The Cholinergic Anti-Inflammatory Pathway

in vitro, animal, and human studies

Matthijs Kox

Colofon

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Contents

Chapter 1

General introduction
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General introduction

Innate immunity is the body's alarm system. Upon recognition of invading pathogens such as bacteria and viruses, innate immune cells release inflammatory mediators signalling the presence of danger which results in the recruitment of inflammatory cells to the site of infection. Furthermore, innate immune cells and inflammatory mediators play a pivotal role in the clearance of pathogens. The innate immune system therefore represents the body's first line of defence and is vital to survival. However, excessive, inappropriate, or persistent activation of the innate immune system, such as in sepsis or autoimmune diseases, can cause organ damage. In this respect, therapeutic measures aimed to limit the innate immune response could be beneficial. Classically, the innate immune response is thought to be self-governing, e.g. not regulated by another system. However, in the past few decades, strong links between the brain, in particular the autonomic nervous system (ANS), and the innate immune system have been established. Through the afferent arm of the parasympathetic vagus nerve, the ANS can sense inflammation in the periphery and relay this information to the brain. The efferent sympathetic division of the ANS has been shown to both stimulate and dampen the innate immune response, and more recently, a novel anti-inflammatory mechanism mediated by the efferent vagus nerve has been identified. This mechanism is called the cholinergic anti-inflammatory pathway, after acetylcholine, the neurotransmitter of the vagus nerve.

This thesis describes interactions between the ANS and the innate immune system, focusing on the cholinergic anti-inflammatory pathway and the effects of stimulation of this pathway. In the remainder of this introduction, some background on the ANS, the innate immune system and the links between these two systems is provided. Furthermore, the aim and outline of this thesis is presented.

The autonomic nervous system

The ANS is called autonomic because it is regarded as a system that we cannot consciously influence. Descriptions of the ANS go back to antiquity. Galen (A.D. 130-200) described the sixth cranial nerve, which comprised what we call today the ninth (glossopharyngeal), tenth (vagus), and eleventh (accessory) cranial nerves, and the sympathetic chain[1]. Galen described that through this sixth cranial nerve, spirits go from one organ to another, producing the phenomenon of 'sympathy': cooperation or coordination of organs. It was not until 1563 that a distinction between the parasympathetic (vagus) and sympathetic nerve was made by Eustachius[2]. The medieval Latin word vagus literally means wandering; it 'wanders' from the brain stem

through organs in the neck, thorax, and abdomen. In the nineteenth century, Langley in Cambridge first developed the notion of antagonism between the sympathetic and parasympathetic systems[3], and one of his disciples later showed that epinephrine has the same general effects as stimulation of the sympathetic nerve. In 1921, German pharmacologist Otto Loewi discovered the release of vagusstoff (later identified as acetylcholine, the principal neurotransmitter of the parasympathetic nervous system) from the vagus nerve upon electrical stimulation, providing the fist evidence of synaptic transmission and the birth of the neurotransmitter[4]. In 1946, von Euler identified norepinephrine as the principal neurotransmitter of the sympathetic nervous system[5].

Except for skeletal muscle, the ANS innervates every organ in the body. The sympathetic and parasympathetic parts of the ANS typically have opposite effects on effector organs; the sympathetic part can be viewed as the body's accelerator ('fight or flight' response) while the parasympathetic part represents the brake ('rest and digest'). The most obvious example of this opposite effect is the heart; sympathetic activity increases heart rate via noradrenergic stimulation of beta-receptors while parasympathetic (vagus nerve) activity slows the heart down via stimulation of muscarinic acetylcholine receptors. In the 90s of the previous century, effects of the sympathetic nervous system on the innate immune response were established. Finally, in the year 2000, a novel pathway was discovered via which the vagus nerve can reflexively attenuate the innate immune response: the cholinergic anti-inflammatory pathway.

The innate immune system

Pattern recognition of pathogen-derived and endogenous ligands

Cells of the innate immune system, such as monocytes and macrophages, recognise conserved components of pathogens, so-called pathogen-associated molecular patterns (PAMPs), via pattern recognition receptors (PRRs)[6]. Upon ligation of PRRs, an acute inflammatory response is orchestrated, characterized by production of proinflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, and recruitment of immune cells, including neutrophils and lymphocytes, to the site of inflammation. Subsequently, pathogens are phagocytosed, leading to production of inflammatory mediators that result in the activation of lymphocytes and more specific adaptive immune responses[7, 8]. In conjunction with this pronounced proinflammatory response, anti-inflammatory mediators such as IL-10 are released to counteract excessive inflammation. The most extensively studied family of PRRs is

the Toll-like receptor (TLR) family. In 1997, the first human homolog of Toll, TLR4, was identified[9]. The human TLR family currently consists of 10 members[6]. These different TLRs recognize different PAMPs; TLR2 is involved in the recognition of peptidoglycan, a cell wall component of Gram positive bacteria, TLR3 recognizes viral double stranded RNA, TLR4 binds lipopolysaccharide (LPS), the major cell wall component of Gram negative bacteria which is the most potent of all PAMPs, TLR5 recognizes bacterial flagellin, TLR7 binds viral single stranded RNA, and TLR9 recognizes bacterial and viral unmethylated CpG DNA[6, 10]. More recently, it has emerged that next to PAMPs, TLRs and other PRRs also recognize DAMPs (damage-associated molecular patterns), endogenous molecules released upon tissue injury[11, 12]. Examples include binding of hyaluronic acid, a major component of the extracellular matrix, to TLR4[13], and recognition of mitochondrial DNA by TLR9[14]. Ligation of DAMPs to PRRs results in generation of a sterile inflammatory response[11].

Therapy aimed to limit the innate immune response

While an effective acute inflammatory response is essential in case of invasion of microorganisms, it must be tightly regulated. Overactivation or persistent activation of the innate immune system can result in a systemic cytokine storm as well as overproduction of reactive oxygen production by neutrophils, leading to shock, tissue damage, and ultimately, multiple organ dysfunction syndrome (MODS) and death. This cascade of events is commonly seen in the early phase of sepsis and accounts for high mortality and morbidity; sepsis is still the leading cause of death in the ICU[15]. As such, many therapies aimed to limit the innate immune response have been investigated in the past, of which only few have proven to be effective. Anti-cytokine therapies such as anti-TNF-α and anti-IL-1β that showed great promise in animal models of sepsis, have yielded disappointing results in patients[16, 17]. Likewise, treatment with antibodies against LPS[18], or a TLR4 antagonist[19], has not resulted in improved outcome in septic shock. These results have led to the current opinion that there is not a single 'magic bullet' to be targeted in sepsis. Furthermore, these studies have signified that dampening the immune response too much may be detrimental; when pathogen clearance is seriously compromised, known as immune paralysis, the body is more vulnerable to secondary infections. This is especially harmful in the later phase of sepsis, when the initial hyperinflammatory state has subsided and the immune system is already in a hyporeactive state[20]. The most compelling evidence for this phenomenon has emerged from the opposing effects of trials employing high versus low doses of corticosteroids; high doses of these immune suppressing drugs resulted in increased mortality[21], whereas low-dose treatment showed favourable

effects on outcome[22]. To date, the only pharmacological therapeutic interventions that have resulted in improved outcome in sepsis are activated protein C (APC) and the abovementioned low-dose corticosteroids[22, 23]. Although both APC- and corticosteroid-treatment is associated with accelerated resolution of the excessive innate immune response in the early phase of sepsis[23, 24], these drugs exert various other effects that might account for the observed improved outcome. Still, these clinical studies indicate that appropriately-timed therapies aimed to reduce the initial overwhelming innate immune response in sepsis can be beneficial.

Another field where limiting the innate immune response could prove to be effective is excessive or inappropriate sterile inflammation. For instance, hyperinflammation similar to that observed in sepsis is known to occur following major trauma[25]. Furthermore, inflammatory processes play a deleterious role in the pathogenesis of ventilator-induced lung injury (VILI), which is another major problem in the ICU[26, 27]. Lastly, in auto-immune diseases such as rheumatoid arthritis, inappropriate and persistent activation of the innate immune system causes major tissue damage[28]. Interestingly, many of the anti-cytokine therapies designed to attenuate the immune response in sepsis but have failed in terms of outcome, have proved to be very effective in autoimmune diseases[29].

Taken together, limiting excessive, inappropriate or persistent activation of the innate immune system is a viable treatment option for a variety of conditions. As described in the first part of the introduction, the ANS can modulate the innate immune response, thereby representing a novel treatment opportunity to limit the innate immune response.

Links between the autonomic nervous system and the innate immune response

Immune-ANS signalling

It has long been known that inflammatory cytokines in an inflamed part of the body signal the brain via the afferent vagus nerve, resulting in fever[30], activation of the stress response consisting of the hypothalamic-pituitary-adrenal (HPA) axis[30], and the sympathetic nervous system[31]. This signalling pathway was elegantly demonstrated in animal studies, where subdiaphragmatic vagotomy reduced the increase in temperature and cortisol response (the end-product of the HPA axis) induced by intraperitoneal administration of LPS[32], IL-1β[33], and TNF-α[33]. In addition, it has been proposed that increased afferent vagus nerve signalling results in increased efferent vagus nerve activity, thereby representing a vago-vagal reflex that may act as a negative feedback loop during infection or inflammation[34, 35].

ANS-immune signalling: sympathetic nervous system

The sympathetic nervous system exerts its effects on innate immunity via binding of cathecholamines to both α- and β-adrenergic receptors present on innate immune cells such as monocytes and macrophages. Short-term stimulation of the β receptors (in particular β2) results in attenuation of the proinflammatory cytokine response and augmentation of anti-inflammatory cytokine levels. This has been extensively investigated *in vitro*; co-incubation with (nor)epinephrine or β-adrenergic agonists has been shown to inhibit TNF-α and IL-6, and enhance IL-10 release in LPS-stimulated human whole blood and isolated monocytes, and this effect was blocked by co-incubation with β-adrenergic antagonists[36-39]. This anti-inflammatory effect of the sympathetic nervous system was shown to be mediated by increased levels of intracellular cAMP[36]. In contrast, long-term (24 hours) incubation of monocytes with β-adrenergic agonists resulted in decreased cAMP levels below baseline and subsequent LPS stimulation showed increased levels of TNF-α[36]. In accordance, 24-hour norepinephrine infusion resulted in increased plasma concentrations of proinflammatory mediators in rats[40]. *In vitro*, α2 receptor stimulation has been demonstrated to enhance LPS-stimulated TNF-α release in mouse macrophages[41]. Effects of catecholamines on the innate immune response have also been investigated *in vivo* in humans. In the human endotoxemia model (LPS administration in healthy volunteers), antecedent epinephrine infusion starting 3 hours before LPS administration resulted in a markedly reduced proinflammatory cytokine response and increased levels of IL-10[39,42]. Interestingly, epinephrine infusion starting 24 hours before LPS administration limited the proinflammatory response to a much lesser extent[39], again emphasizing that there is a strong temporal relation between exposure to catecholamines and the inflammatory response. In addition, as part of a stress response, an increase in sympathetic activity is often accompanied by HPA axis activation and subsequent cortisol release, which is known to inhibit the innate immune response. Moreover, catecholamines can also directly activate the HPA axis[43]. These notions strongly support an overall anti-inflammatory effect of short-term activation of the sympathetic nervous system.

ANS-immune signalling: parasympathetic nervous system; the cholinergic anti-inflammatory pathway

Approximately ten years ago, Kevin Tracey and colleagues discovered that electrical stimulation of the efferent vagus nerve attenuated the LPS-induced inflammatory response in rats, while transecting the vagus nerve (vagotomy) enhanced inflammation[44]. Furthermore, it was shown that acetylcholine, the vagal neurotransmitter, inhibited LPS-induced release of proinflammatory cytokines in human macrophages[44].

As acetylcholine can bind both muscarinic and nicotinic acetylcholine receptors, macrophages were incubated with acetylcholine in combination with both muscarinic and nicotinic acetylcholine receptor antagonists, of which only the nicotinic antagonist α-bungarotoxin was able to block the anti-inflammatory effects of acetylcholine[44]. Further studies using knockout mice for different nicotinic acetylcholine receptors identified the α7 nicotinic acetylcholine receptor (α7nAChR) on macrophages as the principal mediator of the anti-inflammatory effects of the vagus nerve[45]. This mechanism was termed the cholinergic anti-inflammatory pathway. As the afferent arm of the vagus nerve can sense inflammation in the body and the efferent arm limits it, this pathway has been proposed as a reflex-type response to counteract excessive inflammation, an anti-inflammatory reflex[35] (Figure 1). In this vago-vagal reflex, afferent vagus nerve fibres terminating in the nucleus tractus solitarius synapse with fibres in the dorsal motor nucleus, where the efferent vagus nerve originates, a mechanism well-described in regulation of digestive functions[46]. The effects of efferent vagus nerve activity on the heart can be measured by heart rate variability analysis which will be explained later on in this chapter.

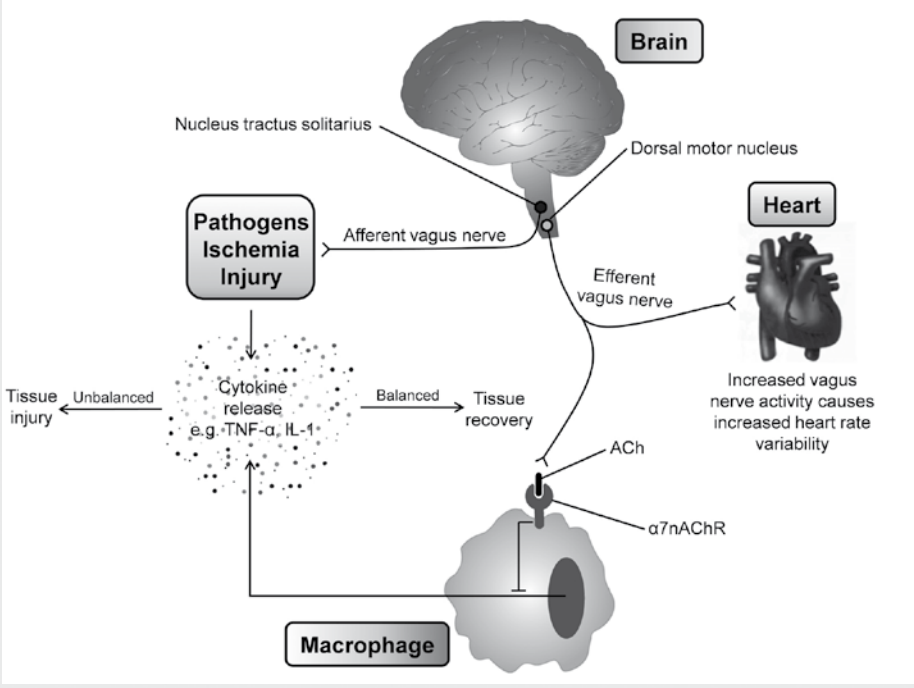

Figure 1 *The anti-inflammatory reflex*

With regard to the anatomical basis of the effects of the cholinergic anti-inflammatory pathway on effector organs, it is thought that in organs that are innervated by the vagus nerve, such as the liver[47], lungs[48], and gut[49], acetylcholine released from cholinergic nerve terminals activates α7nAChRs on tissue resident macrophages[50]. A special role for the spleen, an important cytokine-producing organ, has been proposed. The spleen is not innervated by cholinergic fibres, it solely receives sympathetic input[51]. However, it has been reported to be essential for the anti-inflammatory effects of the vagus nerve[52]. It was suggested by Tracey's group that the effects of vagus nerve stimulation on the spleen rely on synaptic activation of the splenic (sympathetic) nerve by the vagus nerve in the celiac-superior mesenteric plexus ganglion[53]. In turn the splenic nerve releases norepinephrine which can directly attenuate cytokine production in splenic macrophages via β-receptors[54], or enhance splenic acetylcholine levels resulting in α7nAChR activation and inhibition of cytokine production[53]. Furthermore, it has been suggested that via this pathway, circulating leukocytes passing the spleen are 'educated' to release lower levels of inflammatory cytokines[52, 55].

The α7nAChR is widely expressed in the central and peripheral nervous system and has been well-characterized therein[56]. In neurons, the α7nAChR is a pentameric complex composed of five α7 subunits assembled like the staves in a barrel to form an ion channel which is gated by the binding of ligands[57, 58], resulting in membrane depolarization and Ca2+ influx. Impaired α7nAChR activity has been implicated in pathogenesis of Alzheimer's disease, schizophrenia, and depression[59]. The presence of α7nAChR on cells of the innate immune system, such as primary human macrophages and peripheral blood mononuclear cells, has more recently been established[45, 60, 61]. Because these cells are non-excitable, the exact structure and intracellular signalling of the α7nAChR have not been completely elucidated[62]. Nonetheless, the anti-inflammatory effects of α7nAChR agonists, such as nicotine and acetylcholine, in human monocytes and macrophages *in vitro* have been shown by various research groups[44, 45, 60, 63]. Furthermore, the anti-inflammatory effects of electrical vagus nerve stimulation and nicotine have been established in various animal models of inflammation, including endotoxemia, sepsis, postoperative ileus, rheumatoid arthritis, and ischemia/reperfusion injury[44, 64-71]. In addition to the non-specific agonist nicotine, various specific α7nAChR agonists, such as AR-R17779, CAP55, PNU-282987, and GTS-21 have been employed in animal models of inflammation and have shown promising results[71-78]. Alternative methods of activation/stimulation of the cholinergic anti-inflammatory pathway have also been investigated in animal models. Acetylcholinesterase inhibition resulted in improved outcome in murine sepsis, supposedly by enhancing acetylcholine availability and thereby α7nAChR activation[67]. Finally, a novel gut-brain-immune axis

was identified in which lipid- and protein-enriched nutrition results in the release of cholecystokinin (CCK) in the gut, which in turn activates the afferent vagus nerve, leading to increased efferent vagus nerve activity (vago-vagal reflex), and ultimately attenuation of the inflammatory response in hemorrhagic shock[79] (Figure 2). While the aforementioned studies all showed beneficial effects of stimulation of the cholinergic anti-inflammatory pathway, excessive activation of the pathway may dampen the innate immune response to the point of immunosupression. This is supported by a study showing worsened survival in nicotine-treated mice during bacterial sepsis[52]. Likewise, nicotine pretreatment impaired bacterial clearance and increased mortality in murine septic peritonitis[80].

