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Divergent splanchnic sympathetic efferent nerve pathways regulate IL-10 and TNF responses to endotoxaemia.

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Key points

- An endogenous neural reflex, mediated by the splanchnic, but not other sympathetic nerves, moderates the cytokine response to systemic inflammatory challenge. This reflex suppresses the pro-inflammatory cytokine TNF, while enhancing levels of the anti-inflammatory cytokine IL-10.
- The reflex enhancement of IL-10 depends on the splanchnic nerve supply to the adrenal gland and on β_2 adrenoreceptors, consistent with mediation by circulating adrenaline. After splanchnic nerve section it can be rescued by restoring circulating adrenaline.
- The reflex suppression of TNF depends on splanchnic nerve branches that innervate abdominal tissues including, but not restricted to, spleen: it is not blocked by adrenal denervation or β_2 adrenoreceptor antagonism.
- Distinct sympathetic efferent pathways are thus responsible for pro- and anti-inflammatory cytokine components of the reflex regulating inflammation.

Abstract

The efferent branches of the splanchnic sympathetic nerves that enhance interleukin-10 (IL-10) and suppress tumour necrosis factor-α (TNF) levels in the reflex response to systemic immune challenge were investigated in anaesthetized, ventilated rats. Plasma levels of TNF and IL-10 were measured 90 min after intravenous lipopolysaccharide (LPS, 60 μg/kg). Splanchnic nerve section, ganglionic blockade with pentolinium tartrate or β_2 adrenoreceptor antagonism with ICI 118551 all blocked IL-10 responses. Restoring plasma adrenaline after splanchnic denervation rescued IL-10 responses. TNF responses were disinhibited by splanchnic denervation or pentolinium treatment, but not by ICI 118551. Splanchnic nerve branches were cut individually or in combination in vagotomized rats, ruling out any vagal influence on results. Distal splanchnic denervation, sparing the adrenal nerves, disinhibited TNF but did not reduce IL-10 responses. Selective adrenal denervation depressed IL-10 but did not disinhibit TNF responses. Selective denervation of either spleen or liver did not affect IL-10 or TNF responses, but combined splenic and adrenal denervation did so. Finally, combined section of the cervical and lumbar sympathetic nerves did not affect cytokine responses to LPS. Together, these results show that the endogenous anti-inflammatory reflex is mediated by sympathetic efferent fibres that run in the splanchnic, but not other

sympathetic nerves, nor the vagus. Within the splanchnic nerves, divergent pathways control these two cytokine responses: neurally driven adrenaline, acting via β_2 adrenoreceptors, regulates IL-10, while TNF is restrained by sympathetic nerves to abdominal organs including the spleen, where non-β_2 adrenoreceptor mechanisms are dominant.

Introduction

The CNS can have a powerful modulatory influence on immune function. In addition to neuroendocrine mechanisms, both sympathetic and parasympathetic arms of the autonomic nervous system have the capacity to moderate inflammation and immune function (Hori *et al.*, 1995; Borovikova *et al.*, 2000; Elenkov *et al.*, 2000; Nance & Sanders, 2007; Andersson & Tracey, 2012; Bellinger & Lorton, 2014). Evidence garnered over the past 40 years implicates the sympathetic nervous system in moderating innate immune responses to systemic inflammation and infection (Besedovsky *et al.*, 1979; Elenkov *et al.*, 2000; Nance & Sanders, 2007; Seeley *et al.*, 2012; Bellinger & Lorton, 2014; Pongratz & Straub, 2014). One sympathetic branch, the splanchnic nerve, which innervates the adrenal medulla and abdominal viscera, has recently been shown to endogenously modulate the systemic inflammatory response to endotoxaemia (Martelli *et al.*, 2014b; Martelli *et al.*, 2014c; Martelli *et al.*, 2019; Occhinegro *et al.*, 2021) and subsequently shown to modify the innate immune response to systemic infection (Lankadeva *et al.*, 2020).

The clearest evidence that the splanchnic sympathetic nerves exert this endogenous action is the disinhibition of inflammatory responses that is seen when their transmission is interrupted. Following challenge with intravenous lipopolysaccharide (LPS), the circulating levels of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF), interferon gamma and interleukin 6 are enhanced in animals whose splanchnic nerves have been cut: concomitantly, their levels of the anti-inflammatory cytokine, interleukin-10 (IL-10), are reduced. These data have been interpreted as showing that the splanchnic sympathetic nerves comprise the efferent arm of an endogenous reflex that is stimulated by systemic immune challenge, and whose combined action on several cytokines prevents excessive inflammation (Martelli *et al.*, 2014b; Occhinegro *et al.*, 2021; McAllen *et al.*, 2022). This basic reflex evidently applies across species: it has been demonstrated so far in rats, mice and sheep (Martelli *et al.*, 2014c; Lankadeva *et al.*, 2020; Occhinegro *et al.*, 2021).

However, a number of questions remain about this reflex. First, as the splanchnic nerves include both afferent and efferent fibres, it is still unsettled whether the pro-inflammatory effect of cutting the splanchnic nerves is due to disruption of afferent or efferent neural pathways. Second, if it is indeed sympathetic efferent fibres that are responsible for the anti-inflammatory action, are they present in other sympathetic nerves? Third, is their anti-inflammatory action mediated by β_2 adrenoreceptors, as reported for the effects of exogenously applied catecholamines (Elenkov, 2008; Agac *et al.*, 2018). Fourth, given that the spleen is widely considered to be a dominant site where neuro-immune interactions occur (Katafuchi *et al.*, 1993a; Hori *et al.*, 1995; Kees *et al.*, 2003; Buijs *et al.*, 2008; Andersson & Tracey, 2012; Olofsson *et al.*, 2012; Kenney & Ganta, 2014; Liu *et al.*, 2020; Sokal *et al.*, 2021), and it is supplied by the splanchnic nerves, what effect does acute denervation of the spleen have on the splanchnic anti-inflammatory action? Fifth, do other abdominal target organs supplied by the splanchnic nerves participate in the reflex response, and is there a role for the branch to the adrenal gland that is responsible for releasing adrenaline and noradrenaline into the circulation?

Noradrenaline is the main transmitter released by sympathetic nerves, and recent evidence has suggested that a major driver of the anti-inflammatory action of noradrenaline may be IL-10 (Agac *et al.*, 2018), although inhibitory actions on pro-inflammatory cytokines may also contribute (van der Poll *et al.*, 1994; Nance & Sanders, 2007; Elenkov, 2008; Pongratz & Straub, 2014). IL-10 has been implicated as a master regulator of immunity (Couper *et al.*, 2008), and plays a key role in the regulation and resolution of inflammation (Sabat *et al.*, 2010; Saraiva *et al.*, 2020). Accordingly, we focussed on the responses of IL-10 (as well as TNF) in the present survey of reflex anti-inflammatory pathways. Here we address some of these remaining issues by measuring the effects of pharmacological blockade and selective nerve cuts in a standardized model of systemic inflammation.

Methods

Experiments were approved by the Animal Experimentation Ethics Committee of the Florey Institute of Neuroscience and Mental Health (approval 19-033) and adhered to the guidelines of the National Health and Medical Research Council of Australia. Male adult Sprague Dawley rats (body weight 290-520 g, n = 207) supplied by Animal Resources Centre, Perth, Western Australia were used in experiments. They were housed in individual cages and

provided water and pelleted food (Barastoc G+) *ad libitum*. Room temperature was maintained at 22°C and there was a 12:12 h light-dark cycle.

General surgical preparation

Rats were initially anaesthetized with intraperitoneal sodium pentobarbitone (60 mg/kg). After clipping the fur over relevant sites, anaesthesia was continued by delivering 2% isoflurane/O_2 via a nose cone. The trachea was then cannulated and the 2% isoflurane/O_2 was administered by artificial ventilation (Ugo Basile, Gemonio, Italy) at approximately 3 ml tidal volume and 60 inflations per minute. Inflation pressure was measured from a side tube. Core body temperature, measured by a thermocouple inserted 5 cm into the rectum, was maintained around 37 ± 0.3 °C by manual control of a heating mat under the rat. The right femoral artery and vein were cannulated with polyethylene tubing so that arterial pressure could be recorded, arterial blood samples obtained and drugs administered intravenously. The arterial line was filled with heparinised (50 units/ml) saline solution. Arterial pressure, heart rate (derived from the arterial pulse), respiratory pressure and body temperature were recorded for the rest of the experiment on a computer-based chart system (CED 1401 with Spike2 software, Cambridge Electronic Design, Cambridge, U.K.). Surgical procedures to cut nerves for the various experimental protocols were then performed. At the completion of the preparative surgery, anaesthesia was switched to urethane by gradually administering intravenous 20% urethane (6 ml/kg) over 10-20 min while proportionately reducing isoflurane levels to zero. Artificial ventilation of the urethane-anaesthetised animal was maintained on 100% O_2 for the remainder of the experiment. No paralysing agent was given, and ventilation was adjusted if and when necessary to a level that just suppressed spontaneous respiratory effort. A surgical depth of anaesthesia was maintained throughout, as indicated by a lack of spontaneous movement and no withdrawal response to firm pinching. No anaesthetic supplementation was required in any experiment. At the conclusion of the experiment, the anaesthetised animal was killed by an intravenous overdose of 20% urethane (1.5 ml bolus), and death confirmed from the blood pressure trace.

