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# The splanchnic anti-inflammatory pathway: could it be the efferent arm of the inflammatory reflex?

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## **New Findings**

### **What is the topic of this review?**

We review the current literature on the neural reflex, termed the “inflammatory reflex”, which inhibits an excessive release of inflammatory mediators in response to an immune challenge.

### **What advances does it highlight?**

The original model proposed that the inflammatory reflex is a vago-vagal reflex that controls immune function. We posit that, in the endotoxemic animal model, the vagus nerves do not appear to play a role. The evidence suggests that the efferent motor pathway, termed here the “splanchnic anti-inflammatory pathway”, is purely sympathetic; travelling via the greater splanchnic nerves to regulate the ensuing inflammatory response to immune challenges.

## **Abstract**

Exposure to immune challenges results in the development of inflammation. An insufficient inflammatory response can be life-threatening, whereas an exaggerated response is also detrimental as it causes tissue damage and, in extreme cases, septic shock that can lead to death. Hence, inflammation must be finely regulated. It is generally accepted that the brain inhibits inflammation induced by an immune challenge in two main ways: humorally, by activating the hypothalamic-pituitary-adrenal axis to release glucocorticoids; and neurally, via a mechanism that has been termed the inflammatory reflex. The efferent arm of this reflex – the neural-to-immune link – was thought to be the ‘cholinergic anti-inflammatory pathway’. Here we discuss data that support the hypothesis that the vagus nerves play no role in the control of inflammation in the endotoxemic animal model. We have shown and posit that it is the greater splanchnic nerves that are activated in response to the immune challenge and that in turn drive postganglionic sympathetic neurons to inhibit inflammation.

## Introduction

The brain and the immune system communicate and influence each other. The focus of this short review is to critically evaluate the most recent data pertaining to the neural control of the inflammatory response in animal models of endotoxemia.

It is well known that the sympathetic nervous system (SNA) is activated by immune challenges. For example, lipopolysaccharide (LPS), a component of the wall of gram-negative bacteria (Raetz & Whitfield, 2002), induces a general sympathetic activation of the splanchnic and splenic nerves (MacNeil *et al.*, 1997; Iriki & Saigusa, 1998; Martelli *et al.*, 2014c) associated with a rise in noradrenaline and adrenaline plasma content, when given intravenously in rats (Zhou *et al.*, 1991; Zhou & Jones, 1993).

The central nervous system also regulates immune function via the autonomic nervous system. The sympathetic nervous system directly innervates lymph nodes and, together with the parasympathetic nervous system, innervates specific organs where macrophages and monocytes mount the innate immune response to immune challenges (Felten *et al.*, 1985, 1987; Nance & Sanders, 2007). A large body of literature has been produced describing the influence that the sympathetic nervous system has on immune function. Recently, Kox and colleagues (2014) have shown that voluntary activation of the sympathetic nervous system can inhibit the inflammation induced by intravenous injection of LPS in humans. Nakai *et al.* demonstrated that physiological inputs from adrenergic nerves influence lymphocyte dynamics and their distribution (Nakai *et al.*, 2014). This regulation of lymphocyte dynamics also appears to be under circadian control via higher structures in the central nervous system like the suprachiasmatic nucleus that control sympathetic and parasympathetic output to peripheral organs like the liver, adrenal glands and spleen (Buijs *et al.*, 2003; Scheiermann *et al.*, 2012; Guerrero-Vargas *et al.*, 2014). A great deal of interest has been focused on the sympathetic control of immune function specifically by splenic nerves (Straub *et al.*, 2000; Kees *et al.*, 2003; Straub, 2004; Buijs *et al.*, 2008; Vida *et al.*, 2011).

These two concepts, the immune challenge activating the sympathetic nervous system that in turn can influence immune function, constitutes the conceptual framework that gives origin to the theory of a neural reflex able to control immune function.

### The original inflammatory reflex

The concept of a neural reflex, termed 'the inflammatory reflex', that controls inflammation in response to an immune challenge was introduced around 15 years ago (Borovikova *et al.*, 2000; Tracey, 2002). The inflammatory reflex is a classic neural reflex that consists of an afferent-sensory arm, conveying information about the immune status of the body to the brain, and an efferent-motor arm, connecting the brain to the immune cells to provide control of the innate immune system. The inflammatory reflex acts in a negative feedback manner: it inhibits the innate immune system and prevents an excessive release of pro-inflammatory factors. It has been proposed that the inflammatory reflex acts to maintain immune homeostasis.