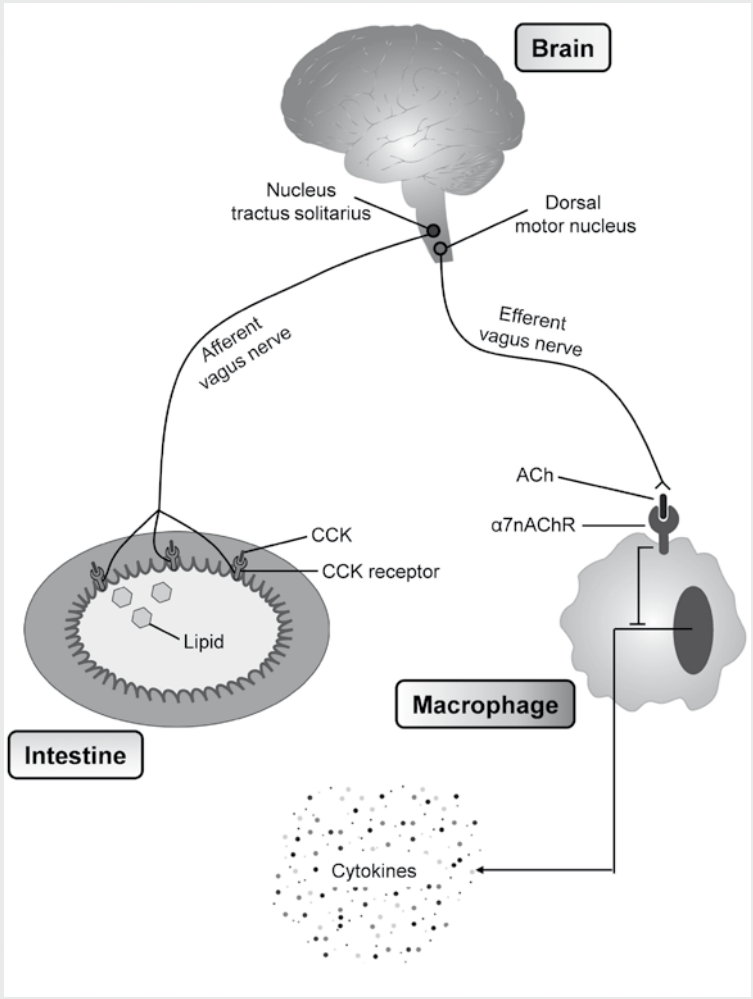

Figure 2 *The gut-brain-immune axis*

Human data regarding the effects of the cholinergic anti-inflammatory pathway on the innate immune response are very scarce. Several reasons can be put forward:
First, investigating the effects of stimulation of this pathway in humans is troublesome. Electrical vagus nerve stimulation has mainly been employed in epilepsy and major depression, but requires surgery[81]. Naturally, this is not feasible in acute situations and therefore represents a less relevant treatment option. Pharmacological stimulation of the pathway with acetylcholine is not possible because it is immediately degraded by acetylcholinesterases upon parenteral administration. The therapeutic potential of acetylcholine is further hampered by its nonspecificity and unwanted side effects such as vasodilatation. Nicotine's therapeutic potential is limited by its lack of pharmacologic specificity, toxic side effects, and the potential to produce physical dependence (addiction). There is one report on the effects of nicotine on the inflammatory response in humans. In this study, transdermal nicotine administration during human endotoxemia did not affect proinflammatory cytokine levels, although it did potentiate IL-10 levels[82]. The lack of an effect on pro inflammation probably resulted from the relatively low plasma nicotine levels achieved by transdermal administration. The most promising and feasible treatment options are specific α7nAChR agonists. As outlined above, many agonists have shown great promise in animal models, however, except for GTS-21, these compounds are at this moment not suitable for human use.
Second, measuring vagus nerve activity in humans *in vivo* is difficult. The only method available in humans is heart rate variability (HRV). This method is based on analysis of the variation in beat-to-beat intervals. The heart is sped up by sympathetic fibres and slowed down by vagus nerve fibres, both synapsing at the sinoatrial node. However, the effects of acetylcholine on the sinoatrial node are much faster than those of catecholamines due to shorter latency and faster decay[83]. Therefore, analysis of fast and slow oscillations in heart rate provides a measure of cardiac parasympathetic (Figure 1) and sympathetic activity. However, ANS outflow might be organ-specific; as such, vagal or sympathetic input to the heart may not represent vagal input to the spleen, lungs, gut, or liver. Furthermore, animal studies have shown that electrical stimulation at levels below the threshold required to change heart rate, and thus alter HRV, are sufficient to activate the cholinergic anti-inflammatory pathway[84]. Another confounding factor is represented by breathing patterns; HRV, and especially the parasympathetic component of HRV (respiratory sinus arrhythmia), is thought to be predominantly mediated by respiration-induced blood pressure changes which are sensed by carotid baroreceptors, leading to (de)activation of cardiac vagal fibers[85, 86]. Therefore, different breathing patterns might significantly affect HRV, which is very

relevant in ICU patients, of which the majority is ventilated. Finally, medication, and especially sedatives, which are used in most ICU patients, are known to affect HRV[87]. HRV analysis as a measure of ANS activity is therefore highly debated[88], however, it is the only tool available to measure activity of both parts of the ANS in humans *in vivo*. Third, the optimal tool to investigate the complex interplay between pro- and anti-inflammatory mechanisms during a systemic innate immune response *in vivo* in humans is the human endotoxemia model. In this model, a standardized dose of LPS is administered to healthy volunteers under controlled conditions; it therefore represents a well-controlled and reproducible model where the timing of the inflammatory stimulus is exactly known. While the human endotoxemia model does not replicate the clinical condition of for instance sepsis, it has been extensively employed to study the acute systemic inflammatory response *in vivo*, and administration of endotoxin affects various systemic physiologic and metabolic processes in a manner similar to the early phase of injury and infection[89]. This makes it a suitable human model for proof-of-principle studies[89]. However, because of the logistic complexity, the need for intensive monitoring, and safety regulations, this model is only operational in a few centres in the world.

The combination of these three barriers is likely the reason why the interplay between the ANS, and particularly the vagus nerve, and the innate immune system has only been sparsely studied in humans *in vivo*.

Aim and outline of this thesis

The aim of this thesis was to perform translational, clinically relevant studies regarding the interplay between the autonomic nervous system and the innate immune response with a distinct focus on the effects of stimulation of the cholinergic anti-inflammatory pathway.

In the first part of this thesis, the interplay between the autonomic nervous system and the innate immune response is assessed, both in patients and healthy volunteers. Patients with traumatic brain injury often suffer from a dysregulated autonomic nervous system and a severely impaired innate immune response. In **chapter 2a**, the hypothesis is presented that (over)activation of the cholinergic anti-inflammatory pathway plays an important role in the observed immune paralysis in patients with traumatic brain injury. In **chapter 2b**, the possible role of the cholinergic anti-inflammatory pathway in patients with subarachnoid haemorrhage, which are suggested to display increased vagus nerve activity, is discussed. In **chapter 2c**, a possible role for the cholinergic anti-inflammatory pathway in the increased mortality observed in critically ill patients receiving nicotine replacement therapy is presented. In **chapter 3**, the relationship between autonomic nervous system activity, measured by heart rate variability, and the innate immune response in patients with brain injury is investigated. In **chapter 4**, this relationship is examined in healthy volunteers during human endotoxemia.

In the second part of this thesis, the effects of stimulation of the cholinergic anti-inflammatory pathway *in vitro*, in animal models and in humans are described. Mechanical ventilation is a lifesaving intervention but can also lead to, or exacerbate pre-existing lung injury, a condition called ventilator-induced lung injury. Ventilator-induced lung injury is a major problem at the ICU, and activation of the innate immune system by mechanical ventilation has been shown to play a pivotal role in this condition. In **chapter 5**, the effects of vagus nerve stimulation and vagotomy in a two-hit model of ventilator-induced lung injury in rats are presented. GTS-21 is the only available specific α7nAChR agonist that can be used in humans, as phase I/II trails in healthy subjects and schizophrenia patients have been conducted[90-92]. The intracellular mechanisms involved in the anti-inflammatory effects of pharmacological α7nAChR stimulation have been sparsely studied, especially in human cells. In **chapter 6**, the effects and intracellular mechanisms of action of GTS-21 and nicotine on the innate immune response in primary human leukocytes are investigated. In **chapter 7**, the effects of GTS-21, acetylcholinesterase inhibition and nAChR blockade are explored in a murine ventilator-induced lung injury model. In **chapter 8**, the potential of GTS-

21 to attenuate the innate immune response in healthy volunteers during experimental endotoxemia is investigated. In **chapter 9**, a novel gut-brain-immune axis, which hitherto has only been described in animal models, is explored in healthy volunteers during experimental endotoxemia. In **chapter 10**, a case study on a Dutch individual known as 'the iceman' is presented. This individual holds several world records with regard to extreme cold tolerance. He claims that he can influence his autonomic nervous system and immune response using a concentration/meditation technique. The effects of this concentration/meditation technique on the ANS and the innate immune response was investigated, both *ex vivo* and during human endotoxemia.

In the third part of this thesis methodological considerations are described. HRV has been suggested to be influenced by respiratory patterns. However, this has only been investigated under static conditions. In **chapter 11**, the influence of different breathing patterns on HRV and its reproducibility in healthy subjects is described during human endotoxemia in which HRV magnitudes greatly fluctuate. Pharmacological intervention trials using the experimental human endotoxemia model are frequently designed in a cross-over manner, using an interval of 1-2 weeks[93-96]. However, repeated endotoxin administration may result in endotoxin tolerance[97] and it is unknown how long this tolerant state persists. Furthermore, *ex vivo* stimulation of leukocytes, for instance after sepsis or major trauma, is generally accepted to reflect a patient's *in vivo* immune status. In **chapter 12a**, the kinetics of repeated endotoxin administration in human volunteers on the innate immune response *ex vivo* and *in vivo* are investigated. In **chapter 12b**, an experimental human endotoxemia study employing a cross-over design is discussed in light of the results reported in chapter 12a.

Finally this thesis is concluded with a summary in **chapter 13** and a general discussion of the findings and future perspectives **in chapter 14**.

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Part one

Autonomic nervous system – innate immune interactions in human health and disease

Chapter 2a

Hypothesis

Increased vagal tone accounts for the observed immune paralysis in traumatic brain injury patients

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Abstract

Traumatic brain injury (TBI) is a leading cause of death and disability, especially in the younger population. In the acute phase after TBI, patients are more vulnerable to infection, associated with a decreased immune response *in vitro*. The cause of this immune paralysis is poorly understood. Apart from other neurologic dysfunction, TBI also results in an increase in vagal activity. Recently, the vagus nerve has been demonstrated to exert an anti-inflammatory effect, termed the cholinergic anti-inflammatory pathway. The anti-inflammatory effects of the vagus nerve are mediated by the α7 nicotinic acetylcholine receptor present on macrophages and other cytokine-producing cells. From these observations, we hypothesize that the immune paralysis observed in patients with TBI may, at least in part, result from augmented vagal activity and subsequent sustained effects of the cholinergic anti-inflammatory pathway. This pathway may counteract systemic proinflammation caused by the release of endogenous compounds termed alarmins as a result of tissue trauma. However, sustained activity of this pathway may severely impair the body's ability to combat infection. Because the cholinergic anti-inflammatory pathway can be pharmacologically modulated in humans, it could represent a novel approach to prevent infections in patients with TBI

Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability in the developed world. In the United States, around 1.5 million people sustain a brain injury annually, of whom approximately 50.000 die[1, 2]. TBI occurs mainly in the younger population, resulting predominantly from motor vehicle accidents, sports injuries, falls and acts of violence[3]. Patients with patients are more vulnerable to infection. As a result, infection is a common and serious complication following TBI[4]. A recent study reported that especially in the first 20 days after injury, the majority of patients with TBI die of sepsis or pneumonia[5]. Other workers reported that 77% of patients developed sepsis or systemic inflammatory response syndrome in the first seven days following TBI[6]. It is thought that the phenomenon called post-traumatic immune paralysis is responsible for the increased chance of developing an infection in these patients.

Immune paralysis in patients with TBI

The innate immune response is the first line of defense against invading pathogens[7]. This tightly regulated system consists of a wide variety of chemokines, cytokines, cell associated receptors, and other mediators orchestrating the initial response to infection. The adaptive immune response is a more specific and focused system comprised of different effector cell types such as B- and T-cells. While these two different systems evolved independently, it has become clear that innate immune signaling plays a critical role in initiating and instructing the adaptive immune response[8]. Therefore, effects on the innate immune response presumably also alter the adaptive immune response. Although limited, there are experimental data concerning the systemic immune paralysis observed in patients with brain trauma. After severe head injury, decreased T-helper cell activation, attenuated lymphokine-activated killer cell cytotoxicity, and depression of proinflammatory cytokine production (IL-2, IFN-γ), have been reported[9-14]. Moreover, *ex vivo* whole blood stimulation with lipopolysaccharide (LPS) shows an attenuated production of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-8, IFN-γ, and IL-12 in polytrauma patients compared with healthy controls[15]. Another study demonstrated decreased TNF-α mRNA levels in human peritoneal macrophages after polytrauma, while the anti-inflammatory cytokine IL-10 was upregulated[16]. Taken together, these results suggest that the early innate immune response, as well as the adaptive immune response, to pathogens is attenuated in patients with TBI. However, the mechanism underlying this immune paralysis following brain injury remains unclear.

The central nervous system and TBI

Dysfunction of the central nerve system (CNS) has been widely reported in patients with TBI. Many reports focus on the non-autonomic nervous system, such as motor deficits[17, 18], while others have evaluated the role of the autonomic nervous system after TBI[19-21]. The autonomic nervous system comprises a collection of afferent and efferent nerve fibers that regulate important vital functions in the body, including blood pressure and heart rate[22, 23]. The autonomic nervous system is composed of two parts, the sympathetic and the parasympathetic (vagal) part. The balance between these two subsystems, the sympathovagal balance, generates the net effect of the autonomic nervous system, of which heart rate is one of the end products[21]. Heart rate variability reflects the balance between the sympathetic and the parasympathetic parts of the autonomic nervous system, therefore, heart rate variability analysis is extensively used to measure autonomic activity (reviewed in[24]).

Measurement of autonomic nervous system activity

Heart variability can be analyzed in the time-domain, yielding normal-to-normal (R-R) intervals or by spectral (frequency domain) analysis of a series of R-R intervals. In the time-domain, heart rate variability is usually expressed as standard deviation of R-R intervals (SDNN) or as the SDNN index (SDNNIDX), which is the average of SDNN per specified interval[25]. The spectral analysis of R-R intervals is especially useful because a distinction can be made between low frequency (LF) and high frequency (HF) components in heart rate variability, providing information about the interplay of sympathetic and parasympathetic activity. Generally, LF spectral power (range 0.04-0.15 Hz) reflects sympathetic input while the HF power (range 0.15-0.4 Hz) represents parasympathetic influence[26, 27]. Therefore, the HF/LF ratio represents a measure of sympathovagal balance. A unique *in vivo* experiment that illustrates this balance was conducted in patients with an implanted electrical vagus nerve stimulator for the treatment of epilepsy[28]. In these subjects, HF power and HF/LF ratio were greatly increased when the stimulator was activated, illustrating that these measures are reliable indicators of vagal activity in humans. Nevertheless, caution should be taken when evaluating these parameters because other workers have indicated that parasympathetic outflow and other factors such as baroreceptor activity also influence LF power, thus stating that LF power represents a combination of sympathetic and parasympathetic input[29, 30]. Moreover, HF power can be influenced by factors like respiratory pattern[30].

Heart rate variability in patients with acute TBI

Although data are limited, a few human studies have assessed heart rate variability after TBI in the acute phase. An increase in HF/LF ratio was reported in the acute phase after TBI, indicating dominance of parasympathetic activity[20, 31]. In these studies, increased intracranial pressure, often present in acute TBI, has been linked to the high HF/LF ratios observed[20, 32]. Along these lines, patients with acute subarachnoid hemorrhage, which is associated with a rapid increase in intracranial pressure[33], displayed significantly increased HF power compared with controls, while LF power did not change[34]. These results suggest that in patients with TBI, parasympathetic (vagal) activity is augmented, resulting in an increased HF power and increased HF/LF ratio and that elevated intracranial pressure may play an important role in this process. In addition to the above described association between TBI/ increased intracranial pressure and heart rate variability as a measure of vagal tone inpatients with TBI, a model of increased intracranial pressure demonstrated a direct link between intracranial pressure and vagal tone. In cats, vagal activity, measured by microneurography, was increased relative to the increase in intracranial pressure induced by inflating a subdural balloon[35]. This suggests that high intracranial pressure directly influences vagal tone. A potential mechanism behind this phenomenon might be compression of higher vagal nuclei, or the brainstem, leading to vigorous firing of the medulla oblongata, the centre where the vagus nerve originates.