Splanchnic nerve section

On the animal's right side, an incision was made in the flank skin, extending 5 cm caudally from the bottom ribs. The retroperitoneal space was exposed by opening the first muscle layer adjacent to the spinal aponeurosis. The perinephric fat was pushed ventrally to reveal the kidney and adrenal gland. The space was held open with 4 hook retractors and a surgical

microscope was used for the following dissection. Using cotton buds to scrape over and retract the adrenal gland with its nerve supply in the caudoventral direction, the adrenal nerves, splanchnic nerves and splanchnic (also called suprarenal) ganglion were revealed. The splanchnic nerves were then cut (or sham-cut) rostral to the splanchnic ganglion, using fine scissors. The nerve supply to the adrenal was sometimes present as a separate, parallel nerve trunk; additional small branches were also seen on occasion to input the splanchnic ganglion: these were all included in the denervation. The same process was then repeated on the animal's left side.

Distal splanchnic nerve section

Using the same exposure as for full splanchnic nerve section, the nerves supplying the adrenal gland were identified. The splanchnic nerve trunk was cut caudal to these branches, just caudal to the splanchnic ganglion. The same process was then repeated on the animal's left side.

Adrenal nerve section

The same surgical procedures as above were used to cut, or sham-cut the adrenal nerves. High magnification was used to ensure that all nerve branches to the adrenal were identified on each side. Effective adrenal denervation was additionally confirmed by subsequently finding plasma levels of adrenaline below the assay's level of detectability (0.1 ng/ml). Four animals with detectable plasma adrenaline levels were excluded on the basis that their adrenal glands had been incompletely denervated.

Vagotomy

Following tracheostomy, the left carotid artery and vagus nerve were exposed low in the neck. The vagus was separated from the carotid and surrounding tissues using longitudinal separation by opening scissor points. The vagus was then lifted clear with fine forceps and cut with sharp scissors. The process was then repeated for the right vagus nerve. For sham vagotomy, the vagus was exposed on each side but not cut.

Cervical sympathetic nerve section

The exposure was the same as for vagotomy, but the cervical sympathetic on each side was identified as a thinner nerve adjacent to the vagus. It was positively identified as the cervical

sympathetic trunk by following its course rostrally to the superior cervical ganglion, and then cut caudal to the ganglion.

Lumbar sympathetic nerve section

With the animal lying on its right side, the left retroperitoneal space was exposed as described above for splanchnic nerve section, but with a larger incision and exposure extending further caudally. The left leg was held extended. Caudal to the left kidney, the anterior spinal muscles were strongly retracted and pulled up so as to expose the abdominal aorta. The left and right lumbar sympathetic trunks were then identified as ganglionated nerves running longitudinally dorsal to the aorta, respectively above and below the segmental arterial branches. The left and right lumbar sympathetic trunks were then cut, caudal to the renal arterial supply.

Denervating the liver

The cervical vagi were cut bilaterally. With the animal supine, a long midline incision was made down the *linea alba*. The xiphisternum was cleared and tied with a silk thread that was then retracted upwards and rostrally. The peritoneum was opened and retracted on both sides with hooks. Using cotton buds, the caudal margin of the liver was flipped upwards and packed rostrally with saline-soaked gauze. The stomach and intestines were held clear with saline-soaked gauze packing. Using a dissecting microscope, the bundle containing the portal vein, bile duct and hepatic artery was identified and, with a little clearing, placed on top of a black plastic footplate 5 mm wide that was attached to the end of a metal arm and held in place with a micromanipulator. The bile duct was then pulled under the toe of the footplate, which rotated the portal vein in a manner that displayed the hepatic artery and the hepatic sympathetic nerves on its upper surface. Using fine forceps and scissors, the nerves were identified and cut, or sham-cut. To complete the denervation process, the connective tissue surrounding the artery and portal vein was minutely examined and stripped to remove any extraneous nerve fibres. The bile duct was then flipped back and the plate removed.

Denervating the spleen

This was also done in vagotomised animals. With the abdomen opened and displayed as described above, the animal was tilted slightly on its right side and, using cotton buds, the spleen was pulled on its pedicle over to the animal's right side and packed into that position with saline-soaked gauze. The splenic artery was then traced to its origin and, following its

course, usually two main nerves could be identified. These were then cut, or sham-cut. The denervation process was completed by stripping the surface of the artery of any potential nerve filaments remaining.

After hepatic and splenic denervations or sham denervations the abdominal incision was closed with sutures.

Experimental protocol

A standard protocol was used in all experimental trials. Five to eight rats were included in each experimental group. Following the completion of surgery and the switch to urethane anaesthesia, rats were left untouched for 30 minutes to stabilise. A blood sample was then drawn for baseline cytokine measurements: if this when assayed showed a baseline TNF >7.8 pg/ml or IL-10 > 28 pg/ml (two standard deviations above their mean resting levels), it was taken to show ongoing inflammation and that animal was excluded from the study. Data from eleven such animals were excluded. Ten minutes after the baseline blood sample, lipopolysaccharide (LPS, 60 µg/kg i.v., derived from *Escherichia coli* 0111:B4; Sigma-Aldrich, St. Louis, MO, USA) was injected via the femoral vein. A second blood sample (1.5 ml) was taken 90 minutes later for the measurement of plasma TNF and IL-10 responses to systemic LPS. That time was chosen to capture their peak plasma levels (Martelli *et al.*, 2014b). The experiment was then terminated and the animal killed.

Pentolinium tartrate was given at a dose of 10 mg/kg in 1 ml/kg saline between the first blood sample and the LPS injection. Pentolinium was selected as the ganglion blocking drug because of its more limited passage across the blood-brain barrier in comparison to other ganglion blockers (Cunningham *et al.*, 2019). ICI 118551 was given at a dose of 0.1 mg/kg immediately before the LPS injection, followed by infusion at 0.1 mg/kg/h in 1 ml/h starting approximately 2 mins after the LPS injection and continued to the end of the experiment. That dose was chosen as appropriate from preliminary experiments; (higher doses caused a drop in heart rate, indicating that they also affected β_1 receptors). For both drugs, matching saline vehicle control experiments were performed.

Adrenaline replacement experiments were performed on animals whose splanchnic nerves had been cut but whose vagi were intact. Adrenaline was infused at either 1.2 or 0.12 ug/kg/min in a volume of 1 ml/h, starting approximately 2 mins after LPS administration and

continuing until the second blood sample. Control animals received isotonic saline vehicle on the same schedule.

Cytokine and catecholamine assays

Blood samples were collected in Eppendorf tubes containing EDTA, chilled on ice then centrifuged at 3000 g for 15 mins in a refrigerated (4^{o}C) centrifuge. Triplicate plasma samples were drawn into Eppendorf tubes and stored at -80^{o}C until assays were performed. The concentrations of TNF-α and IL-10 in plasma were measured in suitably diluted samples by ELISA (R&D systems). The limits of detectability for IL-10 and TNF were 3 and 2 pg/ml, and inter-assay coefficients of variation were 9.9% and 9.7%, respectively. Plasma concentrations of adrenaline were also measured by ELISA (Abnova, Taipei, Taiwan).

Statistical evaluation

GraphPad Prism 9.2 software was used for statistical calculations. Results are expressed as mean and standard deviation. The concentrations of TNF and IL-10 measured in experimental groups were transformed to log10 values and homogeneity of variance tested. For comparison of two samples, all of which had homogeneity of variance, the Student t-test was employed. For comparison of 3 or more samples, a single factor analysis of variance was used; where a significant F value ($p < 0.05$) was obtained, the Tukey multiple comparison test followed.

