 2001), *E. coli*-derived (NIBSC, UK) with an endotoxin content < 4 ng (40 IU) mg^{-1} of protein, as measured in a Limulus Amoebocyte Lysate test (European Pharmacopoeia, 1999); sheep anti-rat IL-10 serum (raised against recDNA rat IL-10^{tyr149}, NIBSC) and PIS (NIBSC), both with endotoxin content < 0.24 ng (2.4 IU) ml^{-1} of protein; purified LPS from *E. coli* (serotype, 0111:B4, Sigma, UK) and killed organisms (cell walls) of *S. aureus* (Sigma). LPS was reconstituted with saline (sterile, pyrogen-free 0.9 % (w/v) saline, Sabax, Johannesburg, South Africa) and injected at a dose of 100 μg kg^{-1}. The cell walls of *S. aureus*, heat-killed and fixed in formalin, were supplied as a suspension in phosphate-buffered saline, and injected at a final dose of 3×10^{11} cell walls kg^{-1}.

Experimental protocol

Experiment 1. Rats were injected I.PO. with rrIL-10 (200 μg kg^{-1}) or saline, immediately followed by an injection (I.PO.) of LPS (100 μg kg^{-1}, $n = 5$), *S. aureus* (3×10^{11} cell walls kg^{-1}, $n = 5$) or saline (1 ml kg^{-1}, $n = 5$). Core body temperature was monitored for 24 h after injection. A minimum of 1 week was allowed to elapse between injections in the same animal. Blood and pouch fluid samples (for assay of cytokines) were collected from separate groups of animals (under terminal anaesthesia with halothane), 4 h after injection of LPS or saline ($n = 5$ per treatment group). Sampling of the inflammatory exudates within the pouch was achieved by lavaging the pouch with 1 ml of sterile saline. The lavage fluid was quickly aspirated, centrifuged (3000 *g*, 4 °C, 10 min), with the resulting supernatant collected and stored at −70 °C until assayed. Blood was collected by cardiac puncture into sterile tubes containing pyrogen-free heparin (10 IU ml^{-1}) and centrifuged (5300 *g*, 4 °C, 10 min). Plasma was stored at −70 °C until assayed.

Experiments 2 and 3. Anti-rat IL-10 serum or PIS was injected (1 ml, I.PO.) into rats at the same time as the injection (I.PO.) of LPS (100 μg kg^{-1}, $n = 5$ per treatment), *S. aureus* (3×10^{11} cell walls kg^{-1}, $n = 5$ per treatment), or saline (1 ml kg^{-1}, $n = 5$). Body temperature was monitored continuously for 72 h. Blood and pouch fluid samples (for assay of cytokines) were collected from separate groups of antiserum-treated animals (under terminal anaesthesia with halothane), 5 and 25 h after injection of LPS, *S. aureus* or saline ($n = 5$ per treatment group).

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of TNF-α, IL-1β, IL-6, IL-1ra and IL-10 in the plasma and pouch fluid were measured using rat-specific sandwich ELISAs (NIBSC), as described previously (Safieh-Garabedian *et al.* 1995; Rees *et al.* 1999*a, b*; Cunha *et al.* 2000; Ball *et al.* 2001). The ELISA for rat IL-1β detects both precursor and mature IL-1β but not TNF-α, IL-1α, IL-6, IL-1ra or IL-10. Similarly, the ELISAs for rat TNF-α, IL-6, IL-1ra and IL-10 do not cross-react with rat IL-1β, rat IL-1α or each other (NIBSC). The sensitivities of the assays were: IL-1β and IL-6, 1.9 pg ml^{-1}; TNF, IL-1ra and IL-10, 3.9 pg ml^{-1}. Given the high concentrations of cytokines at the site of inflammation compared to those in the circulation, some samples required large dilutions. The assay detection limit, which allows for the sample dilution factor, therefore differs between different biological fluids and treatment groups.

Statistical analysis

All data are expressed as means ± S.E.M. for five animals. Temperature responses were plotted as body temperature–time curves. Peak change in body temperature and fever indices (FIs, °C h, calculated as the time integral of the body temperature elevation above the temperature prevailing at the time of injection) were determined. Data were analysed using either ANOVA, followed by a Tukey-Kramer Multiple Comparisons *post hoc* test for differences between more than two groups, or Student's *t* test for differences, at the same time point, between two groups. Cytokine concentrations, at the various time points and in the various biological fluids, were compared with the corresponding concentrations measured in control animals, at the same time and in the same biological fluid using either ANOVA, followed by a Tukey-Kramer Multiple Comparisons *post hoc* test for differences between more than two groups, or Student's *t* test for differences at the same time point, between two groups. Comparisons between different cytokines and/or time points were not analysed. Where cytokine concentrations were undetectable, samples were assigned a value equivalent to the detection limit of the assay. A two-tailed probability $P < 0.05$ was considered statistically significant.

RESULTS

Effect of exogenous IL-10 on LPS-evoked fever and cytokine production

LPS (100 μg kg^{-1}, I.PO.) evoked fever that began approximately 2 h after its injection and reached a maximum value of 39.0 ± 0.1 °C at 3 h, significantly higher than the 37.0 ± 0.2 °C in vehicle-injected animals (Fig. 1, upper panel, $P < 0.001$, ANOVA, Tukey *post hoc* test). Exogenous IL-10 (200 μg kg^{-1}, I.PO.) significantly reduced both the amplitude and duration of the LPS-induced fever ($P < 0.001$, ANOVA, Tukey *post hoc* test): the maximum body temperature in the presence of IL-10 was 37.8 ± 0.1 °C (Fig. 1*A*) and the FI (the area under the fever curve) was reduced by 67 %. Exogenous IL-10 alone had no effect on body temperature: body temperature following IL-10 injection was not different from that of saline-injected animals ($P > 0.05$, ANOVA, Tukey *post hoc* test). IL-10 attenuated the amplitude of the *S. aureus*-evoked fever, on days 2 and 3, but had no effect on the duration of the fever (Fig. 2).