Vagal activity and the cholinergic anti-inflammatory pathway

Experimental evidence in the past several years has demonstrated that activation of the efferent vagus nerve has an inhibitory effect on the innate immune response (the cholinergic anti-inflammatory pathway, reviewed in[36-38]). Several *in vitro* studies have demonstrated that pretreatment of cultured human macrophages with the principal vagal neurotransmitter acetylcholine, significantly attenuates the LPS-induced release of proinflammatory mediators such as TNF-α, IL-6, IL-1β, IL-18, and HMGB1, while release of the anti-inflammatory cytokine IL-10 was unaffected[39, 40]. Moreover, vagus nerve electrical stimulation attenuates serum TNF-α and IL-6 levels in animals after endotoxin administration and prevents the development of shock[39, 41-43]. In contrast, vagotomized animals exhibited elevated levels of proinflammatory cytokines with aggravation of shock[39, 44, 45]. In humans, we know of only one study that evaluated inflammatory mediators in patients with a vagus nerve stimulator[46]. In this study, circulating inflammatory mediators were measured before implantation of the stimulator and after 12 weeks following implantation of the stimulator in a small group of patients with severe depression. After stimulator implantation, the

proinflammatory mediators TNF-α and IL-6 were significantly elevated; however, other proinflammatory mediators such as IL-1 and CRP were not. In addition, levels of the anti-inflammatory cytokine TGF-β were also significantly increased, which makes it difficult to draw any conclusions regarding the manner in which vagus nerve stimulation modulates the immune status in these patients. Moreover, levels of circulating inflammatory mediators in these patients do not actually reflect the status of the immune response, because no inflammatory stimulus was present.

The anti-inflammatory effect of the vagus nerve is mediated by the α7 nicotinic acetylcholine receptor (α7nAChR) expressed on macrophages and other cytokine-producing cells[43]. The identification of this receptor stimulated the use of nicotine instead of acetylcholine in studies exploring the anti-inflammatory effect of the vagus nerve because of its higher efficiency and selectivity, and has led to the search for even more selective α7nAChR agonists[40]. GTS-21, a specific α7nAChR agonist that has already been used in human clinical trials for the treatment of Alzheimer's disease, has been studied in a murine pancreatitis model. GTS-21 decreased pancreatitis severity, associated with reduced IL-6 levels and decreased pancreatic neutrophil accumulation[45]. Furthermore, CNI-1493, a compound exhibiting systemic anti-inflammatory effects currently used in clinical trials for the treatment of Crohn disease, has recently been shown to activate the vagus nerve via a centrally mediated intracerebral effect[47, 48]. CNI-1493 prevented endotoxin-induced shock and attenuated the rise in serum TNF-α levels in rats[47, 48]. Inhibitors of α7nAChR have also been identified. Mecamylamine, a drug used in humans for the treatment of hypertension and tobacco addiction, has been utilized in experimental settings and exhibited effects analogous to vagotomy, such as enhanced serum IL-6 levels and augmented severity of inflammation[45]. The intracellular cascade after α7nAChR activation has been, in part, elucidated by a recent study demonstrating that activation of α7nAChR leads to recruitment and activation of the anti-inflammatory JAK2-STAT3 (Janus kinase-2; signal transducers and activators of transcription-3) cascade[49]. While studies regarding the cholinergic anti-inflammatory pathway have mainly focused on the innate immune system, the adaptive immune response may also be attenuated by vagal outflow because of the vital role the innate immune system plays in initiating the subsequent adaptive immune response.

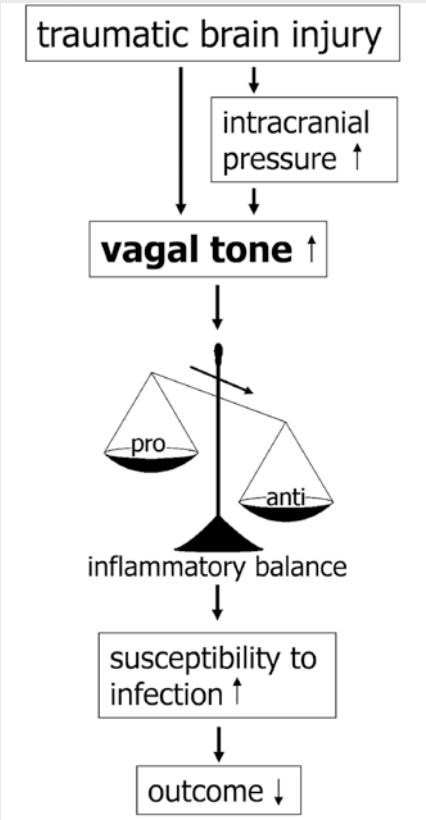
In addition, apart from the anti-inflammatory effects of the vagus nerve, inflammation can also affect vagal tone. Evaluation of heart rate variability during endotoxic shock in humans demonstrated attenuated variability accompanied by a decrease in total spectral power[50]. This may represent a negative feedback mechanism to prevent inhibition of the immune response. In this article we focus on the anti-inflammatory effects of the vagus nerve.

The cholinergic anti-inflammatory response represents an exciting new pathway that modulates the innate, and possibly also the adaptive, immune response and provides novel pharmacological treatment opportunities for situations associated with a disturbed sympathovagal balance.

Summary and hypothesis

In this report we have discussed three separate observations. 1) The immune response is severely impaired in patients after TBI, and is likely to be associated with the high prevalence of infections in these patients. 2) In the acute phase after TBI, vagal activity is augmented, resulting in a shift in the sympathovagal balance, possibly as a consequence of increased intracranial pressure 3) Vagal activity has an inhibiting effect on the innate immune response. These observations have led us to a new hypothesis that could explain the immune paralysis observed in patients with TBI. We hypothesize that the impaired immune response in these subjects may, at least in part, result from increased vagal activity and subsequent suppression of the immune response (Figure 1).

Figure 1 *Traumatic brain injury increases vagal tone directly or as a result of elevated intracranial pressure. Subsequently, vagal activity attenuates inflammation via the cholinergic anti-inflammatory pathway. As a result, susceptibility toward infections increases, resulting in worse outcome.*



Discussion

The hypothesis postulated above is based on evidence obtained from studies with different research objects. To our knowledge, no studies are available that test this hypothesis directly. Because the cholinergic anti-inflammatory pathway can be pharmacologically modulated in humans, such treatment in patients with TBI could represent a novel, promising approach to prevent infections in this specific group of patients.

Although elevated intracranial pressure appears to be an important link in the parasympathetic anti-inflammatory response following TBI, other mechanisms have also been suggested. Recently, the role of endogenous mediators in systemic inflammation in the absence of infection[51] was discussed. Toll-like receptors (TLRs) are well-known for their role in recognition of exogenous pathogens and subsequent triggering of immune responses. Excessive or inappropriate activation of TLRs can lead to systemic inflammation and shock, similar to that observed in septic patients. In addition to recognizing outside invaders, TLRs, as well as other receptors, also recognize endogenous damage signals termed alarmins, such as heat-shock proteins and HMGB1. Tissue trauma, for instance resulting from TBI, elicits release of these damage signals[52-54]. Consequently, trauma can trigger the immune response and this could explain the systemic inflammatory reaction in the absence of infection often observed in these patients. An increase in vagal activity may counteract this mechanism by attenuating the release of endogenous inflammatory mediators. In this context, it is noteworthy that *ex vivo* treatment of human monocytes with nicotine (stimulation of the cholinergic anti-inflammatory pathway) resulted in downregulation of TLR4 expression in human monocytes[55]. This observation may also explain the attenuated production of proinflammatory cytokines in blood of trauma patients that was stimulated *ex vivo* with LPS, the primary ligand for TLR4[15]. In this prospect, increased vagal activity may represent a negative feedback mechanism counteracting the massive systemic proinflammatory response. This notion is supported by animal studies in which increased vagal activity resulted in attenuation of inflammation, prevention of development of shock, and decreased mortality after experimental inflammation[39, 40, 45]. In this view, the resultant and sustained immune paralysis is merely a side-effect of the negative feedback loop. However, the animal studies mentioned before have all been performed using models of sterile inflammation. In animal experiments using live microbial sepsis, activation of the cholinergic anti-inflammatory pathway resulted in worsened survival, impaired migration of neutrophils to the inflamed area, and increased outgrowth of bacteria[41, 44, 56]. Moreover, human data on heart rate variability in the acute phase after TBI indicate that increased HF/LF ratios are associated with poor outcome and higher mortality[57-59]. These results suggest that increased vagal activity is beneficial in sterile inflammation induced by LPS or chemical compounds that evoke inflammation, but may impair the body's ability to combat an infection with live bacteria.

In contrast to aforementioned studies, also a decreased HF power and decreased HF/LF ratios have been reported in patients with TBI[19, 21, 60] . However, these studies

evaluated patients in the post-acute (chronic) phase of TBI (>30 days). Along these lines, patients in the chronic phase of subarachnoid hemorrhage demonstrated similar HF/LF ratios compared with control subjects, while HF/LF ratios were increased in the acute phase, as discussed before[34]. In the chronic phase of TBI or subarachnoid hemorrhage, patients usually do not have an increased intracranial pressure, possibly explaining the absence of increased vagal activity. Moreover, a potential explanation for decreased vagal output in the chronic phase of TBI may be damage to the brainstem and medulla oblongata resulting in impaired activity of the vagus nerve. In the acute phase of TBI, this effect may be overwhelmed by vigorous firing as a consequence of the compression of the brainstem. One study in a very acute setting (immediately after admission to the intensive care unit one 5-minute ECG was recorded) also reported decreased vagal activity and lower HF/LF ratios, indicating sympathetic dominance[61]. However, in this very acute setting, elevated intracranial pressure has probably not developed yet. Thus, time after injury appears to be a very important factor in autonomic dysfunction.

Taken together, we believe that our hypothesis may, at least in part, explain the immune paralysis observed in the first phase following TBI. Because increased vagal tone is associated with increased mortality and poor outcome in patients with TBI, intervening in the cholinergic anti-inflammatory pathway could be of potential benefit to these patients. This possibility warrants further research to elucidate the mechanism of immune paralysis in patients with TBI.

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Chapter 2b

Letter to the editor

Does subarachnoid haemorrhage influence the innate immune response?

Matthijs Kox, Jan C. Pompe, Cornelia W. Hoedemaekers, Peter Pickkers

Intensive Care Medicine 2007; 33(7):1303

Abstract original article

Sympathetic activation and inflammatory response in patients with subarachnoid haemorrhage

Naredi S, Lambert G, Friberg P, Zäll S, Edén E, Rydenhag B, Tylman M, Bengtsson A.
Intensive Care Medicine 2006; 32(12):1955-61

Background The aim of this study was to evaluate the correlation between sympathetic nervous activation and the immune response in patients following subarachnoid haemorrhage (SAH).

Methods Fourteen patients on a neurosurgical intensive care unit with acute non-traumatic SAH were included. Fifteen healthy, age-matched volunteers served as controls for measurement of catecholamine spillover. Blood sampling for C3a, C5b-9, IL-6, IL-8 and norepinephrine kinetic determination was made within 48 h, at 72 h and on the 7th-10th day after the SAH.

Results SAH patients exhibited a profound increase in the rate of norepinephrine spillover to plasma at 48 h, 72 h and 7-10 days after the insult, 3-4 times that in healthy individuals. The plasma levels of C3a, IL-6 and C5b-9 were significantly elevated at 48 h, at 72 h and 7-10 days after the SAH, but the plasma level of IL-6 decreased significantly 7-10 days after the SAH. There was no relationship between the magnitude of sympathetic activation and the levels of inflammatory markers.

Conclusions Following SAH a pronounced activation of the sympathetic nervous system and the inflammatory system occurs. The lack of significant association between the rate of spillover of norepinephrine to plasma and the plasma levels of inflammatory markers indicates that the two processes, sympathetic activation and the immune response, following SAH are not quantitatively linked. In spite of a persistent high level of sympathetic activation the plasma level of IL-6 decreased significantly one week after SAH.

To the editor

Immune suppression following injury to the central nerve system is an important clinical issue because up to 50% of brain-injury patients develop infection, especially in the first few days following the insult[1]. Recently, Naredi *et al.* tried to establish the immunomodulating effects of the sympathetic nervous system following subarachnoid haemorrhage[2]. The authors found increased norepinephrine spillover following subarachnoid haemorrhage, indicating sympathetic activation, and elevated levels of circulating proinflammatory cytokines and complement such as C3a, IL-6, and C5b-9. These parameters were measured to evaluate the relationship between sympathetic nervous system activity and the inflammatory response status, but no quantitative correlation between these two systems was found.

Following tissue trauma, as in subarachnoid haemorrhage, circulating levels of proinflammatory cytokines may not provide a good measure of innate immunity because these levels are mainly the result of the trauma itself[3] and do not reflect the innate immune response to a subsequent trigger. Therefore, the reported rise and subsequent normalisation of the proinflammatory mediators after subarachnoid haemorrhage observed in this study is not surprising. To evaluate the status of the immune response after subarachnoid haemorrhage or other tissue trauma, *ex vivo* stimulation of immune cells obtained from these patients with lipopolysaccharide or another inflammation-provoking compound would be more appropriate. Moreover, in light of the recently identified cholinergic anti-inflammatory pathway, future studies into this field should probably consider not only the *sympathetic* activity, but also the activity of the *parasympathetic* autonomic nervous system (vagal activity) when evaluating the modulation of the immune response[4]. This relatively newly discovered pathway also seems to be relevant in patients with subarachnoid haemorrhage. Apart from an increase in plasma catecholamine concentrations, another study in subarachnoid haemorrhage patients also found a marked increase in parasympathetic activity in the acute phase following subarachnoid haemorrhage[5]. The rise in parasympathetic activity may be the result of the bleeding and brain injury itself or mediated by the subsequent increase in intracranial pressure.

In summary, *ex vivo* cytokine release to an inflammatory stimulus present a better measure of the effect of subarachnoid haemorrhage on the innate immune response than the concentrations of circulating cytokines. Moreover, not only the sympathetic but especially the parasympathetic nerve system may play a pivotal role in the modulation of the innate immune response. Because increased activity of the vagus

nerve has been shown to dampen the immune response in both *in vitro* and animal studies[4], this may represent an interesting new avenue for future research and a promise for new treatment modalities.

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Chapter 2c

Letter to the editor

A possible role for the cholinergic anti-inflammatory pathway in increased mortality observed in critically ill patients receiving nicotine replacement therapy

Matthijs Kox, Jan C. Pompe, Cornelia W. Hoedemaekers, Peter Pickkers, Johannes G. van der Hoeven

Critical Care Medicine 2007; 35(10):2468-9

Abstract original article

The association of nicotine replacement therapy with mortality in a medical intensive care unit

Lee AH, Afessa B.

Critical Care Medicine 2007; 35(6):1517-21

Background Smokers admitted to the intensive care unit may receive nicotine replacement therapy to prevent withdrawal. However, the safety of nicotine replacement in the critically ill has not been studied. The objective of this study was to determine the impact of nicotine replacement on the outcome of critically ill patients.

Methods We performed a retrospective, case-control at the medical intensive care unit of a tertiary academic hospital. Patients who were active smokers at admission to the intensive care unit were included in the study. Those who received nicotine replacement therapy were considered as cases, and those who did not receive nicotine replacement were considered as controls.

Results For each of the 90 cases, one control smoker who did not receive nicotine replacement therapy was selected based on the severity of illness and then age. Outcome was measured by hospital mortality and 28-day intensive care unit-free days, defined as the number of days spent outside of intensive care or without mechanical ventilation by a living patient following admission to intensive care. The mean mortality rate predicted by the Acute Physiology and Chronic Health Evaluation III was 9.2% for the cases compared with 10.3% for the controls ($p = .7127$). The observed hospital mortality rate was 20% in the cases vs. 7% in the control group ($p = .0085$). When adjusted for the severity of illness and invasive mechanical ventilation, nicotine replacement therapy was independently associated with increased mortality (odds ratio, 24.6; 95% confidence interval, 3.6-167.6; $p = .0011$). The mean (sd) 28-day intensive care unit-free days were 20.7 (10.5) in the case group compared with 23.4 (7.1) in the control group ($p = .0488$).

Conclusions Our study shows that nicotine replacement therapy is associated with increased hospital mortality in critically ill patients. However, because of the limitations of the study, a future study based on a better case-control design is warranted.

To the editor

The cholinergic anti-inflammatory pathway is a recently discovered mechanism which implies that activation of the efferent vagus nerve attenuates the innate immune response[1, 2]. The anti-inflammatory effect of the vagus nerve is mediated by the α7 nicotinic acetylcholine receptor (α7nAChR), which is activated by acetylcholine, the principal vagal neurotransmitter[2]. Apart from acetylcholine, nicotinic agonists can also activate the α7nAChR and attenuate the innate immune response[1, 2]. Since these discoveries, various research groups have used nicotine, or other more specific α7nAChR agonists, to modulate diseases with an overactive immune response, such as sepsis or autoimmune disorders. However, sustained activation of the cholinergic anti-inflammatory pathway can also have detrimental effects. As a result of 'immune paralysis', it may render the body more susceptible to infection. For instance, in a mouse model of live microbial sepsis nicotine administration resulted in increased mortality[1]. This effect resulted from impaired influx of immune cells such as neutrophils into the inflamed area, leading to inadequate clearance, and therefore outgrowth, of bacteria.

Recently, Lee *et al.* investigated the impact of nicotine replacement therapy (NRT) on the outcome of critically ill patients[3]. NRT ameliorates withdrawal symptoms in smokers and is therefore frequently initiated in hospitalized patients. The authors reported increased mortality in patients receiving NRT compared with patients that did not. This is quite a remarkable observation, as several clinical trials have proven that NRT is not associated with increased mortality in non-critically ill hospitalized patients[4, 5]. Lee *et al.* speculate that the hemodynamic effects of nicotine, such as increased heart rate, blood pressure, myocardial contractility, and constriction of coronary arteries might be responsible for the increased mortality observed. Especially intensive care unit patients may be more susceptible to these effects.