Results

Is the anti-inflammatory reflex mediated by the splanchnic nerves attributable to sympathetic efferent fibres?

To test for the involvement of an autonomic efferent pathway, the effects of splanchnic nerve section were compared with those of the ganglion blocking drug, pentolinium tartrate. Effective ganglion blockade was demonstrated by the low blood pressure of both sham-operated and splanchnic denervated rats treated with pentolinium (Table 1). As shown in Fig. 1A, animals treated with pentolinium showed similarly reduced levels of IL-10 and increased levels of TNF as seen in animals subjected to splanchnic nerve section. Combining pentolinium with splanchnic nerve section had no further additive effect (Fig. 1B).

Do sympathetic nerves other than the splanchnic mediate a reflex anti-inflammatory action?

To test this, rats were subjected to bilateral combined lumbar and cervical sympathetic nerve section and compared with rats given a sham procedure. As shown in Fig. 2, there was no difference between the cytokine responses to systemic LPS in each animal group.

Does the vagus contribute?

Compared with sham-operated animals, those subjected to bilateral vagotomy showed no difference in the plasma levels of TNF, confirming previous findings (Martelli *et al.*, 2014c). However, the vagotomised animals showed significantly lower levels of IL-10 in response to systemic LPS (Fig. 3A). To distinguish whether this effect of the vagus was independent of the splanchnic nerves (rather than acting reflexly through them (Komegae *et al.*, 2018)), we tested whether the vagi were able to support any release of IL-10 in the absence of the splanchnic nerves. As shown in Fig. 3B, they were not: bilateral vagotomy had no effect after splanchnic nerve section.

Is the splanchnic anti-inflammatory action mediated by β_2 adrenoreceptors?

Given that the suppression of inflammation by catecholamines has been found by others to depend on β_2 adrenoreceptors (Elenkov, 2008; Agac *et al.*, 2018; Murray *et al.*, 2021), animals were subjected to intravenous administration of the β_2 antagonist ICI 118551. This had no effect on blood pressure or heart rate (Table 2). Compared with vehicle treated animals, ICI 118551 treatment reduced plasma IL-10 to low levels, matching those seen after splanchnic nerve section (Fig. 4). These data indicate that splanchnic nerve-dependent IL-10 release is mediated by β_2 adrenoreceptors. By contrast, ICI 118551 failed to enhance TNF responses to LPS (Fig. 4), indicating that the splanchnic nerves regulate TNF by non-β_2 adrenoreceptor mechanisms.

Which splanchnic nerve branches mediate the reflex control of IL-10 and TNF?

The following experiments to investigate the effects of eliminating different components of the splanchnic nerve supply were performed on bilaterally vagotomised animals. This procedure was adopted to remove any confounding effect of abdominal manipulation and surgery, which have been found to initiate an anti-inflammatory reflex mediated by vagal afferent nerve fibres (Komegae *et al.*, 2018).

The first experimental procedure was to cut the splanchnic nerves distal to the adrenal nerves, preserving the adrenal innervation but cutting the supply to all its other target organs. As shown in Fig. 5, distal splanchnic denervation did not significantly affect IL-10 responses to systemic LPS when compared with sham-operated animals, showing that the adrenal nerves were able to support the reflex enhancement of IL-10 without any other splanchnic contribution. By contrast, distal splanchnic nerve section effectively raised the levels of TNF, indicating a critical role of the distal splanchnic branches. For comparison, the IL-10 and TNF responses observed after full splanchnic nerve section in vagotomised animals (from Fig. 3B) are reproduced here in Fig. 5.

What role is played by the sympathetic innervation of the spleen?

Although often considered pivotal for the neural control of inflammation, evidence on the role of the splenic nerves in regulating systemic inflammation has been mixed (Katafuchi *et al.*, 1993b; Meltzer *et al.*, 2003; Martelli *et al.*, 2019; Liu *et al.*, 2020). Here we denervated the spleen surgically and compared these vagotomised animals with those subjected additionally to splanchnic nerve section or sham procedure. Compared with sham operated controls, selective splenic denervation did not affect either plasma IL-10 or TNF levels (Fig. 6). However, additional section of the whole splanchnic nerves then reduced IL-10 and increased TNF levels in the expected manner (Fig. 6).

What role is played by the hepatic nerves?

Section of the hepatic sympathetic nerves has previously been reported to enhance TNF levels in endotoxaemic rats, an effect attributed to interrupting transmission in afferent fibres to the spinal cord (Soto-Tinoco *et al.*, 2020). In vagotomised animals, we therefore cut the hepatic sympathetic nerves (which include the spinal afferent supply, completing liver denervation), and compared these with vagotomised animals given sham hepatic nerve section. These two groups of animals showed no significant difference in their IL-10 or TNF plasma levels 90 mins after systemic LPS (Fig. 7).

What is the effect of adrenal denervation?

Effective denervation of the adrenal glands was assessed by the absence of plasma adrenaline. In sham-denervated animals, plasma adrenaline levels 90 mins after LPS treatment were 1.45 ng/ml (SD 0.66, n=4), while in 16 adrenal-denervated animals they were below the level of detectability (0.1 ng/ml). Plasma noradrenaline levels in sham-denervated

animals were 1.16 ng/ml (SD 0.38, n=4), and in adrenally denervated animals it was 0.33 ng/ml (SD 0.27 ng/ml, n=12). Compared with sham-operated animals, denervation of the adrenal glands, either alone or combined with other organs, consistently lowered the levels of IL-10 in response to systemic LPS (Fig. 8). For TNF, however, isolated adrenal denervation did not significantly disinhibit the response to systemic LPS (Fig. 8).

Combined denervations

When adrenal denervation was combined with splenic denervation, TNF responses to systemic LPS were disinhibited (Fig. 8). This was a consistent finding: it also occurred also in a second series where adrenal and splenic denervations were combined with hepatic denervation (Fig. 8). However, hepatic denervation without splenic denervation was insufficient to disinhibit TNF responses in adrenally denervated animals (Fig. 8). Finally, full splanchnic nerve section was confirmed to inhibit IL-10 and disinhibit TNF responses in otherwise sham-operated, vagotomised animals (Fig. 8).

Is the inflammatory reflex control of IL-10 attributable to circulating adrenaline?

Cutting the nerve supply to the adrenal glands eliminates circulating adrenaline (but only reduces noradrenaline, which is derived also from sympathetic nerve terminal spillover). To answer the above question we infused adrenaline intravenously at 0 (saline vehicle), 0.12 or 1.2 μg/kg/h in splanchnic denervated animals. At 0.12 μg/kg/h, adrenaline infusion produced plasma levels (0.86, SD 0.19 ng/ml) that were close to those seen in LPS-treated animals with intact splanchnic nerves (mean 1.1, SD 0.28 ng/ml), while 1.2 μg/kg/h gave seven-fold higher levels (mean 7.8, SD 2.78 ng/ml). Table 2 shows the effect of these doses on blood pressure and heart rate as the difference between time 0 and 10 mins. As may be seen in Fig.4, infusing adrenaline at the lower rate restored IL-10 responses to match those of animals with intact splanchnic nerves. But it also shows that adrenaline caused a dose-related suppression of TNF responses to LPS.

Discussion

This study provides clear evidence that sympathetic efferent fibres in the splanchnic nerves, but not other sympathetic nerves, mediate the reflex neural control of both pro-and anti-inflammatory cytokines. Moreover, it shows for the first time that the splanchnic neural pathways regulating TNF and IL-10 diverge. The reflex release of IL-10 is prevented by ganglion blockade with pentolinium, β_2 adrenergic antagonism with ICI 118551 and by

denervation of the adrenal glands. This indicates that the reflex control of IL-10 is mediated by neurally-driven release of adrenal catecholamines, most likely adrenaline, acting on β_2 adrenoreceptors. Consistent with this inference, restoring circulating adrenaline restored the IL-10 response to LPS. The endogenous reflex suppression of TNF, by contrast, is largely independent of circulating adrenaline and of β_2 adrenoreceptors. The reflex neural control of TNF is dependent on the splanchnic nerves distal to the adrenal supply, which innervate several visceral organs including the spleen.

Sympathetic efferents mediate the reflex

Ganglion blockade with pentolinium was no more effective than splanchnic nerve section in disabling the inflammatory reflex, as measured by either TNF or IL-10 levels. Further, pentolinium had no additional effect when the splanchnic nerves were cut. Together, these findings indicate that sympathetic efferent fibres within the splanchnic nerves are responsible, and that this efferent pathway accounts for the full endogenous anti-inflammatory neural reflex mediated by the autonomic nervous system. This view is further supported by the lack of effect when other sympathetic nerves (cervical and lumbar) were cut.