The key finding in support of this concept was obtained in experiments using endotoxemic anaesthetised rats. In this setting, bilateral cervical vagotomy induced an over-production of plasma tumor necrosis factor – alpha (TNF- $\alpha$ ) while electrical stimulation of the efferent stump of the

cervical vagus was anti-inflammatory. These initial findings represent the foundation of the original theory of the inflammatory reflex and has been comprehensively described in a recent review (Pavlov & Tracey, 2015). According to this original theory, both afferent and efferent arms of this reflex travel (mainly) via the vagus nerves (Fig. 1A). The efferent motor pathway (also termed the cholinergic anti-inflammatory pathway) consisting of a parasympathetic, a sympathetic and a cellular component, has been elegantly described in a long series of studies (Andersson & Tracey, 2012). According to this line of research, the efferent vagus nerves are activated in response to an immune challenge. The vagus nerves would, in turn, drive the splenic sympathetic nerves to release noradrenaline in the spleen (Rosas-Ballina *et al.*, 2008). Noradrenaline then binds to  $\beta$ 2-adrenoreceptors present on a selected population of T-cells. These T-cells would then release acetylcholine (ACh) that binds to nicotinic receptors containing the  $\alpha$ 7 subunit ( $\alpha$ 7nAChRs) on splenic macrophages (Wang *et al.*, 2003; Rosas-Ballina *et al.*, 2011, see Andersson & Tracey, 2012 for a more comprehensive review of the role of  $\alpha$ 7nAChR on macrophages). The final outcome is the prevention of nuclear translocation of the NF $\kappa$ B transcription factor in the macrophages, resulting in subsequent inhibition of the synthesis and release of TNF- $\alpha$  and other pro-inflammatory cytokines (Yoshikawa *et al.*, 2006).

The experimental model used to provide evidence for the existence of this inflammatory reflex, and to describe each step of its efferent motor arm of the reflex, is the rodent rendered endotoxemic by intravenous injection of lipopolysaccharide (LPS). The endotoxemic model is not a surrogate for all experimental immune challenges. However, this model was used to introduce, develop and describe each step of the efferent motor arm of the original inflammatory reflex (Borovikova *et al.*, 2000, Wang *et al.*, 2003; Rosas-Ballina *et al.*, 2008; Rosas-Ballina *et al.*, 2011) and, as such, is the focus of this review. We therefore propose the following caveat: the conclusions reached in this review might not account for other experimental models involving different immune challenges. We will also focus on the efferent motor pathway of the reflex, originally termed the cholinergic anti-inflammatory pathway, and propose a revised version of it, the splanchnic anti-inflammatory pathway. Furthermore, this review will only touch briefly on the afferent arm of the reflex. For more comprehensive reviews on the cholinergic control of inflammation to immune challenges, including those different from endotoxemia, see McAllen *et al.* (2015).

### **The anatomical link between parasympathetic and sympathetic nerves: is there a synaptic connection between the efferent vagus and splenic neurons?**

The novelty of the original theory of the inflammatory reflex, considered revolutionary at the time, was the synergistic action between the parasympathetic and the sympathetic nervous systems. According to the original theory of the inflammatory reflex the two branches of the autonomic nervous system work together to control immune function. While there is evidence in the literature of a vagal innervation of sympathetic abdominal ganglia (the suprarenal, the coeliac and the superior mesenteric ganglia; Berthoud & Powley, 1993), no evidence of a physical di-synaptic connection between vagal terminals and post-ganglionic sympathetic neurons projecting to the spleen has been described. Such an anatomical connection would represent a vital link for direct activation of splenic nerves from vagal fibres. This lack of evidence led us, almost 5 years ago, to test for the existence of this proposed synaptic connection (Bratton *et al.*, 2012): a connection that represents the backbone of the original theory of the inflammatory reflex.