We believe that the cholinergic anti-inflammatory pathway might also play an important role in the increased mortality associated with NRT in the critically ill. As highlighted previously, nicotine activates the α7nAChR and can therefore attenuate the innate immune response, resulting in immune paralysis. As the major causes of death in the NRT group were pneumonia (n=4) and sepsis (n=3), this suggests that these patients were more susceptible to infections than were the control group, where 2 patients died of sepsis and none of other infections. Thus, the absence of increased mortality after NRT in the non-critically ill in earlier trials may be explained by the fact that these patients were at a much lower risk of acquiring a concomitant infection than intensive care unit patients. Unfortunately, Lee *et al.* do not mention the prevalence of infections in the NRT and the control groups, nor do they provide data on the

amount or concentration of nicotine administered to the patients. This information would certainly help us evaluate the possible role of the cholinergic anti-inflammatory pathway in the increased mortality found in these patients.

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Chapter 3

The effects of brain injury on heart rate variability and the innate immune response in critically ill patients

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Provisionally accepted in Journal of Neurotrauma

Abstract

Background Brain injury and related increased intracranial pressure (ICP) may lead to increased vagus nerve activity and subsequent suppression of innate immunity via the cholinergic ant-inflammatory pathway. This may explain the observed increased susceptibility to infection in these patients. In the present study, we investigated the association between brain injury, vagus nerve activity, and innate immunity.

Methods We determined heart rate variability (HRV) as a measure of vagus nerve activity, plasma cytokines, and cytokine production of *ex vivo* LPS-stimulated whole blood in the first 4 days of admission to the neurological ICU in 34 patients with various forms of brain damage. HRV, immune parameters, and the correlations between these measures were analyzed in the entire group of patients and in subgroups of patients with conditions associated with high (intracranial hemorrhage [ICH]) and normal ICP (subarachnoid hemorrhage [SAH] with an extraventricular drain alleviating ICP). Healthy volunteers were used for comparison.

Results HRV total spectral power and *ex vivo* stimulated cytokine production was severely depressed in patients compared with healthy volunteers ($p<0.05$). Furthermore, HFnu (a HRV parameter corresponding with vagus nerve activity) was higher, and the LF/HF ratio (a HRV parameter corresponding with sympathetic activity) was lower in patients compared with healthy volunteers ($p<0.05$). HFnu correlated inversely with *ex vivo*-stimulated TNF-α production ($r=-0.22$, $p=0.025$). The most pronounced suppression of *ex vivo* stimulated cytokine production was observed in the ICH group. Furthermore, in ICH patients, HFnu correlated strongly with lower plasma TNF-α levels ($r=-0.73$, $p=0.002$).

Conclusions Our data suggest that brain injury, and especially conditions associated with increased ICP, is associated with vagus nerve-mediated immune suppression.

Introduction

Traumatic brain injury (TBI) is the leading cause of death and disability in children and young adults in the Western World[1]. The direct effects of damage to the brain account for high mortality, but in patients who survive the initial head injury, infection is the most frequently observed complication[2-5]. The high prevalence of infectious complications indicates that the immune system is compromised after TBI, a condition called immune paralysis. Immune paralysis is also present in nontraumatic brain injury such as subarachnoid hemorrhage (SAH)[2]. The mechanism behind immune paralysis following (traumatic) brain injury is poorly understood. We recently hypothesized that the autonomic nervous system, and in particular increased activity of the vagus nerve, may play a role in the observed immune paralysis[6]. TBI[7-9] and SAH[10-12] are associated with autonomic dysfunction, and recent evidence indicates that increased vagus nerve activity can limit the innate immune response[13, 14]. In this so-called cholinergic anti-inflammatory pathway, the vagal neurotransmitter acetylcholine, released in organs innervated by the vagus nerve, limits inflammatory cytokine production through the α7 nicotinic acetylcholine receptor present on tissue-resident macrophages[15]. In addition, it has been proposed that vagus nerve signaling to the spleen 'educates' passing circulating leukocytes to release lower levels of inflammatory cytokines[16, 17]. Excessive or persistent activation of this anti-inflammatory pathway might lead to an immune suppressed state that renders the body vulnerable to infection.

In our hypothesis, we proposed a critical role for increased intracranial pressure (ICP)[6]. Elevated ICP is a common feature of TBI[18] and SAH[19]. As a result of high ICP, compression of the brainstem may lead to increased firing of the medulla oblongata, where the efferent vagus nerve originates. Direct evidence for this phenomenon was found in cats, where inflation of a subdural balloon resulted in increased vagus nerve discharges relative to ICP levels[20]. In line, increased parasympathetic predominance has been demonstrated in the acute phase of TBI in patients[8, 21]. Furthermore, significant increases in parasympathetic activity were found in patients in the acute phase of SAH[11], and a recent study reported that parasympathetic predominance is a predictor of mortality in SAH[10].

All of the clinical studies mentioned above have used heart rate variability (HRV) as a measure of autonomic nervous system activity, as this is the only non-invasive tool available to monitor both branches of the autonomic nervous system in humans. Power spectral analysis of short-term (5 minute recordings) HRV provides a measure of autonomic modulation of the heart rate and is widely used to assess autonomic

nervous system activity[22, 23].

The aim of this study was to asses the association between brain injury, vagus nerve activity, and innate immunity. To the best of our knowledge, this has never been studied in humans before. We determined HRV and measures of innate immunity in patients admitted to the neurological ICU with various forms of brain damage. Concentrations of circulating cytokines were analyzed and, in light of the spleen-mediated effects of the vagus nerve on circulating leukocytes, we also assessed the inflammatory response of circulating leukocytes in *ex vivo* stimulation experiments. HRV, immune parameters, and the correlations between these parameters were analyzed in the entire group of patients as well as in subgroups of patients to compare brain injury associated with high and normal ICPs.

Materials and methods

Study population

We performed an prospective observational study in 34 adult patients admitted to the neurological ICU of a tertiary care university hospital in Nijmegen, the Netherlands in 2010 and 2011. Patient inclusion was aided by the help of nurses of the research-ICU department which made rounds along the wards twice daily. The study protocol was approved by the Ethics Committee of the Radboud University Nijmegen Medical Centre and complied with the Declaration of Helsinki including current revisions and the Good Clinical Practice guidelines. The Ethics Committee waived the need for informed consent, because of the study's observational nature. All patients >=18 yrs with traumatic and nontraumatic brain injury were eligible for the study. Patients were excluded in case of known arrhythmias or a pre-existent immune compromised status. One of the objectives of this study was to investigate the relation between conditions associated with increased ICP, vagus nerve activity, and the innate immune response. Therefore, we aimed to analyze three separate subgroups: subarachnoid hemorrhage (SAH), intracranial hemorrhage (ICH) and ICU control patients that suffered from trauma without brain damage (TRAUMA). In our institute, most SAHs are treated with extraventricular drains, thereby alleviating ICP. Therefore, this group of patients represents isolated brain injury without high ICPs. Baseline HRV data obtained in a group of 40 healthy male volunteers that participated in a human endotoxemia experiment (previously published in[24]) were used for comparison. In addition, for comparison of e*x vivo* stimulation data, we performed whole blood stimulation experiments identical to those performed in patients in 4 healthy male volunteers on 4 different days, where each measurement was separated by 5 days.

Data collection

The first measurement was performed within 24 hours of the initial event. Patients were measured every 24 hours for the first 4 days after admission to the ICU (days 0, 1, 2, and 3), unless they died or were discharged from the ICU. At these time points, blood was drawn for cytokine analysis and *ex vivo* stimulation, and HRV was measured. Relevant medications, including antibiotics, sedatives, analgesics, and inotropic drugs were recorded on a daily basis. Furthermore, demographic and clinical data as well as in-hospital mortality was recorded.

Heart rate variability analysis

A 5 minute 3-lead ECG recording was obtained using a Medilog AR12 recorder (Huntleigh Healthcare, Cardiff, UK); patients were not disturbed during the measurement. R-peak position was determined at a sample rate of 4096 Hz. HRV was analyzed using dedicated software (Medilog Darwin HRV, Huntleigh Healthcare, Cardiff, UK). In each 5-min recording, QRS complexes were detected and only normal-to-normal beat (NN) intervals were tabulated, yielding an interval tachogram. Recordings with artifacts such as premature (supra)ventricular beats or other arrhythmias comprising more than 5% of the total epoch were discarded. After linear detrending, power spectral density was determined by fast Fourier transformation (FFT) of interval tachograms using the Welch method and a FFT width of 1024. Very low frequency (VLF) power (0.0033-0.04 Hz) can not be reliably obtained from 5-min recordings and was therefore not analyzed[23]. With regard to the expected differences in total power and large variations in spectral power between patients, only normalized units or ratios are meaningful for comparison. Therefore, high frequency power in normalized units (HFnu), a measure of cardiac vagus nerve activity[23, 25, 26], and the ratio between low frequency and high frequency power (LF/HF), which is regarded to reflect sympathetic activity[27, 28], was used to assess autonomic activity. HFnu was calculated using the formula: (HF/[total power-VLF])*100.

Ex vivo whole blood stimulation

Ex vivo blood stimulation was performed using an custom in-house developed method (ImmuTube). This system consists of tubes prefilled with 2 mL of culture medium or 2 mL of culture medium containing lipopolysaccharide (LPS). Prefilled tubes were stored at -80 °C and brought to room temperature before use. 0.5 mL of lithium-heparin anti-coagulated blood (Vacutainer System, BD, Biosciences, Plymouth, UK) was then added to the prefilled tubes and the tubes were gently inverted to ensure proper mixing. Subsequently, tubes were incubated for 24 hours at 37 °C, centrifuged

(2500g, 4°C, 10 minutes) and the supernatant was stored at -80°C until analysis. In non-stimulated samples, no cytokine production was observed in any of the samples from patients or healthy volunteers, ruling out contamination.

Reagents

Culture medium (RPMI 1640 Dutch modification) was obtained from ICN Biomedicals (Costa Mesa, CA, USA). Culture medium was supplemented with gentamicin 10 μg/mL, L-glutamine 10 mM, and pyruvate 10 mM. *Escherichia coli* LPS (serotype O55:B5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). LPS was further purified as described previously[29] and used at a final concentration of 10 ng/mL.

Cytokine measurements

For plasma cytokine determination, EDTA anticoagulated blood (Vacutainer System, BD, Biosciences, Plymouth, UK) was collected from the patient's arterial catheter and immediately centrifuged at 2000g for 10 minutes at 4 °C. Plasma was stored at -80 °C until analysis. Plasma concentrations of TNF-α, IL-6, and IL-10 were measured using a simultaneous Luminex Assay according to the manufacturer's instructions (Milliplex, Millipore, Billerica, MA, USA). Cytokine concentrations in *ex* vivo stimulated whole blood supernatants were determined by enzyme-linked immunoassay (ELISA) according to the manufacturer's instructions (IL-6 and IL-10: Human IL-6 Sanquin Amsterdam, The Netherlands; TNF-α: R&D systems, Minneapolis, MN, USA).

Statistical analysis

None of the measured parameters was normally distributed (calculated using the Shapiro Wilk test). Therefore, data are represented as median (range). Statistical tests used are indicated in the text and figure legends. Differences between two groups were tested with Mann-Whitney U-tests and differences between three or more groups/days with Kruskal-Wallis tests with Dunn's post-hoc test (corrected for multiple comparisons). Spearman correlations Spearman correlation was used unless specified otherwise. P values of correlation analyses in subgroups were corrected for multiple testing. P values lower than 0.05 were considered significant. Statistical analysis was performed using Graphpad Prism 5 (Graphpad software, San Diego, CA, USA) and SPSS 16.0 (SPSS, Chicago, IL, USA).

Results

Study population

Demographic characteristics of the patient population and healthy volunteers are shown in Table 1. Physiological parameters and relevant medications are listed in Supplementary table 1. The only anti-inflammatory medication used was

dexamethasone, by one patient in the ICH group on days 2 and 3. In the group of healthy volunteers, median HR was 62 (47-97) beats/min and median MAP 96 (80-114) mmHg. In all but one of the SAH patients hydrocephalus was treated upon admission by insertion of an extraventricular drain, thereby alleviating ICP to normal values. No patients with isolated TBI were included to enable direct comparison with the TRAUMA group.

	All patients	SAH	ICH	TRAUMA (no brain damage)	miscellaneous	Healthy volunteers
n	**34**	**8**	**6**	**8**	**12**	**40**
AGE	58 (18-83)	59 (51-77)	68 (57-83)	44 (18-54)	61 (41-73)	22 (18-27)
APACHE-II	20 (8-64)	26 (13-43)	25 (14-64)	13 (8-18)	22 (9-28)	-
SAPS	40 (24-74)	46.5 (25-70)	38 (23-57)	35 (24-45)	48.5 (24-74)	-
GCS	7 (3-15)	8 (3-15)	5 (3-7)	15 (3-15)	6 (3-15)	-
Sex (n [%] male)	20 (59%)	2 (25%)	2 (33%)	6 (75%)	10 (83%)	40 (100%)
In hospital mortality %	8 (24%)	3 (38%)	3 (50%)	0 (0%)	2 (17%)	-

Data are presented as median (range). SAH: subarachnoid hemorrhage, ICH: intracranial hemorrhage. The miscellaneous group consisted of multi-trauma patients with neurological damage (n=3), post-anoxic encephalopathy (n=5), transverse myelitis (n=2), cerebellar infarction (n=1), and intoxication (n=1).

HRV and inflammatory markers in the entire study population

In the entire patient population (n=34), there were no differences in any of the HRV parameters between days. Therefore, data from all 4 days were pooled. HRV total spectral power (an overall measure of variability) was diminished in patients compared with healthy volunteers (Figure 1A). As depicted in Figure 1B and 1C, HFnu (cardiac vagus activity) was significantly higher, and LF/HF (sympathetic activity) was significantly lower in patients compared with healthy volunteers, indicating increased cardiac vagus nerve activity. Similar to HRV data, there were no differences over time *in ex vivo* stimulated cytokine production in both healthy volunteers and patients; data were therefore pooled. *Ex vivo* stimulated whole blood of patients revealed distinct hyporesponsiveness to LPS compared with healthy volunteers (TNF-α shown in Figure 1D; IL-6: healthy volunteers 9254 [3548-17670] pg/mL vs. patients 1800 [8-16885] pg/mL, P<0.0001; IL-10: healthy volunteers 293 [120-404] pg/mL vs. patients 47 [12-366] pg/mL, P<0.0001).

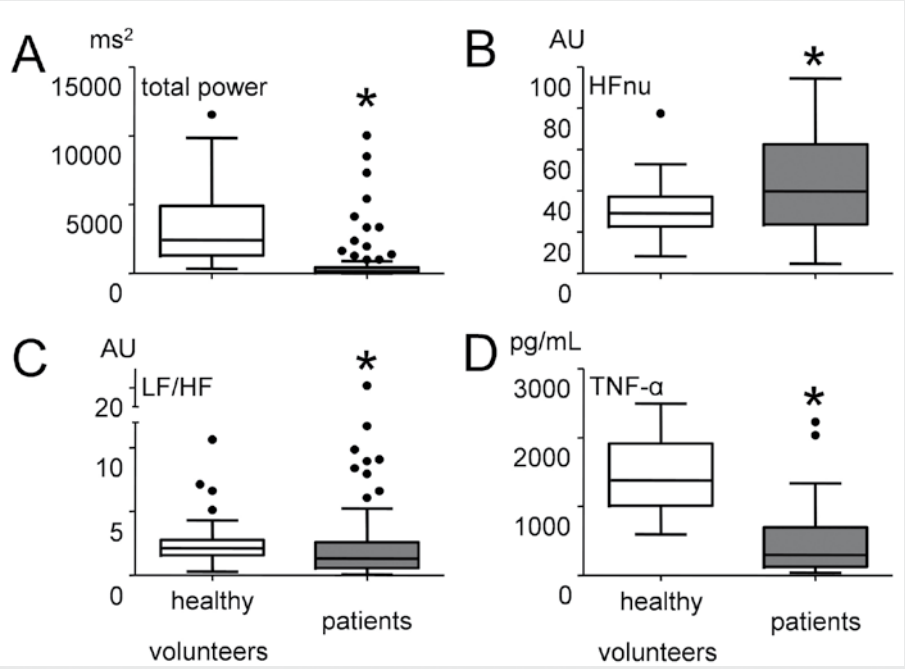

Figure 1 *HRV parameters total spectral power (A), HF normalized units (HFnu, B), and LF/HF (C) in healthy volunteers and patients. D) TNF-α production in whole blood of healthy volunteers and patients ex vivo stimulated with LPS. Data are represented as box and whiskers (Tukey). Ex vivo stimulation was performed on 4 different days in both healthy volunteers and patients and these data were pooled. * indicates p<0.05.*

In all healthy volunteers, plasma cytokine levels were below detection limits. In patients, plasma IL-6 and IL-10 concentrations were significantly higher on the day of admission and decreased afterwards (Figure 2); this pattern was due to the high plasma concentrations in the TRAUMA group on day of admission as described further on in this study. Plasma TNF-α levels were relatively low and did not change over time (6 [2-43] pg/mL).

To investigate the relation between autonomic activity and *ex vivo* stimulated cytokine production or plasma cytokines, we correlated HFnu and LF/HF with these inflammatory parameters and found a significant inverse correlation between HFnu and *ex vivo* stimulated TNF-α production (r=-0.22, p=0.025).