Vagus

As we found previously (Martelli *et al.*, 2014c), bilateral vagotomy had no effect on the TNF response to systemic LPS. Here, however, we found that vagotomy caused a reduction in the IL-10 response. Could this reduction have been due to removal of a vagal *efferent* action, such as the well-described cholinergic anti-inflammatory pathway that, when stimulated electrically or pharmacologically, suppresses the production of TNF (Andersson & Tracey, 2012; Martelli *et al.*, 2014a; Murray *et al.*, 2021; Tanaka *et al.*, 2021)? We do not believe so. This is because the vagi (when intact) were unable to support any reflex release of IL-10 independently of the splanchnic nerves: if the splanchnic nerves were already cut, vagotomy had no effect (Fig 3). The most likely explanation is that vagotomy interrupted some ongoing vagal *afferent* activity that had a reflex connection to the splanchnic nerves. Such a reflex connection from vagal afferents to splanchnic anti-inflammatory neurons has been demonstrated previously (Komegae *et al.*, 2018). Indeed, nutritional stimulation has been shown to exert an ongoing anti-inflammatory influence mediated by vagal afferent fibres (Luyer *et al.*, 2005; Lubbers *et al.*, 2010).

Functional targets

The splanchnic sympathetic nerves innervate a number of abdominal organs, whose roles in the anti-inflammatory reflex response are incompletely understood. In a previous study we removed visceral organs, individually or in combinations, and followed the consequences for the reflex splanchnic nerve-mediated suppression of TNF (Martelli *et al.*, 2019). The present study has yielded complementary data, focussing on the splanchnic nerve branches contributing to the release of IL-10 as well as to the suppression of TNF. For the control of IL-10, the findings were straightforward: as mentioned, they indicate that the adrenal nerves drive circulating adrenaline, which acts via β_2 adrenoreceptors to enhance IL-10 release. β_2 adrenoreceptors are much less sensitive to noradrenaline than adrenaline (Baker, 2010), which could explain why the circulating noradrenaline that remains after adrenal denervation was ineffective. For the reflex suppression of TNF, however, adrenal denervation had no significant effect on its own, indicating that direct neural innervation of visceral tissues is the main effector pathway. The cytokine producing cells that respond to these neural influences have not been determined, but macrophages resident in the spleen and other organs are likely to be involved in the case of TNF (Nance & Sanders, 2007). Circulating cells such as neutrophils, which produce abundant IL-10 but little TNF (Zhang *et al.*, 2009), may be the main target for adrenaline's action: monocytes and lymphocytes could also contribute (Sabat *et al.*, 2010).

Spleen

The spleen receives a branch of the splanchnic sympathetic nerve supply and is a major site of cytokine-generating immune cells (Hori *et al.*, 1995; Kees *et al.*, 2003; Andersson & Tracey, 2012; Kenney & Ganta, 2014; Liu *et al.*, 2020; Sokal *et al.*, 2021). It was therefore a natural focus for the present investigation. Perhaps surprisingly, we found that isolated denervation of the spleen did not measurably affect TNF (or IL-10) levels. This result agrees with, and extends to include the control of IL-10, the earlier finding of Meltzer and colleagues: those workers found that denervation of the spleen had no effect on plasma levels, or splenic expression, of TNF in response to systemic LPS (Meltzer *et al.*, 2003). But the present findings reveal a more complex picture. While neither individual denervation of the spleen or the adrenal glands disinhibited TNF levels, when denervated together, they were effective. This finding was replicated in two independent experimental series (Fig 8). It suggests that the splenic nerves play a role in the reflex, but that circulating adrenaline may be able to compensate, at least partially, for their absence. This finding may be compared with those of a second study by Meltzer and colleagues (Meltzer *et al.*, 2004), where they

found that a combination of splenic nerve cut and adrenal denervation or demedullation was necessary to abrogate stress-induced suppression of the splenic and plasma TNF response to LPS, while either intervention alone was ineffective. We may also compare the present findings with those from our previous study in which combined removal of the spleen, liver, gut, stomach and pancreas did not fully abolish the inflammatory reflex control of TNF, provided the adrenal glands were intact (Martelli *et al.*, 2019). Those procedures of course removed many TNF-secreting cells as well as the nerves controlling them. In the present context, however, we still need to understand where the TNF-producing cells that are influenced by distal splanchnic innervation, but not by circulating adrenaline (Fig. 5), may be located.

Liver

Experiments where the sympathetic nerves to the liver were removed (and its vagal innervation also removed by vagotomy) showed no evidence for their involvement in the regulation of either TNF or IL-10 responses to systemic LPS (Figs. 7,8). This result was surprising, in view of the recent demonstration by Soto-Tinoco and colleagues that spinal afferent fibres in the hepatic sympathetic nerves support an early anti-inflammatory reflex response to systemic LPS (Soto-Tinoco *et al.*, 2020). Several differences in experimental approach may account for this discrepancy: here, we used a higher dose of systemic LPS (60 *vs*. 2 μg/kg) and looked at a later time point. We chose 90 mins because this was when plasma IL-10 and TNF reached their maxima (Martelli *et al.*, 2014b), while they chose 50 mins to reveal the earliest neural response to their stimulus (Soto-Tinoco *et al.*, 2020). Additionally, our model was the anaesthetised, ventilated rat and our denervations were acute rather than chronic. It seems likely that we have been examining a mechanism distinct from that of Soto-Tinoco et al., and that in the present experimental conditions, hepatic afferent signals were overridden by other mechanisms to trigger the reflex. For the reflex studied here, the afferent pathways that trigger the CNS to drive the splanchnic anti-inflammatory pathways still need clarification, as indeed do the central pathways responsible. A component for the reflex control of IL-10 appears to be attributable to afferent fibres in the vagus (discussed above). But at least part of the IL-10 arm and all the TNF arm of the reflex depend on other, unknown, afferent signals. Very recently, a strong case has been made that circulating TNF acts on the carotid bodies to elicit a reflex mediated by the splanchnic nerves to inhibit the further production of TNF (Katayama *et al.*, 2022). Such a pathway could have contributed to the reflex suppression of TNF measured here. Curiously, however, those

authors did not find enhancement of IL-10 levels by the carotid body reflex mechanism that suppressed TNF (Katayama *et al.*, 2022).

Physiology

It is established that exogenously applied adrenaline and noradrenaline act via β (Elenkov, 2008) or specifically β_2 adrenoreceptors (Agac *et al.*, 2018) to suppress TNF and enhance IL-10 responses to inflammatory challenge. What is not established, however, is how this plays out physiologically in the context of acute inflammation or infection. Here we examined the physiological actions of endogenously released sympathetic neurotransmitters and hormones rather than of exogenously applied catecholamines. By this approach we revealed that the IL-10 component of the endogenous reflex is humoral while the TNF component depends on neurotransmission within tissues. Moreover, the two components' responses to a blocking dose of ICI 118551 differed starkly. Why was ICI 118551 ineffective against the TNF response? One possibility could be that the TNF-producing cells reside very close to sympathetic nerve terminals, such that they are exposed to concentrations of noradrenaline sufficiently high, not only to stimulate β_2 adrenoreceptors, but also to out-compete the ICI 118551. Out-competing the blocker seems unlikely, given its thousand-fold greater binding affinity to β_2 adrenoreceptors than that of the sympathetic neurotransmitter noradrenaline (Baker, 2005, 2010). We propose, rather, that TNF-generating cells have 'extrasynaptic' β_2 adrenoreceptors - i.e. beyond the functional reach of neurally released noradrenaline (Fig. 9), though amenable to at least higher levels of circulating adrenaline (Fig. 4). The main sympathetic neural action on TNF-generating cells must then depend on non-β_2 adrenoreceptor transmission, as indicated in Fig. 9; perhaps this is mediated by neurally released noradrenaline acting via non-β_2 adrenoreceptors and/or by co-transmitters with an anti-inflammatory action, such as neuropeptide Y (Straub *et al.*, 2000; Bedoui *et al.*, 2003; Yu *et al.*, 2022).