TNF-α, IL-1β, IL-6, IL-1ra and IL-10 were detected in the pouch (site of injection) 4 h after injection of LPS (Fig. 1*B*). In contrast, only IL-6, IL-1ra and IL-10 were detected in the plasma (Fig. 1*C*). Both local (pouch) and circulating IL-6, and circulating IL-1ra concentrations evoked by LPS were reduced by 48, 39 and 70 %, respectively, 4 h after injection of exogenous IL-10 (Fig. 1*B* and *C*).

Effect of neutralising endogenous IL-10 within the pouch on LPS-induced fever and cytokine concentrations

The temperature responses of control animals (PIS + saline; anti-IL-10 serum + saline) were similar for the duration of the experiment (3 days; $P > 0.05$, ANOVA, Tukey *post hoc* test; Fig. 3*A*). LPS (+ PIS), injected I.PO., evoked fever that began 2–3 h after LPS injection and reached a maximum value of 38.2 ± 0.2 °C, significantly higher than that of 37.2 ± 0.1 °C in controls ($P < 0.001$, Fig. 3*A*). Injection (I.PO.) of sheep anti-IL-10 serum significantly increased the peak fevers evoked by LPS to 38.8 ± 0.2 °C, and fevers were maintained during the day at some 0.5–0.8 °C higher than in animals treated with LPS + PIS for the duration of the experiment (3 days).

Figure 3*B* shows the FIs calculated for 12 h time periods for a total of 72 h after injection. The FI takes into account both the amplitude and the duration of the fever. The 9 h FI (i.e. from injection until lights out, 10.30–19.30 h on day 1) evoked in response to injection of LPS was eightfold higher than in controls (PIS + saline; anti-IL-10 + saline; $P < 0.001$, ANOVA, Tukey *post hoc* test, Fig. 3), but was

similar in animals treated with PIS (horizontal hatched columns) or anti-IL-10 (open columns, $P > 0.05$, ANOVA, Tukey *post hoc* test). Treatment with anti-IL-10 potentiated the 12 h night FI on day 1 (twofold), and both the day and night FIs evoked by LPS on days 2 and 3 after injection (approximately twofold).

Treatment with sheep anti-rat IL-10 serum (I.PO.), at the same time as injection of LPS, also affected concentrations of cytokines (Fig. 4) at the site of injection (pouch) and in the plasma. Treatment with anti-rat IL-10 serum abolished the increase in pouch IL-10 concentrations and augmented the increases in local (pouch) IL-6 (twofold;