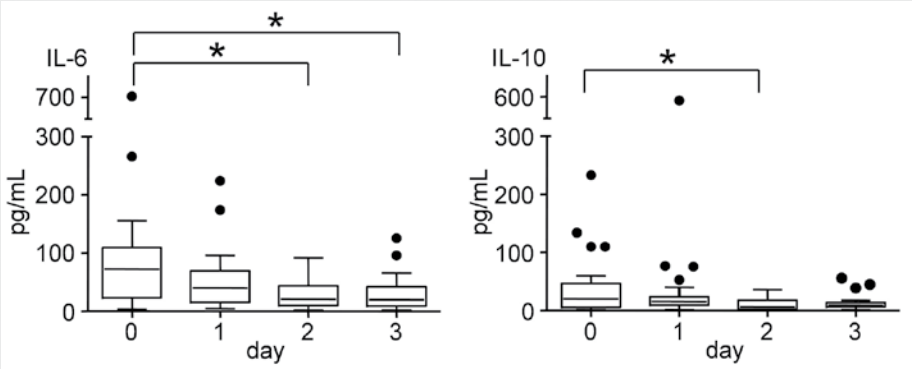

Figure 2 *Concentrations of plasma cytokine IL-6 and IL-10 during the study period in the entire patient population. Data are represented as box and whiskers (Tukey). * indicates p<0.05.*

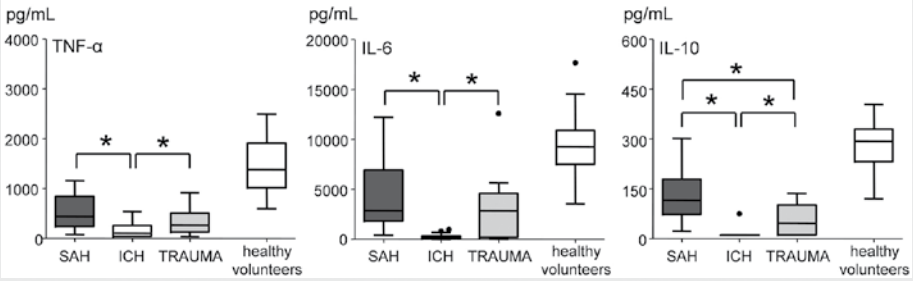

Figure 3 *TNF-α, IL-6, and IL-10 production in whole blood of patients and healthy volunteers ex vivo stimulated with LPS. Data are represented as box and whiskers (Tukey). Ex vivo stimulation was performed on 4 different days in both patients and healthy volunteers and these data were pooled. * indicates p<0.05. Cytokine production in all patient subgroups was significantly lower compared with healthy volunteers but, for reasons of clarity, this is not indicated in the figure.*

HRV and inflammatory markers in subgroups of patients

No differences in HRV parameters between days 0-4 were observed in patients in the SAH, ICH, or TRAUMA subgroups. Therefore, HRV data were pooled. No significant differences in HRV parameters were found between SAH, ICH, or TRAUMA subgroups. Furthermore, no differences in *ex vivo* stimulated cytokine production between days 0-4 were observed within subgroups and data were pooled. *Ex vivo* production of both pro- (TNF-α and IL-6) and anti-inflammatory (IL-10) cytokines in response to LPS stimulation was significantly lower in patients in the ICH group compared with the other two groups (Figure 3). Furthermore, stimulated IL-10 levels were lower in the TRAUMA group compared with the SAH group.

Ex vivo cytokine production in all patient subgroups was significantly lower compared with healthy volunteers. The markedly suppressed *ex vivo* cytokine production in the ICH group compared with the other two subgroups could not be explained by large differences in immunomodulatory covariates such as anti-inflammatory medication or leukocyte counts (Supplementary table 1).

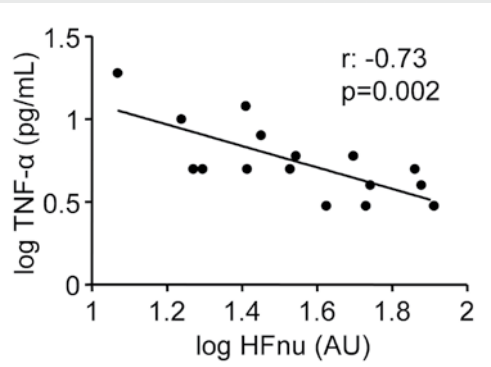

Figure 4 *Correlation between HFnu and plasma TNF-α in ICH patients. For visual purposes, data was log-transformed and Pearson r and p-value is indicated in the graph. Correlation analysis on non-log transformed data: Spearman r: -0.70, p=0.004.*

Within the TRAUMA group, plasma levels of IL-6 were significantly higher on day 0 (146 [83-702] pg/mL) compared with day 2 (48 [13-82] pg/mL, $p<0.05$). In the other two groups, no differences in plasma cytokine levels between days were found. Pooling of the data from the different days revealed significantly higher plasma levels of TNF-α and IL-6 in the TRAUMA group compared with the other two groups (TNF-α: TRAUMA 8 [3-15], SAH 4 [2-11], ICH 5 [3-19] pg/mL; IL-6: TRAUMA 83 [13-702], SAH 26 [2-131], ICH 17 [2-79], $p<0.05$). Plasma IL-10 was higher in the TRAUMA group compared with the SAH group (TRAUMA: 20 [2-110], SAH: 6 [2-110] pg/mL, $p<0.05$). The elevated cytokine levels in the TRAUMA group, especially on the day of admission, were likely due to the trauma insult itself.

We further examined the relation between vagus nerve activity and inflammatory parameters in the ICH group, since this group exhibited a greatly suppressed *ex vivo* stimulated cytokine production. Correlation analysis between HFnu and *ex vivo* stimulated cytokine production was not feasible in light of the very low levels of cytokines in the stimulated samples (approximately 50% of samples were below the detection limit). However, we did find a strong inverse correlation between HFnu and plasma TNF-α levels, indicating that higher cardiac vagus nerve activity is associated with lower plasma TNF-α levels (Figure 4). No such correlations were found in the other subgroups, but this could not be explained by large differences between subgroups in cardiorespiratory parameters or medications that could influence HRV (Supplementary table 1).

Discussion

In the present study, we report increased HFnu levels, a measure of cardiac vagus nerve activity, in patients with brain damage compared with healthy volunteers. Furthermore, these patients exhibited a distinct immune paralysis, reflected by low levels of cytokine production in *ex vivo* stimulated whole blood. Higher levels of HFnu correlated with lower levels of *ex vivo* stimulated TNF-α production. The most pronounced immune paralysis was found in patients with intracranial hemorrhage, a condition associated with high ICP. In these patients, higher levels of HFnu strongly correlated with lower levels of plasma TNF-α. These observations suggest that brain injury, and especially conditions associated with increased ICP, is associated with vagus-nerve mediated immune suppression.

We found markedly lower levels of overall HRV in our patient population compared with healthy volunteers. This is in accordance with previous findings of various investigators showing that brain injury is associated with diminished HRV, and that low HRV predicts unfavorable outcome[7, 30, 31]. The observed decrease in physiological heart rate variability indicates the uncoupling of organ systems generally seen in critically ill patients[27, 32]. Sedatives and analgesics have been shown to contribute to the loss of variability[33, 34], however, as large decreases in HRV are also found during experimental human endotoxemia (LPS administration in healthy volunteers)[24, 27, 35], other factors must also play a role. Brain injury patients exhibited higher HFnu and lower LF/HF ratios compared with healthy volunteers, suggesting a shift in the balance between cardiac autonomic activity towards increased vagus nerve signaling. These results are in line with previous findings of parasympathetic predominance in the acute phase following SAH[11] and severe head injury[21]. Our findings can not be explained by the considerably higher age of the patient population compared with the healthy volunteers, as increased age has been linked to higher LF/HF ratios[36].

Ex vivo stimulation of whole blood with LPS revealed that both pro- and anti-inflammatory cytokine production was severely impaired in patients compared with healthy volunteers. These findings suggest that following brain injury, monocytes (the main producers of the cytokines studied in 24-hour whole blood LPS stimulation experiments[37]) have an reduced capacity to respond to pathogens. Our findings are therefore indicative of a distinct immune paralysis in brain injury patients. To the best of our knowledge, this has never been studied in brain injury patients before. A few studies have investigated effects of severe head injury on T-lymphocytes and found decreased T-helper cell activation, attenuated lymphokine-activated killer cell

cytotoxicity, and depression of PHA-induced proinflammatory cytokine production (IL-2, IFN-g)[38-41].

In our patient population, we found a significant inverse correlation between cardiac vagus nerve activity (reflected by HFnu) and the *ex vivo* TNF-α production in response to LPS. Especially TNF-α production has been shown to be greatly limited by vagus nerve stimulation in animal models[15, 42]. Our findings therefore suggest activation of the cholinergic anti-inflammatory pathway, in which increased vagus nerve input to the spleen results in 'education' of passing circulating leukocytes to release lower levels of inflammatory cytokines[16, 17]. Our findings are in line with a recent study in healthy volunteers, where significant inverse correlations of roughly the same magnitude were found between HF power and *ex vivo* LPS-stimulated TNF-α production[43]. Only one study to date has investigated the effects of electrical vagus nerve stimulation on *ex vivo* cytokine production in humans[44]. This study in refractory epilepsy patients with an implanted vagus nerve stimulator demonstrated a significant reduction in LPS-stimulated IL-8 production after 6 months of vagus nerve stimulation. No significant effects were observed after 3 weeks of stimulation, although, interestingly, a trend towards lower stimulated TNF-α was present.

Our study was strictly observational, therefore, standard care was not altered for study purposes. Unfortunately, ICP was only registered in 2 patients on the day of admission. As this was anticipated, we decided prospectively to create subgroups of brain injury that are associated with increased and normal ICPs. A significantly more pronounced immune suppression was found in the ICH group compared with the SAH and TRAUMA groups. This is supportive of our hypothesis that high ICPs result in more pronounced immune suppression. In the ICH group, high ICPs were suspected based on the space-occupying lesions that were seen on CT-imaging of the brain, whereas in the TRAUMA group without brain damage, and the SAH group in which ICP was alleviated by placement of extraventricular drains in all but one of the patients, intracranial pressure was suspected to be lower. Although HFnu levels in the ICH group were not significantly higher compared with the other groups, we found a strong inverse correlation between HFnu and plasma TNF-α concentrations, indicating anti-inflammatory effects of increased vagus nerve activity. In accordance, correlations between higher vagal HRV parameters and lower plasma levels of inflammatory mediators have been found in both healthy volunteers[45] and coronary heart disease patients[46].

Our study has several limitations. First, HRV only reflects cardiac autonomic activity. At present, it is unclear whether cardiac vagus nerve activity reflects vagal input to other organs that are important in the immune response, e.g. the spleen. Animal and human studies have established that the output of the sympathetic nervous system is highly differentiated[47-49]. No such investigations on the parasympathetic nervous system are known to us, but it appears plausible that differentiated output to organs does exist. Unfortunately, besides HRV, no techniques to measure vagus nerve activity are currently available in humans. Second, the effects of ICP on vagus nerve activity and the immune response could not be properly assessed in this study, because ICP probes were placed in only 2 of our patients. Third, the question remains whether our findings in circulating monocytes can be extrapolated to tissue-resident macrophages, which are the predominant cells involved in the initial innate immune response against invading pathogens. We have recently shown that after administration of LPS to healthy volunteers, *ex vivo* hyporesponsiveness to LPS, reflecting the monocyte compartment, is present in the acute phase but quickly wanes[50]. However, *in vivo* hyporesponsiveness to LPS, reflecting the tissue-resident macrophage compartment, persists for several weeks[50]. In this respect, *ex vivo* hyporesponsiveness might even underestimate the capacity of tissue-resident macrophages to respond to inflammatory stimuli. Finally, another contributor to the observed low HRV in our patient population may be the considerably higher age of the patient population compared with the healthy volunteers, as increasing age is associated with a decreased HRV[36, 51].

In conclusion, in brain injury patients, higher vagus nerve activity is associated with attenuated *ex vivo* LPS-stimulated monocytic TNF-α production. Furthermore, in ICH patients, associated with high ICPs, a pronounced *ex vivo* immune paralysis was observed. In addition, in these patients, higher vagus nerve activity was also associated with lower levels of circulating plasma TNF-α. These findings are suggestive of effects of the cholinergic anti-inflammatory pathway and might contribute to the immune paralysis in brain injury patients.

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Supplementary material

Supplementary table 1 *Physiological parameters and relevant medications*

Parameter	group	Day 0	Day 1	Day 2	Day 3
n	all	34	32	27	23
	SAH	8	8	6	4
	ICH	6	5	5	5
	TRAUMA	8	7	6	5
	misc	12	12	10	9
Mechnical ventilation (n [%])	all	29 (85%)	28 (88%)	19 (70%)	17 (74%)
	SAH	8 (100%)	7 (88%)	3 (50%)	3 (75%)
	ICH	6 (100%)	4 (80%)	4 (80%)	4 (80%)
	TRAUMA	5 (63%)	5 (71%)	4 (67%)	3 (60%)
	misc	10 (83%)	12 (100%)	8 (80%)	7 (78%)
Controlled mode ventilation (n [%])	all	19 (66%)	10 (36%)	5 (28%)	8 (35%)
	SAH	5 (63%)	1 (14%)	0 (0%)	0 (0%)
	ICH	3 (50%)	1 (25%)	0 (0%)	1 (25%)
	TRAUMA	4 (80%)	1 (20%)	0 (0%)	0 (0%)
	misc	7 (70%)	7 (58%)	5 (63%)	6 (71%)
Sedation (n [%])	all	18 (53%)	18 (56%)	12 (44%)	6 (26%)
	SAH	3 (38%)	4 (50%)	2 (33%)	0 (0%)
	ICH	4 (67%)	3 (60%)	2 (40%)	2 (40%)
	TRAUMA	4 (50%)	4 (57%)	2 (33%)	0 (0%)
	misc	7 (58%)	7 (58%)	6 (60%)	4 (44%)
Analgesia (n [%])	all	12 (35%)	17 (53%)	10 (37%)	5 (22%)
	SAH	1 (13%)	4 (50%)	3 (50%)	0 (0%)
	ICH	1 (17%)	1 (20%)	1 (20%)	0 (0%)
	TRAUMA	6 (75%)	6 (86%)	4 (67%)	3 (60%)
	misc	4 (33%)	6 (50%)	2 (20%)	2 (22%)
Inotropes/ vasopressors (n [%])	all	10 (29%)	13 (42%)	8 (30%)	6 (26%)
	SAH	4 (50%)	3 (38%)	0 (0%)	0 (0%)
	ICH	1 (17%)	1 (20%)	1 (20%)	1 (20%)
	TRAUMA	1 (13%)	2 (29%)	2 (33%)	2 (40%)
	misc	4 (33%)	7 (58%)	5 (50%)	3 (33%)
Respiratory rate (breaths/min)	all	15 (8-34)	15 (8-27)	17 (10-28)	18 (10-26)
	SAH	15 (8-18)	14 (8-18)	15 (12-28)	18 (15-19)
	ICH	14 (12-20)	15 (10-21)	19 (12-22)	20 (11-26)
	TRAUMA	16 (8-25)	15 (13-18)	16 (14-24)	16 (10-20)
	misc	17 (12-34)	16 (12-27)	17 (10-24)	19 (10-24)
Heart rate (beats/min)	all	74 (44-122)	81 (43-131)	79 (50-127)	75 (52-131)
	SAH	60 (48-87)	71 (52-120)	75 (62-82)	84 (69-109)
	ICH	60 (54-114)	83 (77-86)	82 (81-107)	75 (69-98)
	TRAUMA	93 (79-122)	98 (83-131)	89 (77-124)	98 (65-131)
	misc	68 (44-82)	79 (43-97)	72 (50-127)	57 (52-99)
Mean arterial pressure (mmHg)	all	91 (62-110)	94 (72-117)	101 (64-127)	101 (71-126)
	SAH	99 (81-110)	101 (90-117)	111 (90-127)	111 (105-120)
	ICH	93 (85-110)	108 (94-116)	116 (88-127)	103 (85-126)
	TRAUMA	75 (62-101)	79 (72-97)	80 (64-113)	99 (90-104)
	misc	90 (69-102)	83 (76-117)	99 (76-113)	98 (71-111)
Temperature (°C)	all	36.5 (32.6 – 39.0)	37.4 (32.6-39.1)	37.5 (33.1-39.0)	37.6 (32.3-39.4)
	SAH	36.8 (35.8 – 37.5)	37.1 (36.5-37.4)	37.6 (37.3-37.9)	38.3 (37.9-38.3)
	ICH	36.4 (35.8 – 37.9)	37.5 (37.0-37.7)	36.8 (35.7-37.8)	37.1 (35.7-37.8)
	TRAUMA	36.1 (35.2 – 37.6)	37.7 (36-39.1)	37.8 (37.0-38.4)	38.2 (37.6-38.8)
	misc	36.2 (32.6 – 39.0)	37.6 (32.6-39.0)	37.5 (33.1-39.0)	35.2 (32.3-39.4)
Leucocytes (10^9/L)	all	13.2 (4.4-25.6)	12.7 (5.6-23.8)	12.6 (4.8-21.5)	11.3 (4.2-15.8)
	SAH	14.6 (8.2-25.6)	17.2 (11.9-23.8)	13.9 (9.6-17.6)	15.2 (13.4-15.5)
	ICH	13.6 (11.2-17.2)	14.0 (11.8-18.8)	15.2 (14.0-15.7)	12.3 (10.3-13.7)
	TRAUMA	7.0 (4.4 – 16.4)	7.9 (5.6-12.5)	8.5 (5.2-13.0)	10.3 (6.5-10.8)
	misc	13.2 (6.1-23.6)	13.7 (5.7-20.0)	10.4 (4.8-21.5)	8.9 (4.2-15.8)

Data are presented as median (range). SAH: subarachnoid hemorrhage, ICH: intracranial hemorrhage. The miscellaneous group consisted of multi-trauma patients with neurological damage (n=3), Cardio-pulmonary resuscitation (n=5), transverse myelitis (n=2), cerebellar infarction (n=1,) and intoxication (n=1).

Chapter 4

Interplay between the acute inflammatory response and heart rate variability in healthy human volunteers

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Shock 2011; 36(2):115-20

Abstract

Background The autonomic nervous system (ANS) and the inflammatory response are intimately linked. Heart rate variability (HRV) analysis is a widely used method to assess cardiac ANS activity, and changes in HRV indices may correlate with inflammatory markers. Here, we investigated whether baseline HRV predicts the acute inflammatory response to endotoxin. Second, we investigated whether the magnitude of the inflammatory response correlated with HRV alterations.