Limitations

Several limitations of this study should be noted. First, it was conducted on animals under general anaesthesia, which is known to depress inflammatory responses (Flondor *et al.*, 2008), although we have previously shown that the inflammatory reflex functions in essentially the same way with and without anaesthesia (Martelli *et al.*, 2014b). Second, the present study followed our previous work (Martelli *et al.*, 2014b; Martelli *et al.*, 2014c; Komegae *et al.*, 2018; Martelli *et al.*, 2019) in using a single, mid-range dose of LPS (< 1%

of the lethal dose in rats (Nezic *et al.*, 2009)). Others have used lower (Meltzer *et al.*, 2003, 2004; Soto-Tinoco *et al.*, 2020) or higher (Borovikova *et al.*, 2000; Meltzer *et al.*, 2003) doses. The cytokine responses seen to 60μg/kg LPS in the present study were clearly amenable to modulation by neural influences, but we cannot say whether other mechanisms might apply in the response to lower or higher doses. A further limitation of the present study is that our surgical denervations were acute, such that they left no histological trace of their completeness or otherwise. In defence of their effectiveness, we can only point to the functional consequence of splenic denervation when combined with adrenal denervation (Fig. 8), and to the loss of plasma adrenaline when the adrenal glands were denervated.

Functional significance

TNF and IL-10 are key regulators of the inflammatory response. Both are secreted early in the inflammatory cascade and have opposing actions both on each other and on the ensuing humoral and cellular responses (Tracey *et al.*, 1989; Couper *et al.*, 2008; Agac *et al.*, 2018; Saraiva *et al.*, 2020). While TNF triggers a range of pro-inflammatory processes, including the release of other cytokines such as IL-6 and interferon gamma, mobilising the body's defences against infection or injury, IL-10 is critical for the protection of tissues from excessive inflammation and for its resolution after injury or infection (Couper *et al.*, 2008; Saraiva *et al.*, 2020). Achieving a balance between pro- and anti-inflammatory mediators that is appropriate for the circumstance may be critical for survival (Seeley *et al.*, 2012). Greater inflammation enhances the ability to clear infections while lesser inflammation protects host organs from damage. The endogenous inflammatory reflex studied here may help to set the right balance under most, but not all circumstances. In severe systemic infection, for example sepsis, the reflex may be unhelpful. In sheep made septic by intravenous infusion of live *E.coli* bacteria, animals with previously cut splanchnic nerves mounted a stronger pro-inflammatory cytokine response. This was expected. Remarkably, however, disabling the inflammatory reflex enabled them to clear their infection and recover much more rapidly than sham-operated animals (Lankadeva *et al.*, 2020).

Summary and conclusions

In sum, the present findings make it clear that the reflex pathways regulating TNF and IL-10, while synergistic in their anti-inflammatory actions, are distinct. The functional target cells for the reflex control of IL-10 are accessed from the circulation rather than by nerves: circulating cells such as neutrophils would be strong candidates. Neutrophils are numerous,

respond to beta$_2$ adrenoreceptor activation (Johnson, 2002; de Coupade *et al.*, 2004) and have the propensity to secrete IL-10 rather than TNF (Zhang *et al.*, 2009). The reflex suppression of TNF, by contrast, is clearly by a direct neural action within abdominal tissues, and is not mediated by β_2 adrenoreceptors. The target cells could be resident macrophages or other immune cells, and the neuro-effector transmission by either noradrenaline acting on other adrenoreceptors and/or by co-transmitters such as neuropeptide Y. Those cells evidently possess 'extrasynaptic' β_2 adrenoreceptors, which are not directly involved in neurotransmission, but enable a minor response to circulating adrenaline. These ideas are outlined in Fig. 9. Finally, if different sympathetic nerve fibres regulate pro-and anti-inflammatory cytokine responses, are they driven by common or distinct central nervous circuits? Future experiments must decide.

Acknowledgements

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Author contributions

Study conceptualization and design: MMcK, DM, RMM; Experimental procedures: MMcK, PTB, RMM; Analysis of samples and data: MMcK, DM, PTB, RMM; Interpretation of data: MMcK, DM, RMM; Manuscript preparation: MMcK, RMM; Editing, revision and approval of manuscript: MMcK, DM, PTB, RMM.

Competing interests

All authors state that they have no conflict of interest.

Data availability

All data are included in the manuscript.

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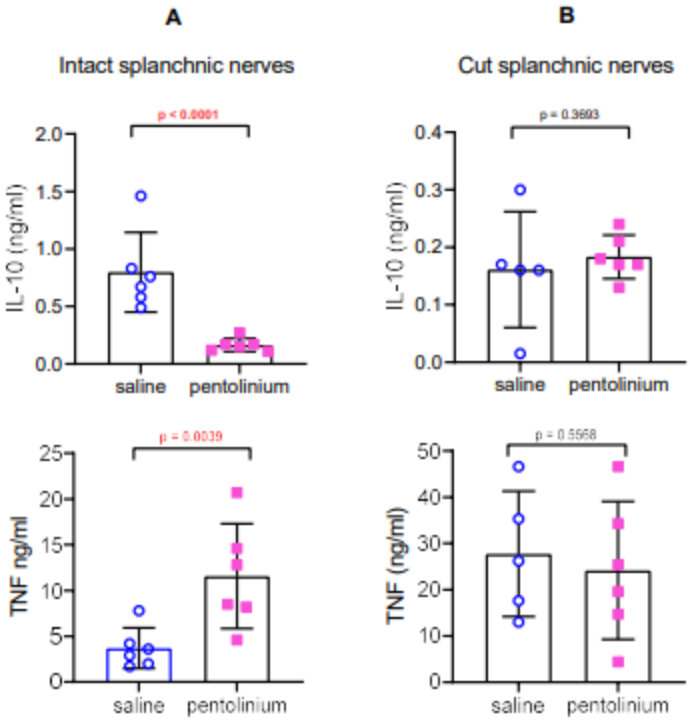


Figure 1. Effect of intravenous injection of isotonic saline (vehicle) or pentolinium tartrate (10 mg/kg) on plasma IL-10 and TNF concentrations at 90 min after intravenous injection of lipopolysaccharide (60 µg/kg) in (A) intact rats (n = 6 per group) and (B) rats with bilateral splanchnic nerve cuts (n = 5 saline group, n = 6 pentolinium group). Student t-test was used to assess differences between saline and pentolinium treatments; p values are shown above the columns.

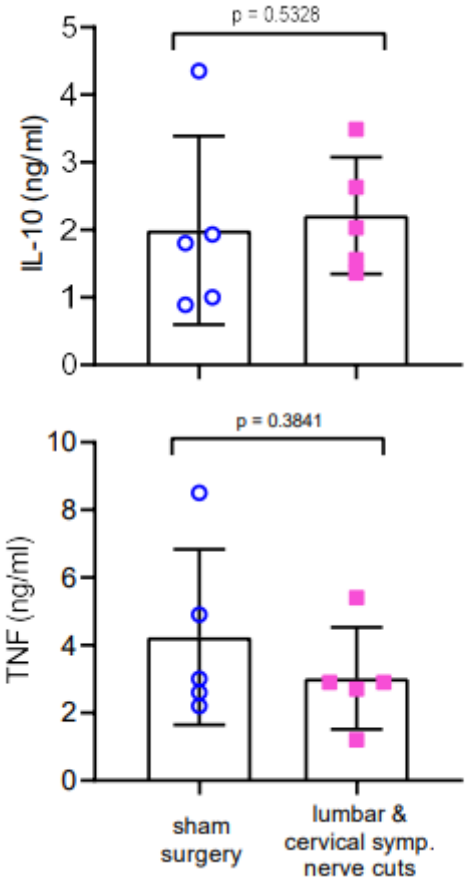


Figure 2. Effect of either sham surgery (n = 5) or bilateral surgical cuts of cervical and lumbar sympathetic nerves (n = 5) on plasma IL-10 and TNF concentrations at 90 min after intravenous injection of lipopolysaccharide (60 µg/kg) in rats. No significant difference was observed (IL-10, p = 0.5328; TNF, p = 0.3841, Student t-test).