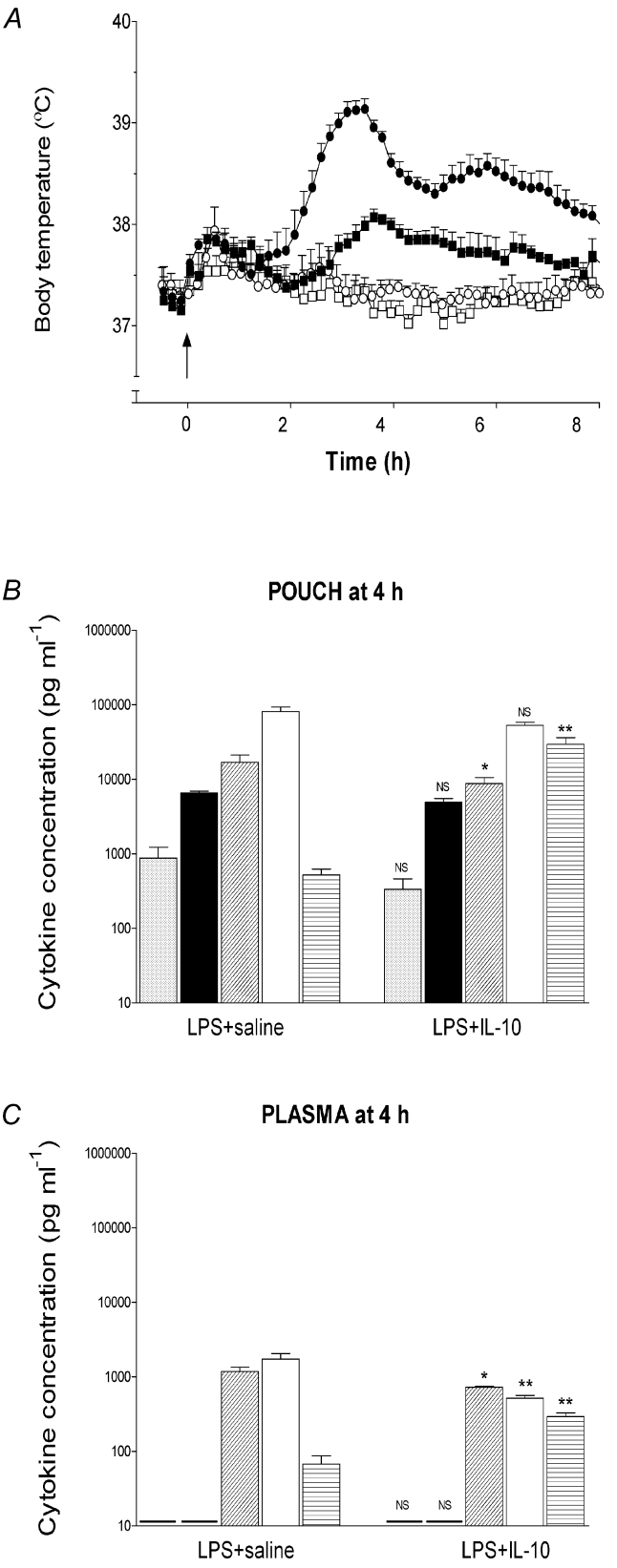

Figure 1. Effect of exogenous IL-10 on LPS-evoked fever and cytokine production

A, fever response of rats injected intrapouch with LPS (100 μg kg^{-1}, filled symbols, $n = 5$) or saline (open symbols, $n = 5$) and IL-10 (200 μg kg^{-1}, squares) or saline (1 ml kg^{-1}, circles) at 10.00 h (arrow). *B* and *C*, concentrations of immunoreactive TNF-α (grey columns), IL-1β (black columns), IL-6 (diagonal hatched columns), IL-1ra (open columns) and IL-10 (horizontal hatched columns) at the site of LPS injection (pouch) and in the plasma, respectively, 4 h after treatment with IL-10. Data are expressed as means ± S.E.M., $n = 5$. — indicates value below the detection limit of the assay. NS, not significant, $^*P < 0.05$, $^{**}P < 0.01$ *vs.* LPS + saline, Student's *t* test.

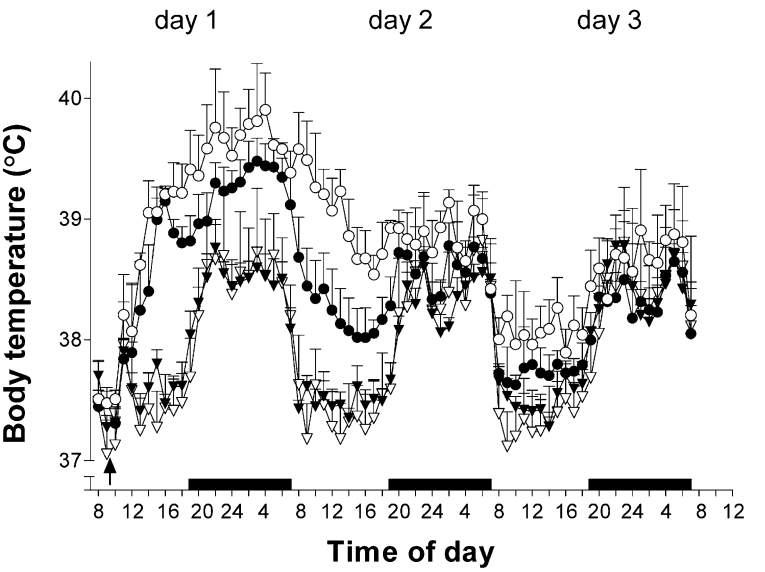

Figure 2. Effect of exogenous IL-10 on *S. aureus*-evoked fever

The fever response of rats injected I.PO. with *S. aureus* (3×10^{11} cell walls kg^{-1}, circles, $n = 10$) or saline (triangles, $n = 10$), and IL-10 (200 μg kg^{-1}, filled symbols) or saline (1 ml kg^{-1}, open symbols). The results are presented as means ± S.E.M., $n = 5$. The arrow indicates the time of injection; the black bars indicate periods of darkness (lights out).

Fig. 4*A*), but had no effect on local and circulating concentrations of TNF-α, IL-1β and IL-1ra, and circulating IL-6, evoked by LPS 5 h after injection (Fig. 4). Pouch fluid concentrations of IL-1β, IL-6, IL-1ra and IL-10, evoked by LPS, remained elevated 25 h after injection of LPS and, except for IL-1ra and IL-10, were even higher in animals in which endogenous IL-10 was neutralised (Fig. 4*B*). In the circulation, only IL-1ra (Fig. 4, lower panel) concentrations were potentiated by treatment with anti-IL-10, 25 h after injection of LPS.