Methods Forty healthy volunteers received a single intravenous bolus of 2 ng/kg endotoxin (LPS, derived from *E coli* O:113). Of these, 12 healthy volunteers were administered LPS again two weeks later. HRV was determined at baseline (just prior to LPS administration) and hourly thereafter until 8 hours after LPS administration. Plasma cytokine levels were determined at various time points.

Results Baseline HRV indices did not correlate with the magnitude of the LPS-induced inflammatory response. Despite large alterations in HRV after LPS administration, the extent of the inflammatory response did not correlate with the magnitude of HRV changes. In subjects who were administered LPS twice, inflammatory cytokines were markedly attenuated after the second LPS administration, whereas LPS-induced HRV alterations were similar.

Conclusions HRV indices do not predict the acute inflammatory response in a standardized model of systemic inflammation. Although the acute inflammatory response results in HRV changes, no correlations with inflammatory cytokines were observed. Therefore, the magnitude of endotoxemia-related HRV changes does not reflect the extent of the inflammatory response.

Introduction

The autonomic nervous system (ANS) and the inflammatory response are intimately linked. The sympathetic nervous system can attenuate the systemic inflammatory response via activation of β2-adrenoceptors[1-3], but can also enhance the inflammatory response locally via stimulation of α2-adrenoceptors[4, 5]. The parasympathetic nervous system, through the afferent vagus nerve can sense inflammation in the periphery and relay this information to the brain, resulting in fever generation and activation of the hypothalamic-pituitary-adrenal axis which in turn leads to cortisol release and stimulation of the sympathetic nervous system[6, 7]. Furthermore, electrical stimulation of the efferent vagus nerve greatly attenuates the inflammatory response in animal models[8]. These findings indicate that there is considerable crosstalk between the ANS and the inflammatory response and that the two branches of the ANS can modulate the inflammatory response.

The ANS innervates virtually every organ in the body. However, outflow of a particular branch of the ANS might be organ-specific. Heart rate variability (HRV) analysis is the only noninvasive tool available in humans to monitor activity of both branches of the ANS[9], but assesses only ANS innervation of the heart. HRV indices (both time and frequency domains) and their physiological correlates[10-13] are listed in Table 1.

Decreased HRV is associated with pathological conditions. In systemically inflamed critically ill patients, such as in sepsis, HRV is completely diminished[14-17] and inversely correlated with disease severity[15, 16], and reduced HRV is a predictor of multiorgan dysfunction syndrome and death[18, 19]. These findings suggest that HRV alterations may reflect the magnitude of the inflammatory response.

In this study, we investigated the interplay between the acute inflammatory response to endotoxin and cardiac ANS activity, measured by HRV. We used the experimental human endotoxemia model, a well-characterized, standardized model of systemic inflammation widely used to study the acute inflammatory response[20]. This model is not hampered by confounding factors such as sedatives, inotropes, and pressors often present in the clinical situation. It therefore provides readily interpretable data regarding the interplay between the acute inflammatory response and HRV. Because ANS-inflammatory interactions are bidirectional, we determined the relation between baseline HRV indices and the inflammatory response as well as the relation between the magnitude of the inflammatory response and HRV alterations.

Table 1 *HRV indices definitions and physiological correlates*

domain	*HRV index (unit)*	*Definition*	*Physiological correlate*
Time domain	SDNN (ms)	Standard deviation of all NN intervals	Sympathetic and parasympathetic activity
	r-MSSD (ms)	Square root of the mean squared difference of successive NN intervals	Parasympathetic activity
	pNN50 (%)	The number of successive NN intervals that differ by more than 50 ms, expressed as a percentage of the total number of NN intervals.	parasympathetic activity
Frequency domain	LF (ms^2)	Low frequency power (0.04-0.15 Hz)	Sympathetic and parasympathetic activity
	HF (ms^2)	High frequency power (0.15-0.4 Hz)	Parasympathetic activity
	total (ms^2)	Total spectral power (0-0.15 Hz)	Sympathetic and parasympathetic activity
	LF/HF (AU)	Ratio between LF/HF	Sympathetic activity
	LFnu (AU)	Normalized LF power (LF/[total-VLF])	Sympathetic activity in situations where total power changes over time
	HFnu (AU)	Normalized HF power (HF/[total-VLF])	Parasympathetic activity in situations where total power changes over time

ms: milliseconds. AU: arbitrary units. VLF: very low frequency power.

Materials and methods

Subjects

HRV was measured in a total of 40 healthy young male nonsmoking volunteers participating in two human endotoxemia studies. Data of 28 subjects were obtained from a study (Clinical Trial Register number NCT00513110) where LPS (endotoxin) was administered once; the other 12 subjects participated in a human endotoxemia study (Clinical Trial Register number NCT00783068) in which they received LPS twice (LPS visits 1 & 2) with a mean interval of 14 days (range, 11-18 days). The study protocols were approved by the Ethics Committee of the Radboud University Nijmegen Medical Centre and comply with the Declaration of Helsinki including current revisions and the Good Clinical Practice guidelines. Written informed consent was obtained from all study participants. The findings of the physical examinations, electrocardiography, and routine laboratory studies on all the volunteers before the start of the experiment showed normal results. Volunteers were not taking any prescription medications, and they were negative for hepatitis B surface antigen and HIV infection.

Human endotoxemia model

Subjects refrained from food 12 hours before the start of the experiment, and caffeine- or alcohol-containing substances 24 hours before the start of the experiment. The experiments were performed at the research unit of the intensive care department, with subjects in supine position. After administration of local anesthesia (lidocaine HCl 20 mg/mL) the radial artery was cannulated using a 20 Gauge arterial catheter (Angiocath, Becton Dickinson, Sandy, Utah) and connected to an arterial pressure monitoring set (Edwards Lifesciences LLC, Irvine, CA, USA), connected to a Phillips IntelliVue MP70 monitor (Philips Medical Systems, Eindhoven, The Netherlands). A cannula was placed in the antecubital vein to permit infusion of 2.5% glucose/ 0.45% saline solution; subjects received a bolus of 1.5 L during one hour before LPS infusion (prehydration), followed by 150 ml/h until 6 hours after LPS infusion and 75 ml/h until the end of the experiment. U.S. Reference *Escherichia coli* endotoxin (LPS derived from *E. coli* O:113; Clinical Center Reference Endotoxin, National Institute of Health, Bethesda, MD, USA) was used. Ec-5 endotoxin, supplied as a lyophilized powder, was reconstituted in 5 ml saline 0.9% for injection and vortex-mixed for at least 10 minutes after reconstitution. The endotoxin solution was administered as an intravenous bolus injection at a dose of 2 ng/kg of body weight. Vital signs, including heart rate (HR) and mean arterial pressure (MAP) were monitored continuously throughout the study protocol.

HRV measurements

HRV was measured hourly by 5-min recordings starting just before LPS administration (T=0 or baseline) up to 8 hours after LPS administration. HRV was measured in supine position and during quiet circumstances. A 3-lead electrocardiogram signal was obtained using a Medilog AR12 recorder (Huntleigh Healthcare, Cardiff, UK). R-peak position was determined at a sample rate of 4096 Hz. HRV was analyzed using dedicated software (Medilog Darwin HRV, Huntleigh Healthcare, Cardiff, UK). In each 5-min recording, QRS complexes were detected, and only normal-to-normal beat (NN) intervals were tabulated, yielding an interval tachogram. Recordings with artifacts such as premature (supra)ventricular beats or other arrhythmias comprising more than 5% of the total epoch were discarded. After linear detrending, power spectral density was determined by fast Fourier transformation of interval tachograms using the Welch method and a fast Fourier transformation width of 1024. Very-low-frequency (VLF) power (0.0033-0.04 Hz) can not be reliably obtained from 5-min recordings and was therefore not analyzed[11].

Cytokine measurements

EDTA anticoagulated blood was collected from the arterial line and immediately centrifuged at 2000g for 10 minutes at 4 °C. Concentrations of TNF-α, IL-6, IL-10, and IL-1RA in plasma were measured using a simultaneous Luminex Assay according to the manufacturer's instructions (Bio-plex cytokine assay, Bio-Rad, Hercules, CA, USA). Samples from both LPS visits of the 12 subjects who received LPS twice were analyzed on the same day.

Calculations and statistical analysis

Based on distribution (calculated by the Shapiro-Wilk test), data are represented as medians or means as indicated. Non-normally distributed HRV indices and cytokine values were successfully log transformed. Statistical tests used are indicated in the figure legends. A p-value <0.05 was considered significant. Statistical analysis was performed using SPSS 16.0 (SPSS, Chicago, IL, USA) and Graphpad Prism 5 (Graphpad software, San Diego, CA, USA).

Results

Correlation between baseline HRV and the LPS-induced cytokine response

LPS administration resulted in a typical transient inflammatory cytokine response, decreased MAP and increased HR (Figure 1). We did not find any significant correlations between basal HRV indices and the Area Under Curve (AUC; all correlations listed in Table 2; most relevant indices shown in Figure 2) or peak levels (Supplementary Table 1) of any of the cytokines measured.

Correlation between the LPS-induced cytokine response and HRV alterations

LPS administration resulted in characteristic HRV alterations (SDNN, LF/HF, LFnu, and HFnu depicted in Figure 3; other indices are shown in Supplementary Figure 1), similar to earlier reports[2, 10, 21, 22]. To determine whether the magnitude of the inflammatory response was correlated with the magnitude of changes in HRV indices, we correlated peak and AUC cytokine levels with maximum/minimum levels of HRV after LPS administration. No significant correlations were found (Supplementary Table 2&3). Furthermore, we correlated the peak and AUC cytokines to the increase or decrease in HRV indices (represented as slope of increase/decrease calculated between baseline and time of maximum/minimum value). Apart from one weak correlation between IL-1RA and the decrease in LF power, the magnitude of the cytokine response (AUC) did not correlate to changes in HRV indices as well (Table 3); this was also true when peak cytokine levels were used for the calculations (Supplementary Table 4).

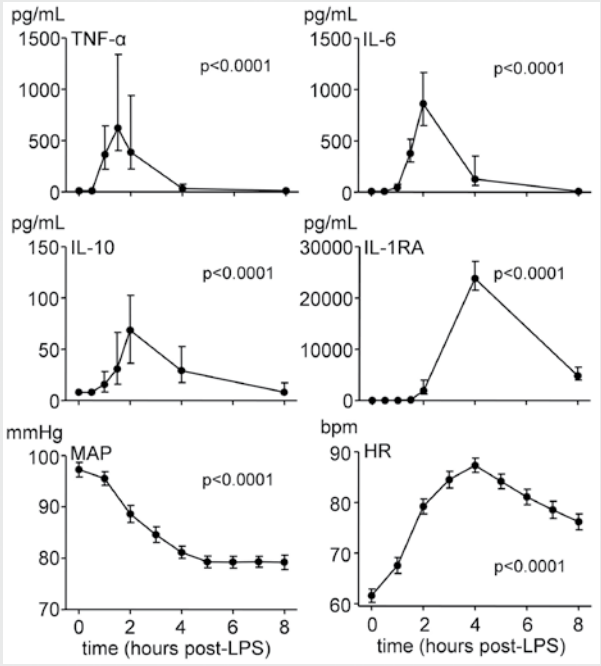

Figure 1

Plasma concentrations of cytokines (TNF-α, IL-6, IL-10 and IL-RA), mean arterial pressure (MAP) and heart rate (HR) during human endotoxemia. Data are expressed as median ± interquartile range (cytokines) or mean ± SEM (MAP & HR) of 40 subjects. p-value was calculated by Friedman test (cytokines) or one-way ANOVA (MAP & HR).

Table 2 *Correlations between baseline HRV indices and the LPS-induced cytokine response*

HRV measure	Correlation coefficient (Pearson *r*)			
	log TNF-α	*log IL-6*	*log IL-10*	*log IL-1RA*
log SDNN (ms)	0.154	0.147	-0.153	-0.012
log r-MSSD (ms)	0.073	0.118	-0.235	-0.055
pNN50 (%)	0.005	0.016	-0.264	-0.080
log LF (ms^2)	0.070	0.034	-0.263	-0.139
log HF (ms^2)	-0.007	0.020	-0.259	-0.064
log total (ms^2)	0.023	0.002	-0.278	-0.035
log LF/HF (AU)	0.086	0.001	0.170	-0.036
LFnu (AU)	0.174	0.043	0.162	0.015
HFnu (AU)	-0.048	-0.033	0.168	0.040

Baseline HRV indices were calculated at t=0, just prior to LPS administration. Cytokine response is defined as the Area Under Curve (expressed in pg/mL.hr) of plasma cytokine concentrations over time. ms indicates milliseconds. AU indicates arbitrary units. n=40 subjects. p-values are not reported because none of the listed correlations were statistically significant.

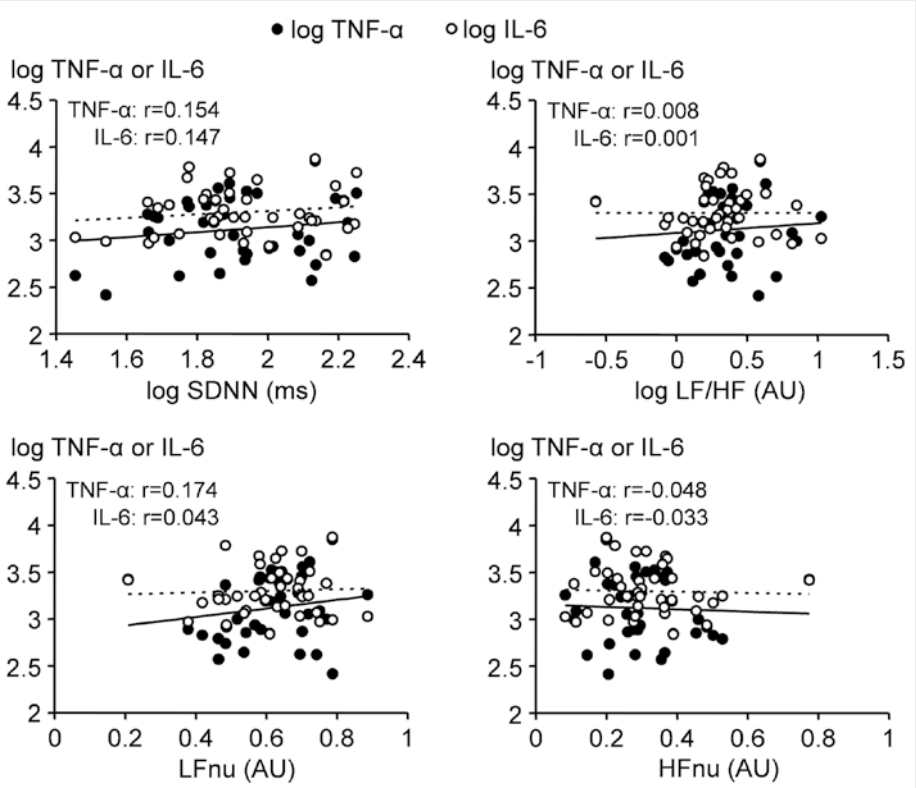

Figure 2 *Scatterplots of the association between basal HRV indices (calculated at t=0, just prior to LPS administration) and the Area Under Curve of the LPS-induced proinflammatory cytokine response (TNF-α and IL-6, expressed in pg/mL.hr) of 40 subjects. ms indicates milliseconds; AU indicates arbitrary units. Solid and dashed lines represent TNF-α and IL-6 regression lines, respectively. Pearson correlation coefficients (none statistically significant) are indicated in the plots.*

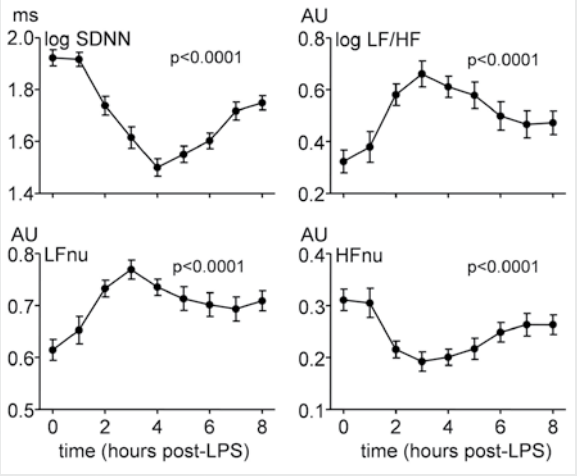

Figure 3
HRV indices during human endotoxemia. Data are expressed as mean ± SEM of 40 subjects. ms indicates milliseconds; AU indicates arbitrary units. p-values were calculated by one-way ANOVA.