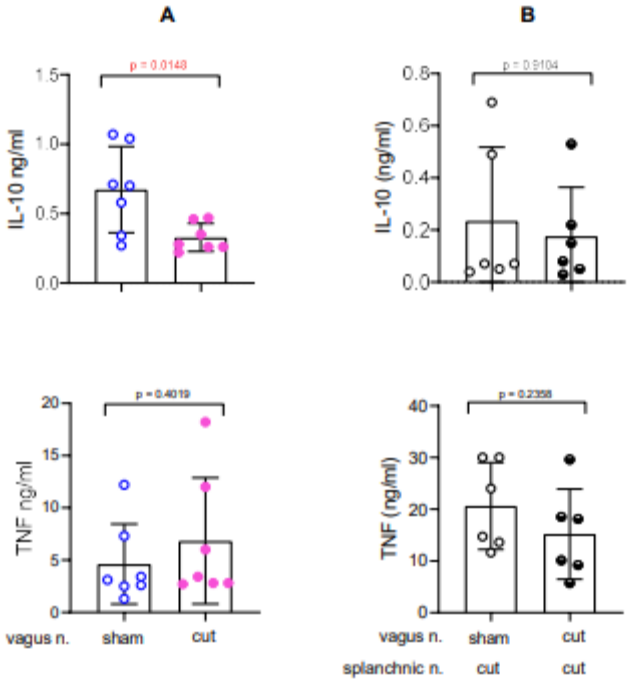


Figure 3. Effect of either bilaterally cutting the vagus nerve or sham vagal surgery on plasma IL-10 and TNF concentrations at 90 min after systemic injection of lipopolysaccharide (60 µg/kg) in rats with either (A) intact splanchnic nerves (n = 7 per group) or (B) rats with the splanchnic nerve cut bilaterally (n = 6 per group). Student t-test was used to assess differences between groups; p values are shown above the columns.

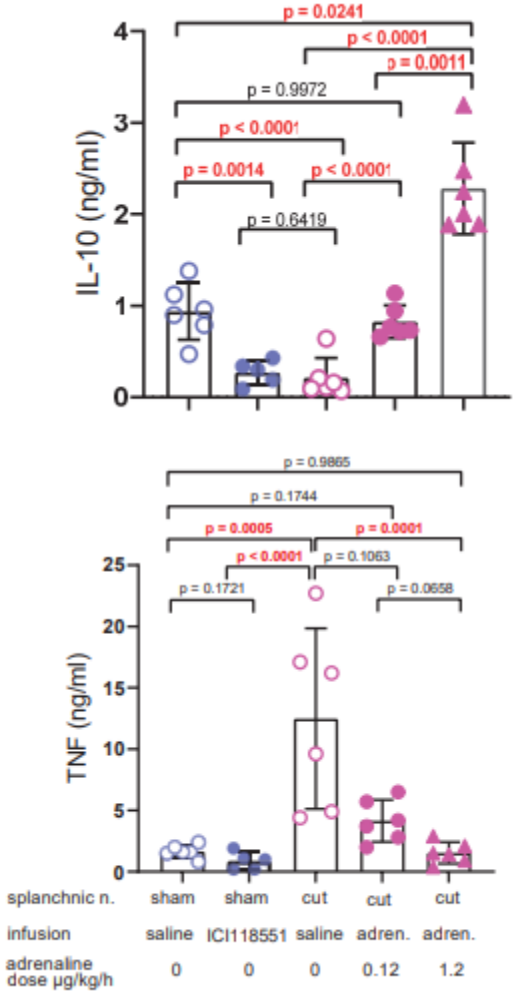


Figure 4. Effect of infusion of saline vehicle (open symbols, n = 6), the β_2 adrenergic antagonist ICI 118551 (0.1 mg/kg/h, filled blue circles, n = 5) or adrenaline (0.12 or 1.2 µg/kg/h, filled magenta symbols, n = 6) on plasma IL-10 and TNF concentrations at 90 min after systemic injection of lipopolysaccharide (60 mg/kg) in rats that had undergone either sham splanchnic nerve surgery or bilateral splanchnic nerve cuts. IL-10 and TNF values were tested by single factor ANOVA. For IL-10, $F_{4,24} = 28.22$, $p < 0.0001$. For TNF values, $F_{4,24} = 15.00$, $p < 0.0001$. Subsequent Tukey multiple comparisons test p values are shown above the columns.

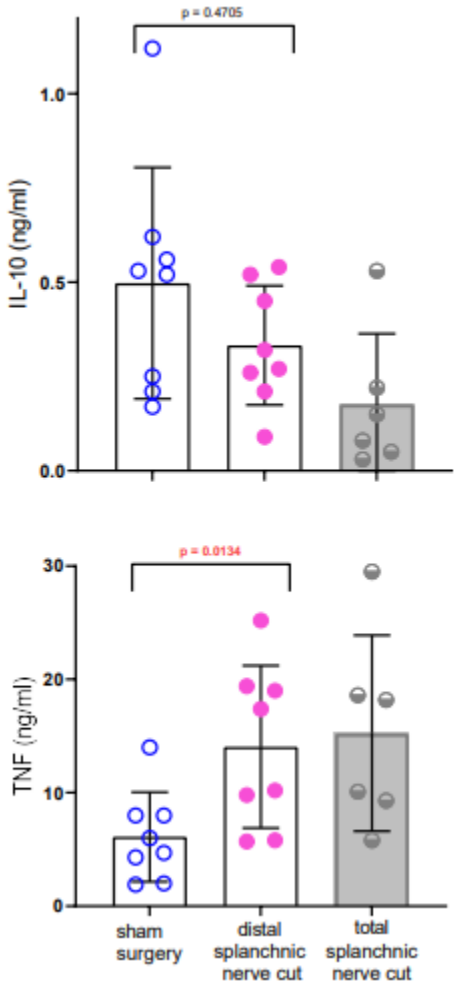


Figure 5. Effect of either sham surgery (n = 8) or section of the distal branches of the splanchnic nerve (n = 8), leaving the adrenal nerves intact, on plasma IL-10 and TNF concentrations at 90 min after systemic injection of lipopolysaccharide (60 µg/kg) in vagotomised rats. A significant difference (Student t-test) was observed in plasma TNF (p = 0.0134) but not in IL-10 concentration (p = 0.4705) between these two groups. For visual comparison, the TNF and IL-10 responses to lipopolysaccharide seen after total splanchnic nerve section in vagotomised rats are shown here in the shaded columns (data copied from Figure 3B).

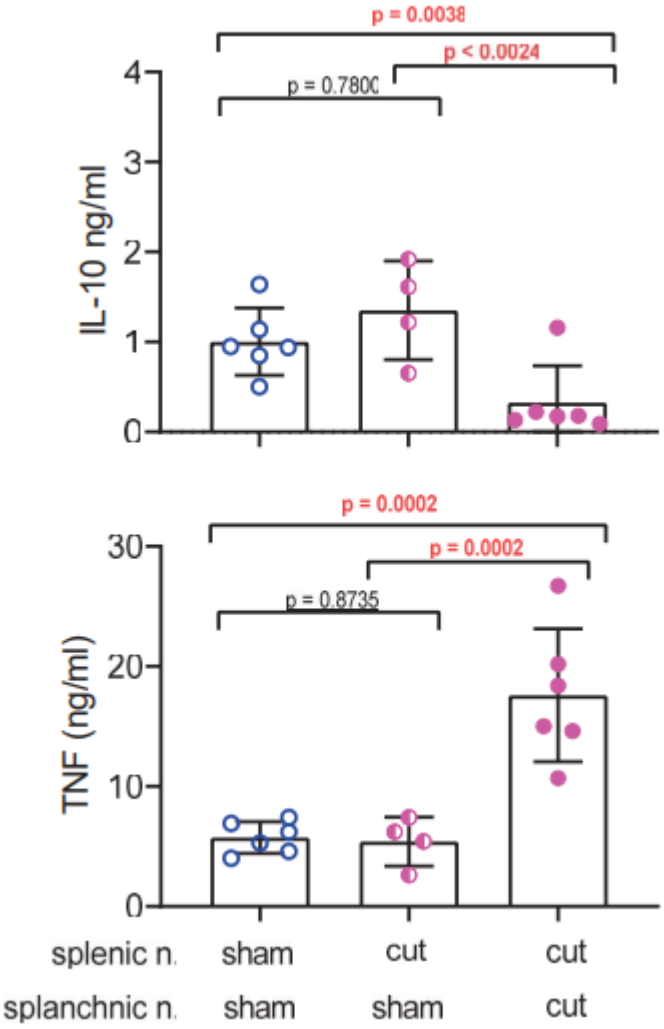


Figure 6. Effect of either sham surgery (n = 6) or surgical denervation of the spleen (n = 4) on plasma IL-10 and TNF concentrations at 90 min after systemic injection of lipopolysaccharide (60 µg/kg) in vagotomized rats with sham splanchnic nerve surgery (left and middle columns). The effect of splenic denervation in vagotomized rats with splanchnic nerves cut bilaterally (n = 6) is shown in the right column. IL-10 and TNF values were tested by single factor ANOVA; $F_{2,13} = 11.93$, $p = 0.0011$ for IL-10 and $F_{2,13} = 22.80$, $p = 0.0001$ for TNF. The Tukey test followed; p values are shown above the columns.