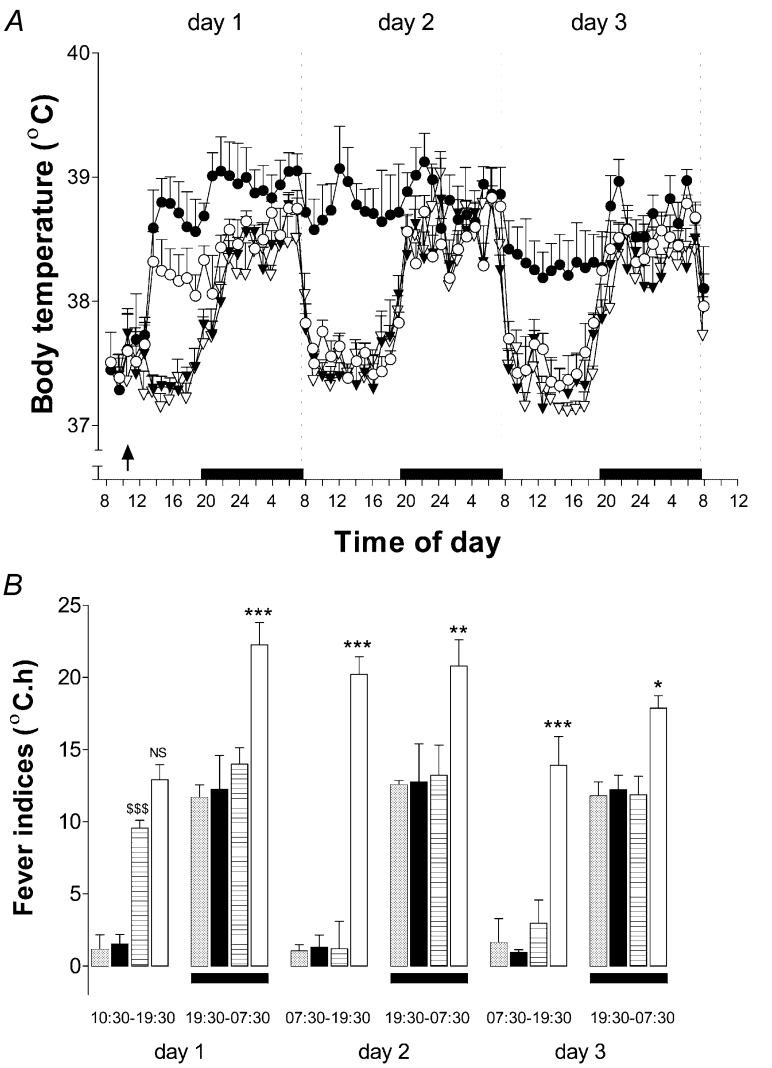

Figure 3. Treatment with anti-IL-10 serum prolongs the duration of LPS-evoked fever

A, I.PO. injection of sheep anti-IL-10 serum (1 ml, filled symbols) or sheep pre-immune serum (PIS, 1 ml, open symbols) followed by injection (I.PO.) of LPS (100 μg kg^{-1}, circles) or saline (1 ml kg^{-1}, triangles). The results are presented as means + S.E.M., $n = 5$. The arrow indicates the time of injection; the black bars indicate periods of darkness (lights out). *B*, fever indices (FIs, °C h, means ± S.E.M., $n = 5$) calculated from the time of injection for rats receiving: PIS + saline (grey columns); anti-IL-10 serum + saline (black columns); PIS + LPS (horizontal hatched columns); anti-IL-10 serum + LPS (open columns). FIs are time integrals of the change in body temperature from the pre-injection body temperature prevailing on the day of injection. Black bars indicate periods of darkness (lights out). NS, not significant; ***$P < 0.001$ PIS + LPS *vs.* anti-IL-10 + LPS; ANOVA (Tukey *post hoc* test) for the same time frame. $$$ $P < 0.001$ PIS + saline *vs.* PIS + LPS; ANOVA (Tukey *post hoc* test) for the same time frame.

Effect of neutralising endogenous IL-10 on *S. aureus*-induced fever and local and circulating cytokine concentrations

I.PO. injection of *S. aureus* (3×10^{11} cell walls kg^{-1}, I.PO.) evoked fever approximately 2 h after injection, with an initial peak of 38.9 ± 0.2 °C at 6 h (Fig. 5, upper panel). Treatment with anti-rat IL-10 serum significantly potentiated the fever peak to 39.3 ± 0.3 °C, and fevers following *S. aureus* + anti-IL-10 were maintained some 0.5 °C higher than in animals treated with *S. aureus* + PIS (Fig. 5, upper panel). Treatment with anti-IL-10 serum also significantly potentiated the mean night-time body temperatures evoked by *S. aureus* from 38.8 ± 0.1 to 39.2 ± 0.1 °C, and body temperatures were higher than in appropriate controls (i.e. PIS + saline: mean, 38.2 ± 0.1 °C and anti-IL-10+saline: mean, 38.3 ± 0.1 °C). The sharp fall in body temperature observed with the onset of daytime (lights on, day 2) was abolished in animals treated with *S. aureus* + anti-IL-10, and temperatures evoked by *S. aureus* + anti-IL-10 were sustained about 1 °C higher than in animals treated with *S. aureus* + PIS (38.4 ± 0.1 °C) and 2.5 °C higher than in control animals (PIS + saline, 36.9 ± 0.1 °C; anti-IL-10 + saline, 36.9 ± 0.1 °C). A similar pattern was observed on day 3.

The lower panel on Fig. 5 shows the 12 h FIs calculated for the 3 day period following injection of *S. aureus* (or saline) in animals treated with PIS or anti-IL-10. The FI