Table 3 *Correlations between LPS-induced cytokine response and alterations in HRV indices.*

HRV measure	Correlation coefficient (Pearson *r*)								
	log SDNN (ms)	*log r-MSSD (ms)*	*pNN50 (%)*	*log LF (ms^2)*	*log HF (ms^2)*	*log total (ms^2)*	*log LF/HF (AU)*	*LFnu (AU)*	*HFnu (AU)*
log TNF-α	0.011	-0.082	-0.019	-0.020	-0.029	0.108	-0.106	-0.167	0.053
log IL-6	-0.118	-0.137	-0.054	-0.099	-0.157	-0.051	0.040	0.010	-0.017
log IL-10	0.110	0.140	0.270	0.050	0.133	0.128	-0.187	-0.203	0.194
log IL-1RA	0.235	0.184	0.066	0.324*	0.225	0.218	0.183	0.104	-0.173

*Cytokine response is defined as the Area Under Curve (expressed in pg/mL.hr) of plasma cytokine concentrations over time. LPS-induced HRV alterations were defined as the slope of increase/decrease of the interval between baseline (t=0, just prior to LPS administration) and the LPS-induced maximum/minimum value. LPS administration resulted in a decrease in SDNN, r-MSSD, pNN50%, LF, HF, total (minimum values at t=4) and HFnu (minimum value at t=3). LPS administration resulted in an increase in LF/HF and LFnu (maximum values at t=3). ms indicates milliseconds. AU indicates arbitrary units. n=40 subjects. * indicates p=0.045.*

LPS-induced HRV alterations in subjects who received LPS twice

The lack of correlation between the magnitude of the inflammatory response and HRV alterations was strengthened by HRV data obtained from subjects who received LPS twice with an interval of approximately two weeks. During the second visit, median peak levels of TNF-α, IL-6, IL-10 and IL-1RA were 43% (p=0.0024), 41% (p=0.0024), 54% (p=0.0038) and 8% (p=0.11) lower compared with the first visit 3(Figure 4, upper left panel) due to the development of endotoxin tolerance[23]. This phenomenon allowed us to investigate the relation between the inflammatory response and HRV within the same subjects. Despite the remarkably attenuated inflammatory response, LPS-induced HRV alterations were not different between both visits. The time-courses of SDNN, LFnu, and HFnu during both endotoxemia visits are shown in Figure 4. Other changes in HRV indices were equally similar between both visits (Supplementary Figure 2).

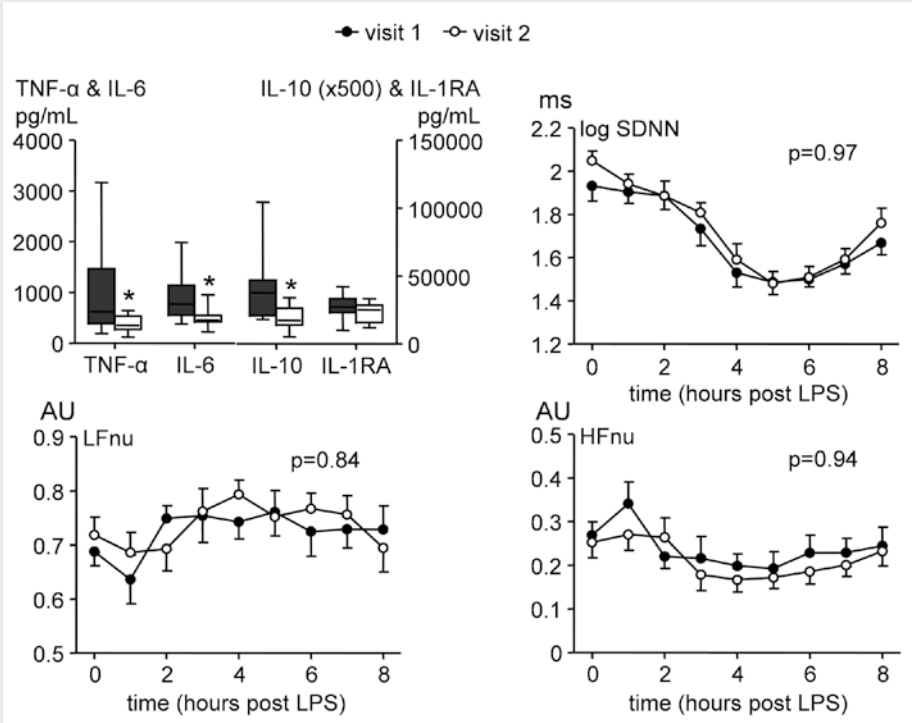

Figure 4 *Peak cytokine levels (upper left panel) and HRV indices (other panels) during human endotoxemia of subjects who received LPS on two separate occasions (visit 1 and visit 2) with an interval of approximately two weeks. Cytokine data are expressed as box and whiskers (whiskers indicating range), HRV indices are expressed as mean ± SEM. n= 12 subjects. ms indicates milliseconds; AU indicates arbitrary units. p-values of peak cytokine levels were calculated by Mann Whitney U test. p-values of HRV indices were calculated by two-way ANOVA (interaction term). * indicates p<0.05.*

Discussion

In the present study, we investigated the interplay between the LPS-induced acute inflammatory response and ANS outflow to the heart, determined by HRV in a large group of healthy male subjects who participated in human endotoxemia experiments. We did not find any correlations between basal HRV indices and the inflammatory response or between the magnitude of the inflammatory response and HRV alterations.

We first set out to investigate the relation between baseline HRV and the inflammatory response. None of the baseline HRV indices, determined before LPS administration, correlated with the magnitude of the LPS-induced inflammatory response in our

study. In contrast, weak (r-values of 0.4 to 0.43) but significant correlations between baseline HRV indices and the LPS-induced inflammatory response were recently reported using a similar experimental protocol, resulting in HRV changes identical to our study[22]. In our study, all indices of HRV except LFnu, HFnu, and pNN50 displayed a markedly skewed distribution, as did the peak and AUC cytokine levels. This is consistent with other HRV studies[24-27]. Therefore, we log transformed the skewed variables, which probably explains the contrasting conclusions between the two studies. In addition, considering the well-established anti-inflammatory effects of vagus nerve stimulation[8, 28, 29], there is no plausible mechanism to explain why the reported higher levels of vagal HRV indices are correlated with elevated TNF-α levels[22]. Especially TNF-α has been extensively studied in the context of the cholinergic anti-inflammatory pathway and is greatly reduced by vagus nerve stimulation or cholinergic agonists in animals and *in vitro*[8, 29-33]. The results from our prospective study in a relatively large group using a standardized model of inflammation suggest that there is no relation between basal cardiac ANS activity and the inflammatory response. A possible explanation is that vagus nerve innervation of the heart does not reflect outflow to other organs that play an important role in the inflammatory response. For example, a key effector organ of the cholinergic anti-inflammatory pathway is the spleen, one of the major cytokine-producing organs[34, 35]. As basal vagal input to the spleen may be different from vagal input to the heart, HRV might not be an appropriate method to assess activation of the cholinergic anti-inflammatory pathway. However, because no other measures of ANS activity were determined in this study, we can not provide a definitive answer to this question. Moreover, although we did not find correlations between HRV and plasma cytokines, it is conceivable that cardiac ANS activity is related to other measures of inflammation, such as immune cell subsets.

To further characterize the interplay between the inflammatory response and HRV, we examined the effects of inflammation on cardiac ANS outflow by correlating the magnitude of the LPS-induced inflammatory response to the magnitude of HRV alterations. Again, we could not demonstrate a relation between these parameters. The most compelling evidence for a lack of this relation was observed in subjects who received LPS on two occasions with an interval of two weeks. Whereas inflammatory cytokine levels were roughly halved during the second LPS administration due to the development of LPS tolerance, changes in HRV were identical to the first endotoxemia experiment, indicating that although the HRV alterations are associated with systemic inflammation, the magnitude of their change is not a measure of the extent of the

inflammatory response. Our findings are corroborated by a recent human endotoxemia study investigating the effects of hydrocortisone on the inflammatory response[21]. Hydrocortisone treatment greatly reduced LPS-induced inflammatory cytokine levels, but did not influence the LPS-induced decrease in HRV. To our knowledge there is only one human study demonstrating effects of immunomodulation on HRV. In this study, epinephrine attenuated the inflammatory response associated with further reduced vagal HRV indices after LPS administration[2]. This is likely due to epinephrine's sympathomimetic properties rather than through an immunomodulatory effect, especially because attenuation of the inflammatory response is anticipated to result in less reduction of HRV indices. Taken together, these findings indicate that changes in cardiac ANS outflow determined by HRV alterations do not reflect the magnitude of the inflammatory response in a controlled model of systemic inflammation, which is not confounded by factors such as sedatives, inotropes, and pressors often present in the clinical situation. In contrast, in septic patients, rather strong inverse correlations between log IL-6 levels and log LF (r=-0.76) and log HF (r=-0.53) power were found, suggesting that HRV does reflect the extent of inflammation in these patients[36]. Possibly other inflammation-associated factors that are not induced during the milder human endotoxemia model account for the observed changes in HRV in septic patients, but at present, this discrepancy cannot be explained.

With regard to the validity of our data, the LPS-induced changes in HRV observed in our study are analogous to those observed in similar human endotoxemia experiments[2, 10, 21, 22]. All 'raw' time-domain and frequency-domain indices were severely depressed after LPS administration, with minimum values observed at 4 hours post-LPS. In this dynamic setting (where total power gradually decreases in time), normalized HRV indices and the LF/HF ratio probably provide more meaningful information about the relative contributions of the two branches of the ANS[11]. Both LFnu and LF/HF are regarded to reflect sympathetic activity while HFnu corresponds to parasympathetic activity[10, 11]. After LPS administration, both LFnu and LF/HF increased; by analogy, HFnu decreased. These findings indicate that LPS administration results in cardiac sympathetic predominance, probably as a result of the stress-response induced by LPS administration. In accordance, increased levels of the stress-hormone cortisol and catecholamines have been reported during human endotoxemia[21, 37, 38] and interestingly, peak levels of cortisol and epinephrine were observed at 3-4 hours after LPS administration, coinciding with maximum/minimum levels of HRV indices[21, 37, 38]. Future studies are warranted to evaluate the relationship between cortisol/catecholamines and HRV after LPS administration. As opposed to

the cardiac sympathetic predominance, muscle sympathetic nerve activity (measured in the peroneal nerve) was found to be suppressed after LPS administration[37]. These findings strengthen the notion that autonomic outflow can not be regarded as a general response, but appears to be organ-specific.

In conclusion, we demonstrate that there is no relation between HRV and the inflammatory response and vice versa in a standardized model of systemic inflammation. This suggests that cardiac ANS activity may not be representative for ANS outflow to other organs involved in the inflammatory response and indicates that changes in HRV are not a reliable surrogate measure for the extent of the inflammatory response.

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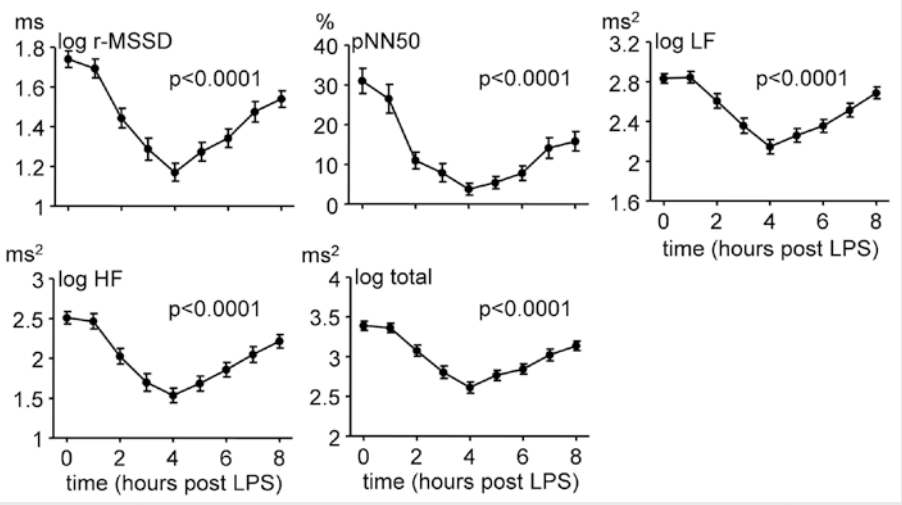
Supplementary material

Supplementary table 1 *Correlations between baseline HRV indices and the LPS-induced peak cytokine levels.*

HRV measure	*Correlation coefficient (Pearson r)*			
	log TNF-a	*log IL-6*	*log IL-10*	*log IL-1RA*
log SDNN (ms)	0.149	0.140	-0.128	0.033
log r-MSSD (ms)	0.108	0.091	-0.224	-0.032
pNN50 (%)	-0.005	-0.024	-0.266	-0.040
log LF (ms^2)	0.063	-0.002	-0.244	-0.089
log HF (ms^2)	0.019	0.003	-0.244	-0.013
log total (ms^2)	0.062	0.000	-0.234	0.017
log LF/HF (AU)	0.034	-0.008	0.166	-0.072
LFnu (AU)	0.081	0.025	0.150	-0.022
HFnu (AU)	0.034	-0.017	-0.186	0.084

Baseline HRV indices were calculated at t=0, just prior to LPS administration. ms indicates milliseconds. AU indicates arbitrary units. n=40 subjects. p-values are not reported because none of the listed correlations were statistically significant.



Supplementary figure 1 *HRV indices during human endotoxemia. Data are expressed as mean ± SEM of 40 subjects. ms indicates milliseconds. p-values were calculated by one-way ANOVA.*

Supplementary table 2 *Correlations between LPS-induced peak cytokine levels and LPS-induced maximum/minimum levels of HRV indices.*

HRV measure	Correlation coefficient (Pearson *r*)								
	log SDNN (ms)	*Log r-MSSD (ms)*	*pNN50 (%)*	*log LF (ms^2)*	*log HF (ms^2)*	*log total (ms^2)*	*log LF/HF (AU)*	*LFnu (AU)*	*HFnu (AU)*
log TNF-α	0.062	-0.032	-0.053	0.011	-0.079	0.051	0.104	0.133	-0.126
log IL-6	0.123	0.109	0.014	0.122	0.032	0.116	0.120	0.136	-0.154
log IL-10	0.043	-0.102	-0.196	-0.095	-0.104	-0.063	-0.074	-0.099	0.118
log IL-1RA	0.306	0.244	0.161	0.297	0.215	0.252	0.105	0.117	-0.139

LPS administration resulted in a decrease in SDNN, r-MSSD, pNN50%, LF, HF, total (minimum values at t=4) and HFnu (minimum value at t=3). LPS administration resulted in an increase in LF/HF and LFnu (maximum values at t=3). ms indicates milliseconds. AU indicates arbitrary units. n=40 subjects. p-values are not reported because none of the listed correlations were statistically significant.

Supplementary table 3 *Correlations between LPS-induced cytokine response and LPS-induced maximum/minimum levels of HRV indices.*

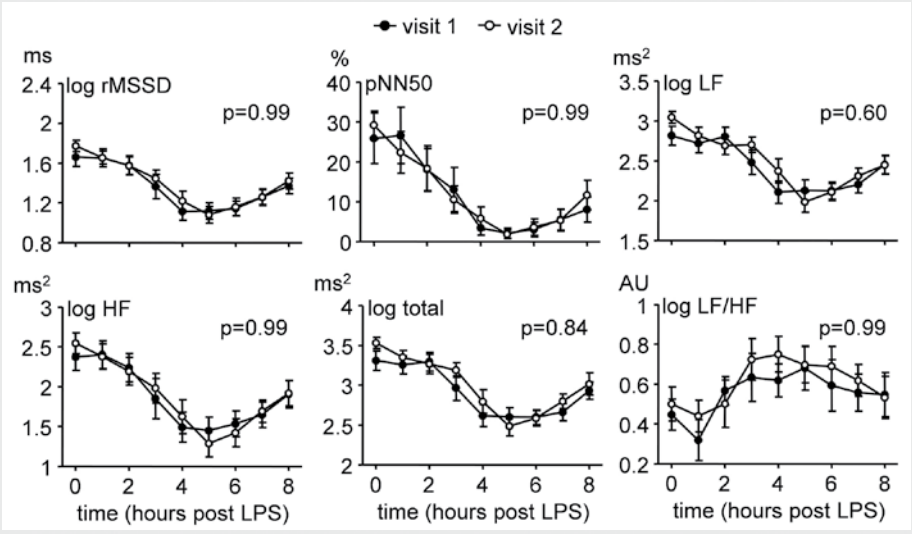
HRV measure	Correlation coefficient (Pearson *r*)								
	log SDNN (ms)	*log r-MSSD (ms)*	*pNN50 (%)*	*log LF (ms^2)*	*log HF (ms^2)*	*log total (ms^2)*	*log LF/HF (AU)*	*LFnu (AU)*	*HFnu (AU)*
log TNF-α	0.089	-0.016	-0.014	0.026	-0.065	0.096	0.028	0.006	-0.045
log IL-6	0.008	0.007	-0.010	-0.023	-0.101	-0.015	0.089	0.093	-0.127
log IL-10	-0.040	-0.140	-0.189	-0.140	-0.172	-0.128	0.019	-0.018	-0.007
log IL-1RA	0.261	0.211	0.138	0.268	0.184	0.230	0.175	0.183	-0.215

Cytokine response is defined as the Area Under Curve (expressed in pg/mL.hr) of plasma cytokine concentrations over time. LPS administration resulted in a decrease in SDNN, r-MSSD, pNN50%, LF, HF, total (minimum values at t=4) and HFnu (minimum value at t=3). LPS administration resulted in an increase in LF/HF and LFnu (maximum values at t=3). ms indicates milliseconds. AU indicates arbitrary units. n=40 subjects. p-values are not reported because none of the listed correlations were statistically significant.