Initially, we performed a series of anatomical studies, injecting an anterograde tracer bilaterally into the dorsal motor nucleus of the vagus (the origin of the efferent vagus nerve) and a retrograde tracer in the spleen parenchyma and looked for juxtaposition of the two tracers to confirm the existence of a direct synaptic connection in the abdominal ganglia. We were able to confirm previous findings on the parasympathetic innervation of abdominal sympathetic ganglia. We also described how the majority of neural inputs (approximately 80%) to the spleen were from the suprarenal ganglia and only 15-20% from the coeliac ganglia. However, an anatomical connection between parasympathetic nerve terminals and the neurons projecting to the spleen was never found (Bratton *et al.*, 2012). Furthermore, we performed electrophysiological studies to determine the effects of electrically stimulating the efferent vagus (using the same parameters and procedure used in the original study describing the pathway; Borovikova *et al.*, 2000) on the normal discharge rate of single spleen-projecting sympathetic neurons. Our results showed that electrical stimulation of the efferent vagus did not influence the discharge rate of any spleen-projecting neurons. Together, these results, while contradicting earlier studies that described how the great majority of splenic projecting neurons were located in the coeliac ganglion (Bellinger *et al.*, 1989), cast some doubts on the postulated existence of a synaptic connection between vagal fibres and postganglionic neurons innervating the spleen.

#### **An alternative inflammatory reflex: the splanchnic anti-inflammatory pathway.**

The study discussed above (Bratton *et al.*, 2012) lacked a direct investigation of the electrophysiological properties of the spleen-projecting sympathetic neurons under an immune challenge. Therefore, a fundamental question remained unanswered: is it possible that the vagus nerve, either directly or indirectly, drives splenic sympathetic nerves only in response to an immune challenge? This has been demonstrated in rats, where intraportal injection of interleukin-1 beta (IL1 $\beta$ , a pro-inflammatory cytokine) was shown to activate the afferent hepatic branch of the vagus nerve and in turn the splenic sympathetic efferent nerves (Niiijima, 1996). For this reason, we set out to test the possibility that, in response to an immune challenge, the vagus nerve drives the activation of the splenic nerves and that this neural activation is responsible for the inhibition of an excessive release of TNF- $\alpha$  and other pro-inflammatory cytokines. At the same time, we proposed an alternative hypothesis for the inflammatory reflex, namely that the efferent motor pathway is purely sympathetic, with no parasympathetic influence. Also, that it is comprised of preganglionic sympathetic fibres that are part of the greater splanchnic nerve that drive chromaffin cells in the adrenal glands and postganglionic sympathetic neurons innervating the spleen, the liver and the gut (Fig. 1B).

In order to test these hypotheses, we performed a series of electrophysiological experiments to study the electrical activity of the splenic sympathetic nerves and their preganglionic sympathetic supply: the greater splanchnic nerve. We confirmed previous findings that both splenic and splanchnic nerves are strongly activated in response to intravenous LPS (MacNeil *et al.*, 1997; Iriki & Saigusa, 1998). We studied the role of the vagus nerves in this sympathetic activation. After waiting 90 minutes from the intravenous injection of LPS, we performed a bilateral cervical vagotomy and observed its effects on the sympathetic activity of the splenic and splanchnic nerves. According to the original theory of the inflammatory reflex, cutting both vagus nerves and hence the putative

preganglionic inputs to the splenic immuno-efferent postganglionic neurons, should have abolished or at least reduced splenic nerve activity. Our results, however, showed that this intervention did not induce any major changes in splenic sympathetic activity. In fact, splenic nerve activity continued to increase for 5-10 minutes after the vagotomy. Unsurprisingly, cutting the left splanchnic nerve was sufficient to return splenic sympathetic nerve activity to baseline levels, comparable to that observed prior to the intravenous LPS challenge. The effect was instantaneous; the drop of the splenic sympathetic activity was concomitant with severing of the splanchnic nerve. This result demonstrated that each action potential that travels in the postganglionic splenic neurons is evoked by excitatory postsynaptic potentials triggered by pre-ganglionic inputs from the splanchnic sympathetic nerve; not the vagus.