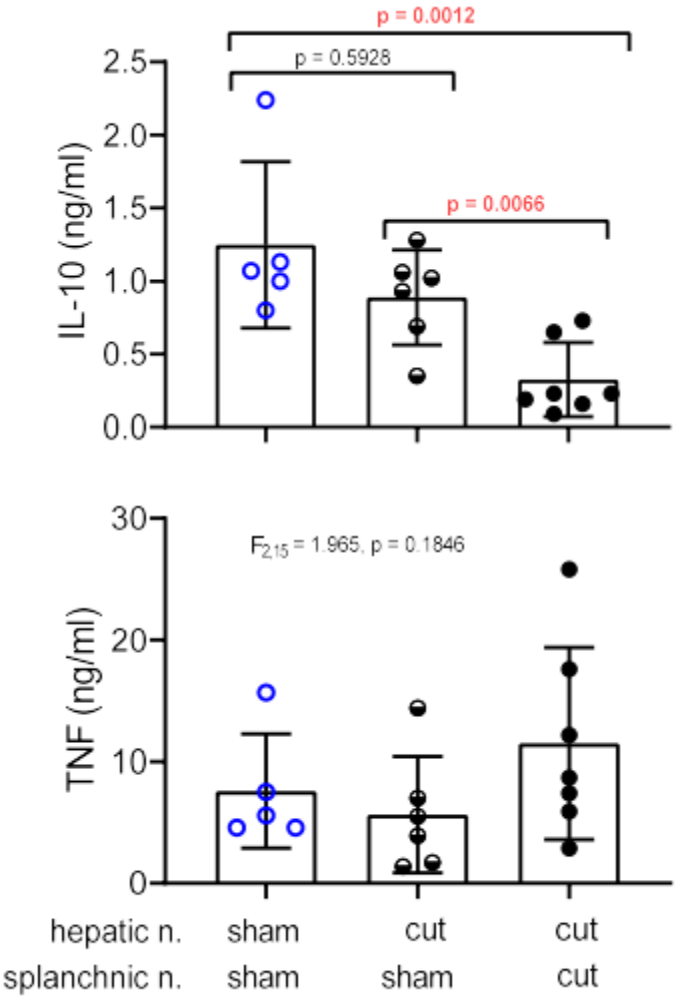


Figure 7. Effect of either sham surgery (n = 6) or surgical denervation of the liver (n = 6) on plasma IL-10 and TNF concentrations at 90 min after systemic injection of lipopolysaccharide (60 µg/kg) in vagotomized rats with sham splanchnic nerve surgery (left and middle columns). The effect of liver denervation in vagotomized rats with splanchnic nerves cut bilaterally (n = 7) is shown in the right column. IL-10 and TNF values were tested by single factor ANOVA; $F_{2,15} = 1.965$, p = 0.1846 for TNF; $F_{2,15} = 11.74$, p = 0.0009 for IL-10. This result for IL-10 was followed by the Tukey test; p values are shown above the columns.

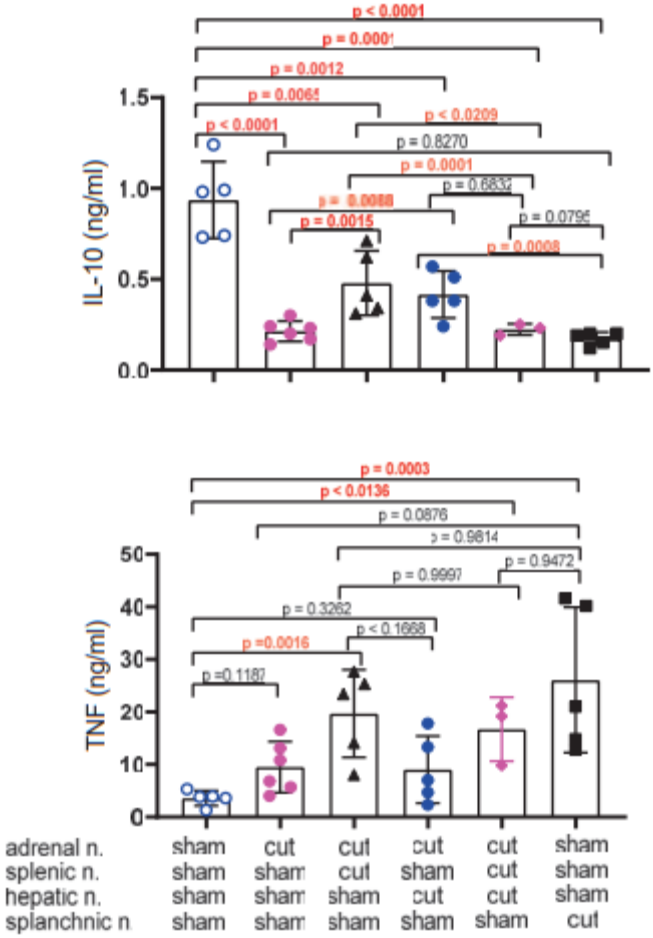


Figure 8. Comparison of effects of cutting combinations of adrenal, splenic and hepatic nerves, or full splanchnic nerve section, on plasma IL-10 and TNF concentrations at 90 min after systemic injection of lipopolysaccharide (60 µg/kg). From the left, columns show effect of: sham surgery to all nerves (n = 5); bilateral section of adrenal nerves with sham surgery to other nerves (n = 6); cut adrenal and splenic nerves with sham surgery to others (n=5); cut adrenal and hepatic nerves with sham surgery to others (n=5); cut adrenal, splenic and hepatic nerves with sham splanchnic nerve section (n= 3); bilateral splanchnic nerve section with sham surgery to other nerves (n=5). IL-10 and TNF values were tested by single factor ANOVA; $F_{5,23} = 25.49$, $p < 0.0001$ for IL-10, and $F_{5,23} = 7.475$, $p < 0.0003$ for TNF. The Tukey test followed for both cytokines; p values are shown above the columns.