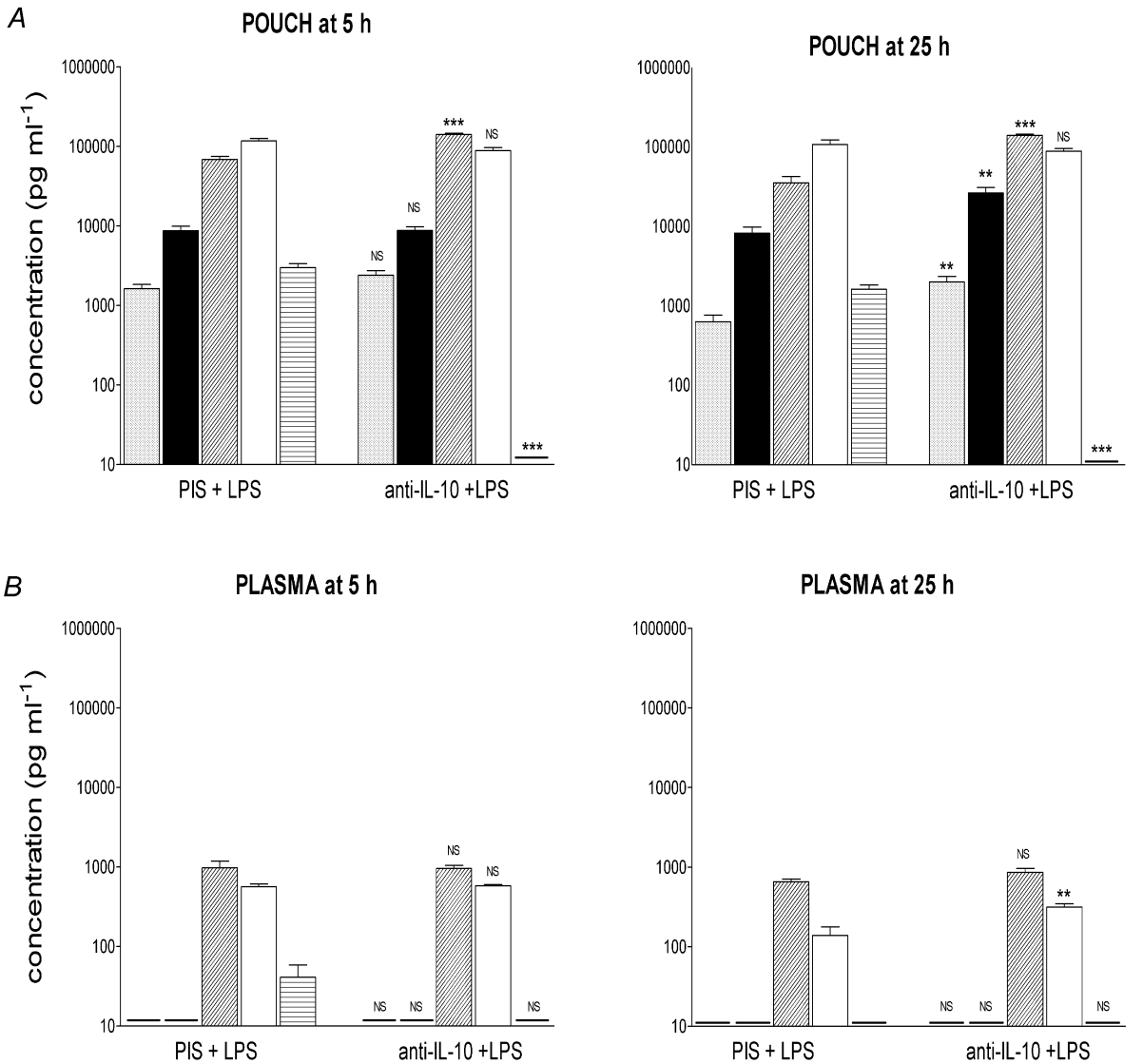

Figure 4. Effect of treatment with anti-IL-10 serum on LPS-induced cytokine production

Concentrations of immunoreactive TNF-α (grey columns), IL-1β (black columns), IL-6 (diagonal hatched columns), IL-1ra (open columns) and IL-10 (horizontal hatched columns) at the site of injection (pouch, *A*) and in the plasma (*B*), 5 and 25 h after injection (I.PO.) of PIS + LPS (100 μg kg^{-1}) or anti-IL-10 + LPS. Data are expressed as means ± S.E.M., $n = 5$. — indicates value below the detection limit of the assay. NS, not significant; $^{**}P < 0.01$, $^{***}P < 0.001$; PIS + LPS *vs.* anti-IL-10 + LPS, ANOVA (Tukey *post hoc* test).

(10.30–19.30 h) for the (daytime) period of 9 h immediately subsequent to injection of *S. aureus* was eightfold higher than in controls (PIS + saline; anti-IL-10 + saline; Fig. 5*B*) and remained so for the entire 3 days following injection. Treatment with anti-IL-10 had no effect on the FI evoked by *S. aureus* for the first 20 h after injection (Fig. 5*B*), but potentiated the 12 h (daytime) FI (by 75 %) on day 2 after injection. By day 3, there was no difference between the two groups. The 12 h (night-time FI) evoked by *S. aureus* was 1.6-fold higher than in controls on day 1 after injection (Fig. 5*B*), but was similar in animals injected with anti-IL-10 or PIS. By the evening of day 2, there was no difference between control animals and animals injected with *S. aureus* ($P > 0.05$, ANOVA, Tukey *post hoc*).

Treatment with sheep anti-rat IL-10 serum, at the same time as injection of *S. aureus*, also had effects upon concentrations of cytokines (Fig. 6) at the site of injection (pouch) and in the plasma. Treatment with anti-IL-10 abolished the increase in pouch IL-10 concentrations evoked by *S. aureus* (as expected) and potentiated the increases in TNF-α (4-fold), IL-1β (2.5-fold), and IL-6 (4.8-fold) at the site of injection (Fig. 6*A*; $P < 0.01$, ANOVA, Tukey *post hoc* test); and increased circulating concentrations (approximately 4-fold) of IL-6 ($P < 0.01$, ANOVA, Tukey *post hoc* test) and IL-1ra ($P < 0.01$, ANOVA, Tukey *post hoc* test; Fig. 6*B*) 5 h after injection. Pouch concentrations of TNF-α, IL-1β, IL-6, IL-1ra and IL-10 remained elevated 25 h after injection of *S. aureus*. However, only pouch IL-6 concentrations were increased significantly (5-fold) at this time point in animals treated with anti-IL-10 ($P < 0.001$, ANOVA, Tukey *post hoc* test; Fig. 6*A*), and pouch IL-10 concentrations still were undetectable in this same group of animals. Treatment with anti-IL-10 had no effect on circulating cytokine concentrations 25 h after injection of *S. aureus* (Fig. 6*B*).