In another set of electrophysiological experiments, we also demonstrated that cervical vagotomy did not have any effect on splanchnic sympathetic nerve activity (Martelli *et al.*, 2014c). We then wanted to study the effects of cutting the splanchnic and/or vagus nerves on LPS-induced TNF- $\alpha$  release. Cervical vagotomy, performed prior to an LPS challenge, did not influence the TNF- $\alpha$  released in response to LPS. On the other hand, cutting the splanchnic nerve induced an exaggerated release of TNF- $\alpha$  into the bloodstream that was 4-fold higher in these animals compared with sham or vagotomised rats. We also went on to show that cutting the splanchnic nerves not only affected TNF- $\alpha$  release but also other important pro- and anti-inflammatory cytokines. For example, 90 minutes after LPS injection the plasma levels of the anti-inflammatory cytokine interleukin-10 were much lower in the group of animals with the splanchnic nerves cut, compared with sham-operated rats. Furthermore, 6 hours after the immune challenge, the group of animals with cut splanchnic nerves also had a 2-fold increase in plasma interleukin-6 and interferon- $\gamma$  compared with the sham group (Martelli *et al.*, 2014b). These results show that the efferent arm of the inflammatory reflex runs in the greater splanchnic nerves, not the vagi, and support our alternative hypothesis that the efferent arm of the inflammatory reflex is purely sympathetic. Therefore, we termed this motor arm the splanchnic anti-inflammatory pathway.

Finally, we wanted to confirm that what we were studying was a true, physiological neural reflex that is activated in response to an immune challenge. The great majority of the studies that deal with the inflammatory reflex, including ours, were performed in anaesthetised animals and anaesthesia is known to be anti-inflammatory (Kotaniidou *et al.*, 1996; Helmy & Al-Attiyah, 2000; Sun *et al.*, 2004; Fuentes *et al.*, 2006; Boost *et al.*, 2007; Yang *et al.*, 2007; Rodríguez-González *et al.*, 2013). Thus, we designed a study with the intention of distinguishing between the anti-inflammatory effects of the inflammatory reflex and those of general anaesthesia. We, therefore, bilaterally sectioned the greater splanchnic nerves one week prior to administering an LPS challenge in conscious rats. We found that cutting the splanchnic nerves had identical effects on TNF- $\alpha$  production in conscious compared to anaesthetised rats. These results confirm that the splanchnic anti-inflammatory pathway is the efferent arm of a neural reflex that physiologically controls inflammation (Martelli *et al.*, 2014b).

### **The vagus versus the splanchnic, which is the anti-inflammatory efferent nerve?**

Our findings differ from those reported by Borovikova *et al.* (2000) in which the vagal concept of the inflammatory reflex was first hypothesised. The main differences were those observed in vagotomised rats compared with sham-operated counterparts. We did not see any effect of cutting the vagus nerves on the TNF- $\alpha$  response to LPS. Conversely, Borovikova and colleagues observed an approximate 20% higher level of plasma TNF- $\alpha$  in the vagotomised rats compared to the sham-operated animals. Many variables might have caused this discrepancy in the two studies. For example, different rat strains and doses of LPS were used in the studies. However, there is one detail that might explain why we were not able to replicate findings originally reported by Borovikova *et al.* (2000). In their study, plasma corticosterone levels, in addition to TNF- $\alpha$ , were measured in each experimental group. Corticosterone is released in response to an immune challenge and its function is to damp down the inflammatory response (Besedovsky *et al.*, 1986; Nakano *et al.*, 1987). The vagotomized group had significantly lower levels (~33%) of plasma corticosterone compared to the vagus nerve intact group. We were unable to recapitulate these findings (Martelli *et al.*, 2014b). In our hands, all experimental groups, whether vagotomised or not, had similar levels of plasma corticosterone. We believe that this difference is sufficient to explain the discrepancy between the two conflicting studies.

In support for an anti-inflammatory role for the vagus nerve, Schulte *et al.* (2014) showed that TNF- $\alpha$  and interleukin-1 $\beta$  plasma levels were significantly higher in the vagotomised versus sham operated rats. However, the authors did not find any differences between LPS-sham vagotomised and vehicle-sham vagotomised animals, raising some doubts as to the actual inflammatory status of the LPS-treated rats. Others have described a pro-inflammatory effect of unilateral cervical vagotomy (Song *et al.*, 2012). However, in this case the LPS was given intraperitoneally (as opposed to intravenously). As such, the afferent vagus could have had a role in the activation of the inflammatory reflex in this endotoxemic animal model. Kox *et al.* (2014) also described a pro-inflammatory effect as a consequence of cervical vagotomy. They studied the plasma and pulmonary TNF- $\alpha$  and interleukin-10 responses to LPS given intravenously in rats. They found that vagotomy induced an augmented TNF- $\alpha$  pulmonary response in spontaneously breathing animals. However, they also found that cutting the cervical vagi did not have any effects on TNF- $\alpha$  or interleukin-10 plasma levels.