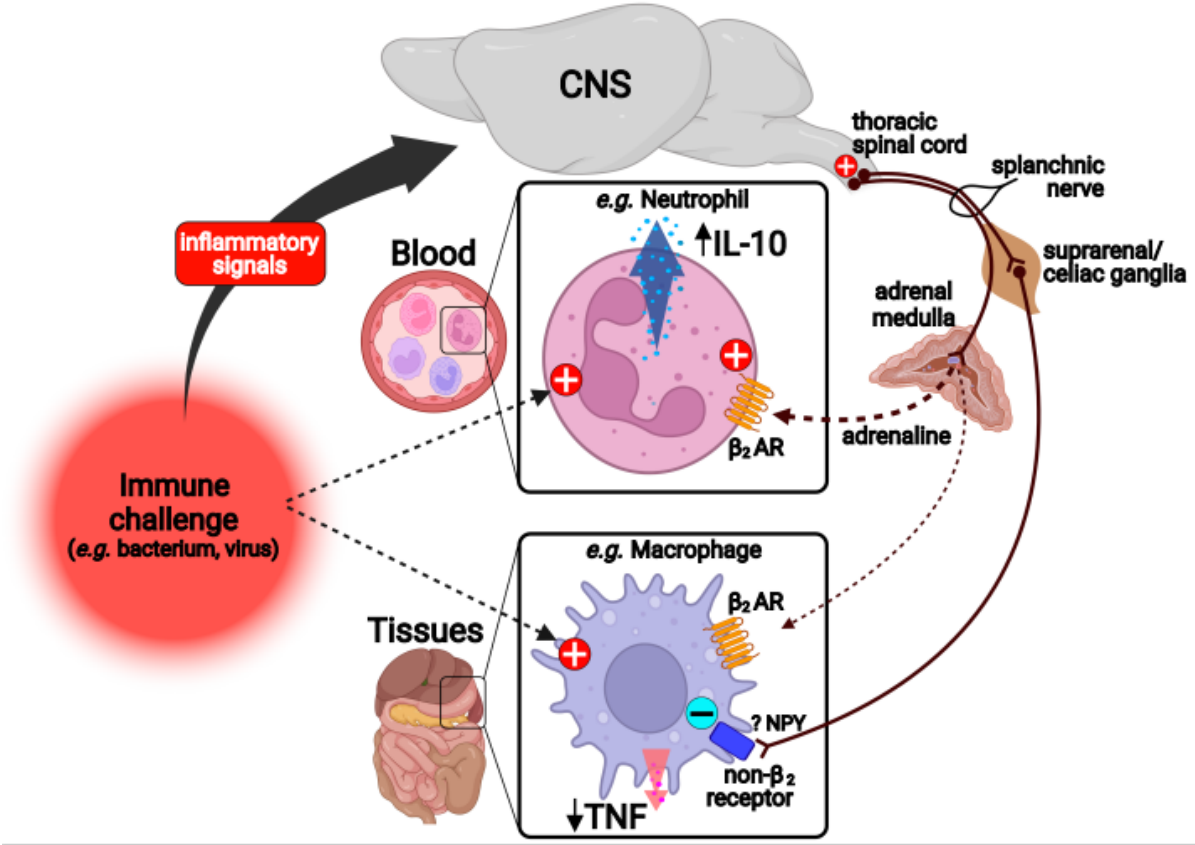
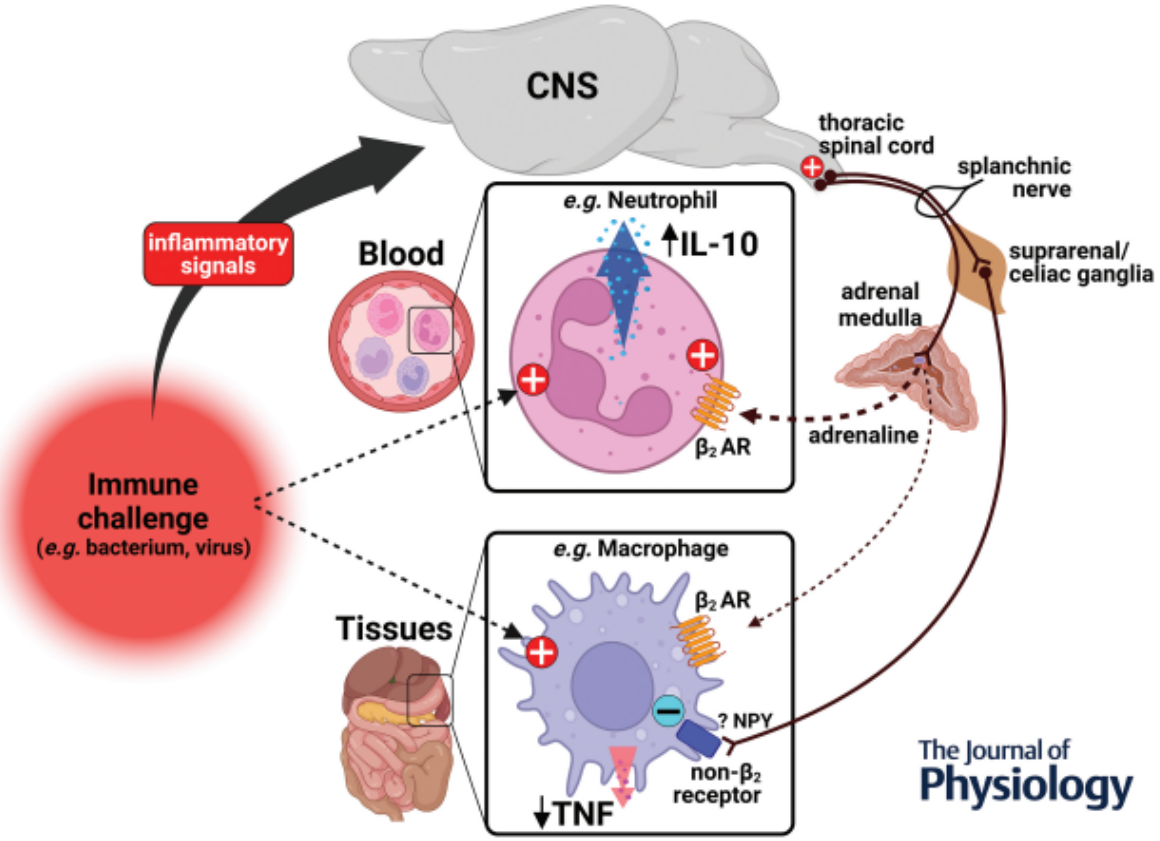


Figure 9. Diagram of the Inflammatory Reflex, outlining the two proposed efferent pathways that respectively enhance IL-10 and suppress TNF responses to inflammation. Created with BioRender.com.



Abstract Figure

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Table 1. Arterial pressure and heart rate immediately before and 90 minutes after administration of lipopolysaccharide to animals of each experimental series. The corresponding figures showing their cytokine measurements are indicated in the left column. (Cardiovascular data for the experiments shown in Fig. 4 are given in Table 2.) Mean (SD) are shown: n indicates the number of rats in each case. Abbreviations: SplancX, bilateral splanchnic nerve section; cervicalX, bilateral cervical sympathetic nerve section; lumbarX, bilateral lumbar sympathetic trunk section; splenX, splenic nerve section; hepX, hepatic sympathetic nerve section; adrX, bilateral adrenal nerve section.

Fig.	**Treatment**	n	**Mean arterial pressure mm Hg**		**Heart Rate beats/min**	
			0 min	90 min	0 min	90 min
1A	vehicle	6	133 (13)	95 (11)	403 (35)	391 (83)
	pentolinium	6	66 (6)	72 (14)	350(20)	379 (19)
1B	splancX + vehicle	5	84 (14)	77 (11)	356 (50)	446 (34)
	splancX + pentolinium	6	52 (10)	56 (8)	340 (15)	382 (22)
2	sham surgery	5	118 (21)	94 (11)	401 (41)	442 (67)
	cervicalX + lumbarX	5	104 (14)	91 (22)	370 (37)	489 (25)
3A	sham vagotomy	7	110 (20)	93 (12)	376 (38)	401 (47)
	vagotomy	6	99 (16)	94 (10)	404 (41)	459 (37)
3B	sham vagotomy + splancX	6	80 (6)	87 (5)	399 30)	395 (26)
	vagotomy + splancX	6	70 (10)	83 (9)	383 (48)	432 (36)
5	sham splancX	8	103 (12)	93 (12)	387 (26)	440 (27)
	distal splancX	8	87 (18)	86 (15)	397 (27)	427 (39)
6	sham splenX + sham splancX	6	94 (21)	96 (12)	408 (28)	479 (22)
	splenX + sham splancX	4	98 (17)	88 (19)	386 (21)	459 (10)
	splenX + splancX	6	74 (11)	63 (17)	401 (37)	462 (34)
7	sham hepX + sham splancX	5	104 (16)	86 (11)	396 (5)	460 (29)
	hepX + sham splancX	6	99 (15)	91 (10)	401 (23)	476 (36)
	hepX + splancX	7	67 (10)	66 (14)	389 (36)	431 (83)
8 (i)	sham +sham +sham +sham	5	103 (11)	90 (10)	413 (27)	463 (27)
(ii)	adrX +sham +sham +sham	6	95 (10)	86 (11)	384 (19)	459 (25)
(iii)	adrX +splenX +sham +sham	5	89 (16)	86 (15)	394 (45)	459 (25)
(iv)	adrX +sham +hepX +sham	5	108 (8)	99 (12)	425 (21)	453 (33)
(v)	adrX +splenX +hepX +sham	3	89 (10)	87 (11)	380 (24)	495 (17)
(vi)	sham +sham +sham +splancX	5	65 (10)	73 (6)	411 (27)	446 (28)

Table 2. Mean arterial pressure and heart rate at 0, 10 and 90 min after lipopolysaccharide injection in the series of experiments in which ICI118551, adrenaline or vehicle were given to sham operated or splanchnic denervated (SplancX) rats. The 0 min values show baseline levels while the 10 min values show the acute effects of ICI118551, adrenaline or vehicle.

Treatment	n	Mean Arterial Pressure Mean (SD)			Heart Rate Mean (SD)		
		0 min	**10 min**	**90 min**	**0 min**	**10 min**	**90 min**
sham surgery + vehicle	6	108(19)	107(20)	96(8)	382(38)	376(35)	387(35)
sham surgery + ICI118551	5	106(17)	104(21)	107(14)	349(44)	341(43)	391(53)
splancX + vehicle	6	69(15)	66(16)	72(18)	390(42)	384(40)	439(46)
splancX + adrenaline (0.12 µg/kg/h)	6	72(14)	57(8)	79(11)	358(38)	358(29)	409(21)
splancX + adrenaline (1.2 µg/kg/h)	6	67(17)	80(4)	71(14)	355(31)	415(28)	425(22)