DISCUSSION

The cloning of recDNA rat IL-10^{tyr149}, a stable IL-10 analogue with full biological activity (Ball *et al.* 2001), and the generation of a sheep antiserum neutralising this molecule have permitted the study of the role of

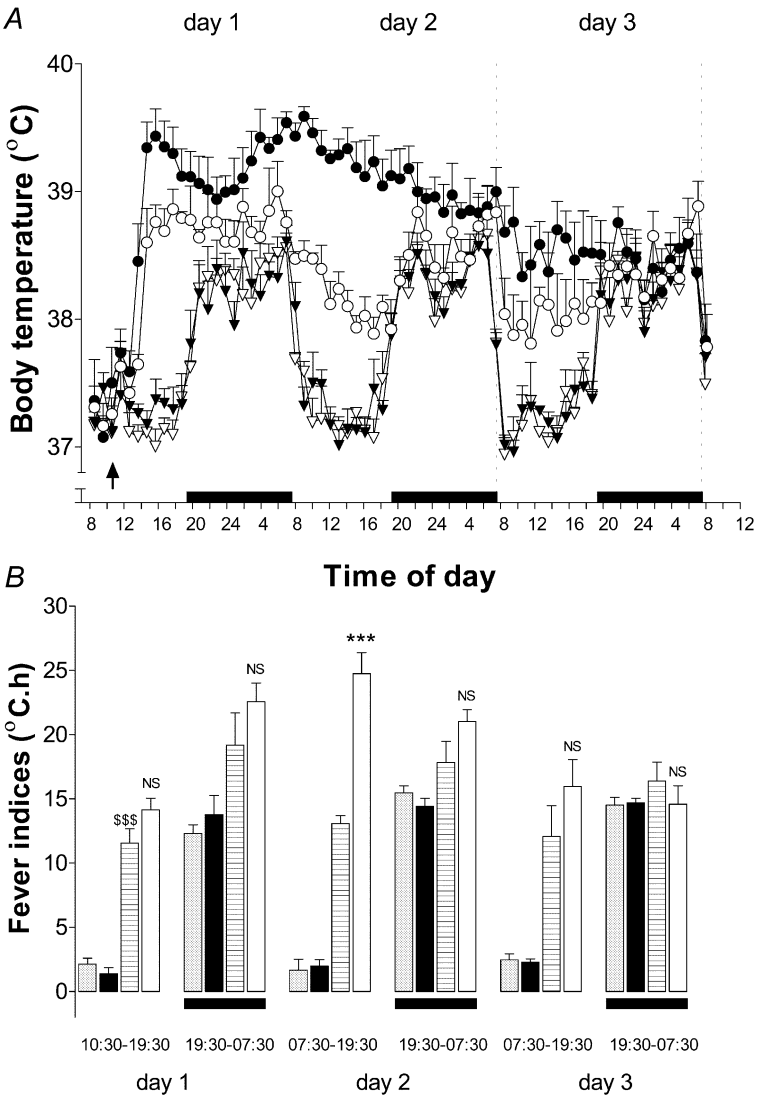

Figure 5. Treatment with anti-IL-10 serum exacerbates the magnitude of *S. aureus*-evoked fever

A, I.PO. injection of sheep anti-IL-10 serum (1 ml, filled symbols) or PIS (1 ml, open symbols) immediately followed by injection (I.PO.) of *S. aureus* (3×10^{11} cell walls kg^{-1}, circles) or saline (1 ml kg^{-1}, triangles). The results are presented as means ± S.E.M., $n = 5$. The arrow indicates time of injection; bars indicate periods of darkness (lights out). *B*, FIs (°C h, means ± S.E.M., $n = 5$) calculated from the time of injection for rats receiving: PIS + saline (grey columns); anti-IL-10 serum + saline (black columns); PIS + *S. aureus* (horizontal hatched columns); anti-IL-10 serum + *S. aureus* (open columns). FIs were calculated as in Fig. 2. Bars indicate periods of darkness (lights out). Data are expressed as means ± S.E.M. NS, not significant; ***$P < 0.001$ PIS + *S. aureus vs.* anti-IL-10 + *S. aureus*; ANOVA (Tukey *post hoc* test) for the same time frame. $$$$P < 0.001$ PIS + saline *vs.* PIS + *S. aureus*; ANOVA (Tukey *post hoc* test) for the same time frame.

endogenous IL-10 in limiting the fevers caused by local injections of *E. coli* LPS and *S. aureus* toxin. We report that exogenous rat IL-10 is a potent inhibitor of the fevers caused by local injections of LPS and *S. aureus*, which is consistent with previous studies using only LPS as the inflammatory stimulus (Nava *et al.* 1997; Pajkrt *et al.* 1997; Leon *et al.* 1999: Ledeboer *et al.* 2002) and raises the possibility that IL-10 may be useful in treating inflammation and fever. The marked inhibitory effect of IL-10 on the amplitude of fever and, in the case of LPS, on the duration of fever, contrasted with its much less dramatic effects on the concentrations of pyrogenic cytokines in the pouch and of IL-6 in the blood. It is possible that IL-10 had a more marked effect on concentrations of pyrogenic cytokines in the pouch at times before we made our first measurements. In addition, actions by blood-borne IL-10, concentrations of which were elevated following its administration into the pouch, may have contributed to its antipyretic effect. Since the ELISA for rat IL-10 does not discriminate between endogenous and injected (i.e. tyr^{149}) IL-10, it is not possible to say with certainty that the IL-10 immunoreactivity detected in the plasma was derived from the pouch. The effect of IL-10 was unlikely to have involved the induction of IL-1ra by IL-10 (Cassatella *et al.* 1994; Jenkins *et al.* 1994). Indeed, IL-10 actually reduced the concentrations of IL-1ra in the plasma.