There have been a number of other studies that have reported a lack of effect on systemic inflammatory status in LPS challenged animals after vagotomy (e.g. Bernik *et al.*, 2002; Mihaylova *et al.*, 2012). Among these, there is the one report that describes the anti-inflammatory action of electroacupuncture in a mouse model of sepsis (Torres-Rosas *et al.*, 2014). The authors showed that the anti-inflammatory action of electroacupuncture requires the intact vagus, is independent from the spleen and is mediated by dopamine released from chromaffin cells of the adrenal glands. In support of our previous findings they also showed that vagotomy had no influence on systemic TNF- $\alpha$  levels *per se* in response to LPS. A similar result was also achieved by de Haan and colleagues recently (de Haan *et al.*, 2013). These authors investigated the effects of a lipid-rich enteral nutrition on the inflammatory response to LPS in mice. The inflammatory index analysed was the plasma levels of mouse mast cell protease I, an index of mucosal mast cell activation in the gut. The authors showed that the fat diet was anti-inflammatory and that this anti-inflammatory effect was negated by chlorisondamine and  $\alpha$ -bungarotoxin (nicotinic acetylcholine receptor blockers) and subdiaphragmatic vagal denervation. Hence, disengaging the cholinergic anti-inflammatory pathway blocked the anti-inflammatory action of the lipid-rich diet. Intriguingly, both vagotomy and

administration of the  $\alpha$ -bungarotoxin did not exacerbate the inflammatory status of mice fed with a normal diet, showing that the cholinergic anti-inflammatory pathway was activated by the lipid-rich diet but not in response to the immune challenge.

### **Conclusion**

The series of findings reviewed in this manuscript support the existence of a neural reflex that influences immune function. This neural reflex acts to damp down the inflammation developed in response to LPS when given intravenously. We believe that the original model of the inflammatory reflex needs to be revised. In our view, the reflex may not involve the vagus nerves since vagotomy, a procedure that compromises both afferent and efferent fibres, appears not to have any effect on the inflammation developed in response to an LPS challenge.

According to our proposed revised model (Fig. 1B), the inflammatory reflex consists of an afferent arm that conveys the information to the brain on the immune status of the body. It also comprises an efferent-motor arm, the splanchnic anti-inflammatory pathway, consisting of the greater splanchnic nerves. Future studies are needed to identify the humoral and neural factor(s) used by the body to trigger the splanchnic anti-inflammatory pathway. We also need to elucidate the target organ of the splanchnic anti-inflammatory pathway: the site at which sympathetic post-ganglionic nerves talk to and inhibit the innate immune system.

Finally, the role of the splanchnic anti-inflammatory pathway needs to be investigated in other types of immune challenges and not solely on the LPS-induced endotoxemia model we described in order to achieve more clinically relevant findings. This might represent a novel therapeutic target that could be used to treat pathologies characterised by uncontrolled inflammatory responses. A number of treatments based on the cholinergic anti-inflammatory pathway are currently undergoing development.

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DM, DGSF and STY all contributed to the writing of this review.

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## Figure Legend

### Figure 1. The Inflammatory Reflex.

In response to lipopolysaccharide (LPS), the inflammatory reflex is activated to dampen down the ensuing inflammatory response. **A: Original model of the inflammatory reflex:** the afferent and the efferent (the cholinergic anti-inflammatory pathway) arms travel via the vagus nerves. The efferent vagus nerve synapses with splenic sympathetic neurons in abdominal sympathetic ganglia and drive them to inhibit the release of inflammatory cytokines in the spleen. **B: Revised model of the inflammatory reflex:** the efferent arm is the splanchnic anti-inflammatory pathway, which inhibits the excessive release of inflammatory cytokines in the spleen but also in other organs innervated by postganglionic sympathetic neurons that are activated by splanchnic nerves following LPS administration.