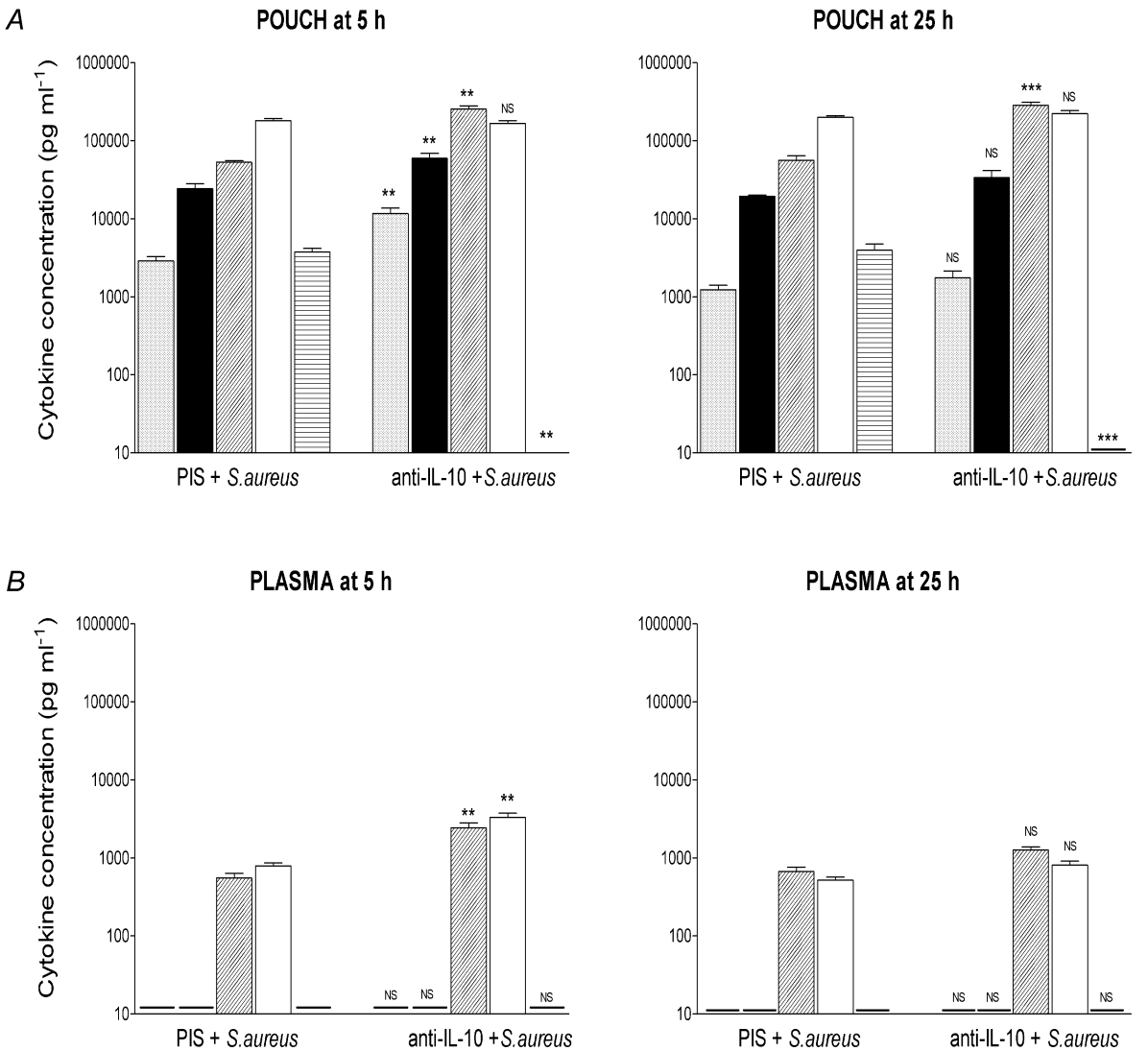

Figure 6. Effect of treatment with anti-IL-10 serum on *S. aureus*-induced cytokine production

Concentrations of immunoreactive TNF-α (grey columns), IL-1β (black columns), IL-6 (diagonal hatched columns), IL-1ra (open columns) and IL-10 (horizontal hatched columns) at the site of injection (pouch, *A*) and in the plasma (*B*) 5 and 25 h after injection (I.PO.) of PIS + *S. aureus* (3×10^{11} cell walls kg^{-1}) or anti-IL-10 + *S. aureus*. Data are expressed as means ± S.E.M., $n = 5$. – indicates value below the detection limit of the assay. NS, not significant; $^{**}P < 0.01$, $^{***}P < 0.001$; PIS + *S. aureus vs.* anti-IL-10 + *S. aureus*, ANOVA (Tukey *post hoc* test).

The profiles of the fever responses to LPS and *S. aureus* (in the absence of anti-IL-10) were different. The response to *S. aureus* was of much longer duration, > 48 h, compared with about 10 h for the response to LPS. In the case of the *S. aureus*-induced fever, which lasted into the night-time of day 2, the nychthemeral rhythm (increased body temperature during the hours of darkness; see Luker *et al.* 2000) obscured the night-time fever response. In spite of this, neutralisation of endogenous IL-10 in the pouch had profound effects upon the fever responses to both LPS and *S. aureus*. Anti-IL-10 increased the amplitude and greatly increased, from about 10 h to > 72 h, the duration of LPS-evoked fever, whereas anti-IL-10 increased only the amplitude, but markedly so, of the already long-lasting *S. aureus*-induced fever. Comparison of pouch and plasma IL-10 concentrations at 5 and 25 h after injection of LPS or *S. aureus* alone, revealed no significant differences between the two time points, despite the dramatic increase in body temperature in response to toxin + anti-IL-10 at 25 h. Nevertheless, our data suggest strongly that locally produced IL-10 has a role as an endogenous antipyretic. In contrast, in another widely used model of local inflammation, in which turpentine was injected subcutaneously, endogenous IL-10 did not appear to have a role in the fever. IL-10-knockout mice (which lack the functional IL-10 gene in all tissues in the body) developed the identical, typical 4–8 h fever observed in wild-type mice (Leon *et al.* 1999), whereas I.P. (systemic) injection of LPS in IL-10-knockout mice evoked fever that was observed on both the 1st and 2nd day after injection (Leon *et al.* 1999). It is plausible, however, that redundancies of cytokine actions *in vivo* have developed in the mediation of fever to turpentine in knockout mice (Paul, 1989; see Kluger, 1991).

Despite the profound effects on fever of neutralisation of IL-10 observed in the present study, the effects of neutralisation of IL-10 on concentrations of pyrogenic cytokines were less dramatic. Enhanced plasma levels of IL-6 in IL-10-knockout mice at 4 h (corresponding with maximum fever) but not the following day (also a time point of maximum fever) have been reported by Leon *et al.* (1999) and led the authors to hypothesise that IL-10 has an endogenous antipyretic action during LPS-induced fever due to its capacity to inhibit the production of endogenous IL-6. Our data in part support such a role for endogenous IL-6. Exogenous IL-10 administration significantly decreased both pouch and plasma IL-6 concentrations 4 h after injection of LPS and, despite the absence of a significant increase in plasma IL-6 concentrations in animals treated with LPS + anti-IL-10 compared to animals injected with LPS alone, local IL-6 concentrations (that is, in the pouch) were increased at both 5 and 25 h after injection, time points at which exacerbated fever occurred. I.PO. administration of *S. aureus* + anti-IL-10 similarly resulted in significant increases in pouch IL-6 concentrations at both 5 and 25 h after injection, and in plasma IL-6 concentrations at 5 h, lending further support to the hypothesis proposed by Leon and colleagues. Of particular note was the finding that following injection of LPS + anti-IL-10, body temperature remained elevated in the absence of increases in plasma concentrations of IL-6 over and above those obtained with LPS given alone, suggesting that plasma IL-6 was not solely responsible for the prolonged fevers evoked by LPS in the presence of anti-IL-10. This result was unexpected since there is evidence to suggest that IL-6 is the final circulating mediator of fever in this model of localised LPS-induced infection/inflammation (Cartmell *et al.* 2000). Interestingly, IL-6-knockout mice failed to develop fever in response to I.P. injection of LPS (Chai *et al.* 1996), although at high doses of LPS fever was evoked, probably through the production of additional endogenous mediators that compensated for the lack of IL-6 (Kozak *et al.* 1998). Also worthy of note is that in the present study IL-6 concentrations were significantly elevated in the absence of fever (Fig. 4, LPS + PIS, 25 h). Either this observation calls into question the role of IL-6 in fever or it implies that IL-6 in the periphery needs to act in concert with another mediator to be pyrogenic.

In the present study, anti-IL-10 had no effect on the concentrations of TNF-α and IL-1β in the pouch at 5 h after LPS injection, by which time the fever had reached its peak, but did increase concentrations of TNF-α and IL-1β in the pouch at 25 h after LPS injection. The opposite was true for *S. aureus*: anti-IL-10 increased concentrations of TNF-α and IL-1β in the pouch at 5 h after *S. aureus* injection, again by which time the fever had reached its peak, but did not increase concentrations of TNF-α and IL-1β in the pouch at 25 h after *S. aureus* injection. Similarly, anti-IL-10 treatment had no significant effect on circulating concentrations of TNF-α or IL-1β, at both 5 and 25 h. In fact, the only cytokine that was increased significantly in the plasma in response to treatment with either LPS or *S. aureus* and anti-IL-10 was IL-1ra. This finding was surprising since IL-10 has been shown to induce the expression of IL-1ra (Cassatella *et al.* 1994; Jenkins *et al.* 1994). The mechanism underlying the prolonged fever response to LPS in animals in which IL-10 had been neutralised remains to be resolved. It is plausible that another cytokine or chemokine might be produced in the pouch in response to exogenous pyrogens, and find its way into the blood. Our preliminary data suggest that cytokine-induced neutrophil chemoattractant-1, which is structurally similar to the pyrogenic human chemokine IL-8 (Zagorski & DeLarco, 1993), might be produced in the pouch in response to exogenous pyrogens. It has been proposed that both IL-6 and IL-8 have important roles in transforming acute into chronic inflammation (Marin *et al.* 2001).

Alternatively, vagal afferents may convey communication between pyrogen-sensitive cells in the periphery and the brain (Dantzer, 1994; Watkins *et al.* 1995), especially if pyrogens are present at low doses in the abdomen rather than in the blood (Bluthé *et al.* 1996; Romanovsky *et al.* 1997). The evidence for such a pathway has burgeoned over the last 5 years (see Zeisberger, 1999; Romanovsky, 2000). However, the importance of this route, and the question of whether or not it has a special role in abdominal pyrogen assault, remains controversial (see Romanovsky, 2000). This pathway of communication certainly cannot be advanced as a general mechanism by which pro-inflammatory cytokines trigger the cascade of thermoregulatory events taking place in response to systemic pyrogenic challenge (Rivest *et al.* 2000). In a view similar to that advanced for vagal signalling, modest evidence has been provided recently for the participation of cutaneous afferents in the transport of immune information from the skin to the brain, in the genesis of fever (Ross *et al.* 2000).

For the present, we can report that locally produced IL-10 has an important role as an endogenous antipyretic, and that it is unlikely to achieve its antipyretic effect primarily by inhibition of the pro-inflammatory cytokines TNF, IL-6 and IL-1. It is likely that other endogenous pyrogens, and or chemokines, contribute to the generation of fever to localised LPS or *S. aureus* administration.

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