Supplementary table 4 *Correlations between LPS-induced peak cytokine levels and alterations in HRV indices.*

HRV measure	Correlation coefficient (Pearson *r*)								
	log SDNN (ms)	*log r-MSSD (ms)*	*pNN50 (%)*	*log LF (ms^2)*	*log HF (ms^2)*	*log total (ms^2)*	*log LF/HF (AU)*	*LFnu (AU)*	*HFnu (AU)*
log TNF-α	-0.035	-0.144	-0.025	-0.043	-0.084	0.019	0.004	-0.019	-0.007
log IL-6	-0.019	-0.023	-0.005	0.059	-0.020	0.056	0.082	0.051	-0.057
log IL-10	0.184	0.187	0.281	0.104	0.204	0.170	-0.247	-0.222	0.280
log IL-1RA	0.242	0.209	0.038	0.333*	0.218	0.203	.0175	0.106	-0.181

*LPS-induced HRV alterations were defined as the slope of increase/decrease of the interval between baseline (t=0, just prior to LPS administration) and the LPS-induced maximum/minimum value. LPS administration resulted in a decrease in SDNN, r-MSSD, pNN50%, LF, HF, total (minimum values at t=4) and HFnu (minimum value at t=3). LPS administration resulted in an increase in LF/HF and LFnu (maximum values at t=3). ms indicates milliseconds. AU indicates arbitrary units. n=40 subjects. * indicates p=0.036.*



Supplementary figure 2 *HRV indices during human endotoxemia of subjects who received LPS on two separate occasions (visit 1 and visit 2) with an interval of approximately two weeks. Data are expressed as mean ± SEM. n= 12 subjects. ms indicates milliseconds; AU indicates arbitrary units. p-values were calculated by two-way ANOVA (interaction term).*

Part two

Limiting the innate immune response through the cholinergic anti-inflammatory pathway; *in vitro*, animal, and human studies

Chapter 5

Effects of vagus nerve stimulation and vagotomy in a two-hit model of ventilator-induced lung injury in rats

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Submitted

Abstract

Background Pulmonary inflammation contributes to ventilator-induced lung injury (VILI). Sepsis-induced pulmonary inflammation (first hit) may be potentiated by mechanical ventilation (MV, second hit). Electrical stimulation of the vagus nerve has been shown to attenuate inflammation in various animal models through the cholinergic anti-inflammatory pathway. We determined the effects of vagotomy (VGX) and vagus nerve stimulation (VNS) in a two-hit VILI model.

Methods Male Sprague-Dawley rats were i.v. administered lipopolysaccharide (LPS) and subsequently underwent VGX, VNS, or a sham operation. 1 hour following LPS, MV with low (8 mL/kg) or moderate (15 mL/kg) tidal volumes was initiated, or animals were left breathing spontaneously (SP). After 4 hours of MV or SP, rats were sacrificed. Cytokine and blood gas analysis was performed.

Results MV with 15, but not 8 mL/kg, potentiated the LPS-induced pulmonary proinflammatory cytokine response (TNF-α, IL-6, KC: $p<0.05$ compared with LPS-SP), but did not impair oxygenation. VGX enhanced the LPS-induced pulmonary proinflammatory cytokine response in spontaneously breathing, but not in MV animals (TNF-α, IL-6, KC: $p<0.05$ compared with sham-operated animals) and resulted in decreased pO_2 ($p<0.05$ compared with sham-operated animals). VNS did not affect any of the studied parameters in both SP and MV animals.

Conclusions MV with moderate tidal volumes potentiates the pulmonary inflammatory response elicited by systemic LPS administration. No beneficial effects of vagus nerve stimulation performed following LPS administration were found. These results questions the clinical applicability of stimulation of the cholinergic anti-inflammatory pathway in systemically inflamed patients admitted to the ICU where MV is initiated.

Introduction

Patients with severe sepsis and septic shock often suffer from respiratory failure, placing them in need of mechanical ventilation (MV). While this intervention is lifesaving, MV can lead to, or worsen lung injury; a condition called ventilator-induced lung injury (VILI)[1]. MV-induced cyclic stretch and/or overinflation elicits a pulmonary inflammatory response (biotrauma) characterized by cytokine production, neutrophil recruitment, and lung edema, resulting in impaired lung function[2, 3]. Patients with systemic inflammation, such as in sepsis, are at increased risk for developing VILI[4]. It is thought that the additional inflammatory insult, or second 'hit' induced by MV, synergizes with the underlying systemic inflammatory process, resulting in detrimental effects on the lungs and other organs (multiple-hit theory)[5, 6]. This cascade of events can ultimately lead to multiple organ dysfunction syndrome (MODS), associated with high mortality[7]. In clinical practice, treatment aimed to limit the initial overwhelming systemic proinflammatory state in sepsis has not proved very successful[8]. However, limiting the second inflammatory hit caused by MV may represent a viable therapy to reduce lung injury and subsequent multi-organ failure in systemically inflamed patients.

Recently, it was demonstrated that the vagus nerve can reflexively limit the innate immune response via binding of its neurotransmitter acetylcholine to the α7 nicotinic acetylcholine receptor (α7nAChR) present on macrophages[9, 10]. This mechanism was named the cholinergic anti-inflammatory pathway. Electrical stimulation of the vagus nerve has shown to attenuate inflammation and improve outcome in several animals models[11-13]. However, this pathway has been relatively sparsely studied in VILI. We have recently shown that pre-treatment with the selective α7nAChR agonist GTS-21 ameliorates VILI in mice[14]. In addition, a very recent investigation demonstrated that electrical vagus nerve stimulation attenuates VILI in a two-hit model (haemorrhagic shock followed by MV), whereas transection of the vagus nerve (vagotomy) resulted in worse outcome[15]. However, in these studies, stimulation or ablation of the cholinergic anti-inflammatory pathway was performed before the initial insult[14], or twice, before the first and second insult[15], thereby limiting its clinical applicability.

In the present study we investigated the effects of vagus nerve stimulation and vagotomy on inflammation and lung injury using a two-hit model of VILI in rats. We used lipopolysaccharide (LPS) administration as the first hit, and MV using low and moderate tidal volumes to model the sepsis patient that is admitted to the ICU, in a hyperinflammatory state, where MV is initiated. As mitigating the initial

proinflammatory hit is often not possible in daily practice, we applied vagus nerve stimulation or vagotomy after LPS administration and before the start of mechanical ventilation.

Materials and methods

Reagents

Lipopolysaccharide (LPS, derived from *E. coli*, serotype 0111:B4) was obtained from Sigma-Aldrich (St Louis, MO, USA) and was dissolved in 0.9% NaCl. The LPS solution was sonicated for a minimum of 30 minutes prior to use. Alfaxalone was purchased from Vétoquinol (Buckingham, UK).

Animals

All procedures described were in accordance with the requirements of the Dutch Experiments on Animals Act, the EC Directive 86/609 and approved by the Animal Ethics Committee of the Radboud University Nijmegen Medical Center. Male Sprague-Dawley rats (Charles River, Sutzfield, Germany) weighing 300-450 gram were used in all experiments.

Experimental protocol

The tail vein was cannulated for anaesthesia induction and maintenance. Anaesthesia was induced by infusion of alfaxalone (15 mg/kg) and maintained by continuous infusion of 33 mg/kg/hour alfaxalone throughout the experiment. The right femoral artery was cannulated to allow continuous blood pressure monitoring, Ringer's lactate administration (5 mL/kg/hour, containing 4 IE/mL heparin) and blood sampling. LPS (10 mg/kg) or placebo (0.9% NaCl) was administered via the tail vein (time point T=-1). Next, a cervical midline incision was made and the left vagus nerve was exposed. In sham-animals (SHAM), the wound was covered with moist gauze for 10 minutes. In vagus nerve stimulation animals (VNS), the vagus nerve was prepared free from the carotid artery and, without transecting the nerve, placed on a bipolar platinum electrode (Plastics One, Roanoke, VA, USA) connected to a Grass S88 stimulator with a SIU5 stimulus isolation unit. The nerve was stimulated for 3 minutes at 5 V, 5 Hz, 2 ms[16, 17]. In vagotomy animals (VGX), both the left and right vagus nerves were transected (bilateral vagotomy). Subsequently, rats were tracheotomized and a tracheal cannula was inserted and fixed with ligatures. Animals were left spontaneously breathing using a nose cone supplying air with an FiO_2 of 40% until 1 hour after LPS/placebo administration, when mechanical ventilation (MV) was started (T=0). Animals were ventilated using a Ugo Basile UB7025 ventilator (Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany). Ventilation

parameters: tidal volume 8 or 15 mL/kg, frequency 75 (8 mL/kg) or 40 (15 mL/kg) breaths/min, PEEP 1 cmH_2O, FiO_2 40%. Spontaneously breathing (SP) animals were not connected to the ventilator, but continued breathing air with an FiO_2 of 40% using the nose cone. Rectal temperature was monitored throughout the experiment and was kept between 36.5° C and 37.5° C using a heating pad and blankets. All animals were sacrificed by exsanguination after 4 hours of mechanical ventilation/ spontaneous breathing (T=4). The experimental protocol is illustrated in Figure 1.

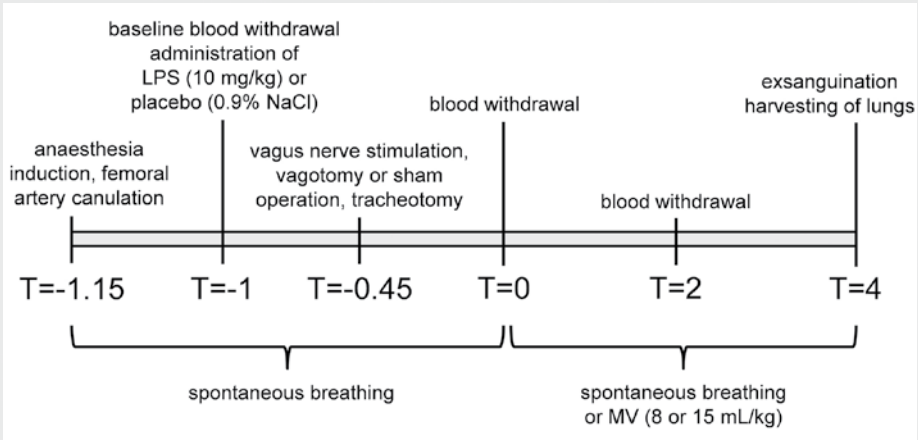

Figure 1 *Experimental procedures*

Blood gas and cytokine measurements

Blood gas parameters pH, pO_2, pCO_2, BE, HCO_3, and lactate were determined with an i-STAT Blood Gas Analyzer (Abbot, Hoofddorp, The Netherlands). EDTA anticoagulated blood was centrifuged for 5 minutes at 14000 rpm and plasma was stored at -80° C until analysis by simultaneous Luminex assay (Milliplex, Billerica, MA, USA), according to the manufacturer's instructions. Immediately after exsanguination, the lungs were carefully removed. The upper part of the left lung was snap-frozen in liquid nitrogen and stored at -80° C until analysis. Lungs were homogenized in T-PER (Thermo Fisher scientific, Rockford, IL, USA) supplemented with protease inhibitor (complete EDTA-free tablets, Roche, Woerden, The Netherlands) in M-Tubes using a GentleMACS dissociater (protein_01 program, Miltenyi Biotec, Utrecht, The Netherlands). M-tubes with homogenates were centrifuged (4000g, 5 min, 4 °C), after which the supernatant was transferred to an eppendorf tube and centrifuged again (14000g, 10 min, 4 °C). Cytokines in supernatants were measured by ELISA (Duoset, R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Calculations and statistical analysis

Data are expressed as mean±SEM. The Grubbs test (extreme studentized deviate method) was performed and significant outliers were excluded from analysis (maximum of one exclusion per dataset). Statistical differences between groups were detected by one-way ANOVA with Bonferroni post-hoc test. A p-value less than 0.05 was considered statistically significant. All tests were performed with Graphpad Prism 5 (Graphpad Software, La Jolla, USA).

Results

MV with 8 mL/kg does not elicit a pulmonary inflammatory response or amplify the LPS-induced inflammatory response

Compared with spontaneously breathing animals, MV with a tidal volume of 8 mL/kg did not result in an increase in pulmonary levels of the proinflammatory cytokine TNF-α or the anti-inflammatory cytokine IL-10 (Figure 2). LPS administration in spontaneously breathing animals significantly increased pulmonary cytokine levels. However, MV did not enhance the LPS–induced cytokine response (Figure 2). A similar pattern was observed for other proinflammatory cytokines (IL-6 and KC, data not shown).

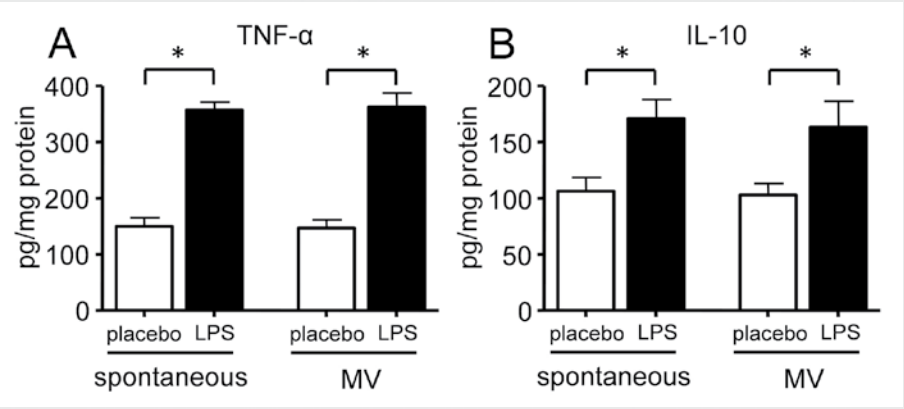

Figure 2 *Pulmonary concentrations of pro-(A: TNF-α) and anti-inflammatory (B: IL-10) cytokines in placebo- and LPS-treated spontaneously breathing and mechanically ventilated (MV) rats. Pulmonary cytokine levels are normalized to the total amount of protein in each lung homogenate. Data are represented as mean ± SEM of 8-9 animals per group. * indicates p<0.05, one-way ANOVA with Bonferroni post-hoc test.*

MV did not result in increased levels of plasma cytokines (Figure 3). As expected, LPS administration resulted in high plasma cytokine levels, however, similar to the

lung data, MV did not further increase or sustain cytokine levels. Again, this pattern was similar for IL-6 and KC (data not shown).

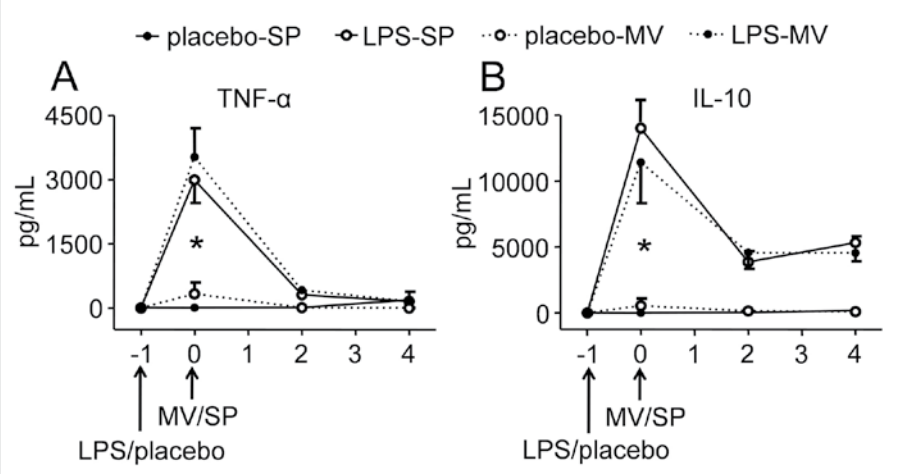

Figure 3 *Plasma concentrations of pro-(A: TNF-α) and anti-inflammatory (B: IL-10) cytokines in placebo- and LPS-treated spontaneously breathing (SP) and mechanically ventilated (MV) rats. Data are represented as mean ± SEM of 8-9 animals per group. * indicates p<0.05 between placebo- and LPS-treated groups (one-way ANOVA with Bonferroni post-hoc test). There were no differences between the placebo-SP and placebo-MV or the LPS-SP and LPS-MV groups.*

In placebo-treated animals, MV resulted in increased pH and decreased pCO_2 compared with spontaneously breathing animals (Table 1). LPS administration resulted in significantly lower arterial blood pressure compared with placebo in both spontaneously breathing and MV animals. Furthermore, the increase in pH observed upon ventilation in placebo-treated animals was not present in LPS-treated animals. In spontaneously breathing rats, LPS administration resulted in lower pCO_2 levels compared with placebo, probably as a result of inflammation-induced hyperventilation. No differences in oxygenation were observed between any of the groups.

MV with 15 mL/kg does not elicit an inflammatory response, but amplifies the LPS-induced pulmonary inflammatory response

Similar to MV with 8 mL/kg, MV with a tidal volume of 15 mL/kg did not result in pulmonary cytokine production (TNF-α shown in Figure 4A). LPS administration in spontaneously breathing animals significantly increased pulmonary cytokine levels, and MV significantly enhanced LPS–induced proinflammatory (TNF-α, IL-6, and KC; TNF-α shown in Figure 4A), but not anti-inflammatory (IL-10) pulmonary cytokine levels. MV with 15 mL/kg did not result in increased plasma cytokine levels (data not

shown). Similar to the data presented in Figure 3, LPS administration resulted in high plasma cytokine levels, however, MV did not affect plasma cytokine levels (data not shown).

Table 1 *Cardiorespiratory parameters in placebo and LPS-treated (10 mg/kg) rats ventilated with 8 ml/kg or left breathing spontaneously.*

Parameter	group	T=-1	T=0	T=2	T=4
Mean arterial pressure	placebo-SP	133 ± 2	98 ± 4 *	100 ± 8 *	115 ± 8 *
	LPS-SP	128 ± 3	71 ± 5	82 ± 4	73 ± 6
	placebo-MV	124 ± 5	97 ± 4	128 ± 7 *	125 ± 9 *
	LPS-MV	133 ± 3	81 ± 6	91 ± 3	89 ± 6
pH	placebo-SP	7.36 ± 0.01	7.31 ± 0.01	7.36 ± 0.02 *	7.40 ± 0.01 *
	LPS-SP	7.36 ± 0.01	7.33 ± 0.02	7.35 ± 0.02	7.40 ± 0.02
	placebo-MV	7.34 ± 0.02	7.29 ± 0.01	7.49 ± 0.01 *	7.49 ± 0.02 *
	LPS-MV	7.36 ± 0.01	7.31 ± 0.01	7.40 ± 0.01	7.40 ± 0.02
pCO_2	placebo-SP	59 ± 3	68 ± 3 *	64 ± 4 * *	57 ± 3 * *
	LPS-SP	59 ± 5	52 ± 3	51 ± 3	44 ± 4
	placebo-MV	62 ± 3	72 ± 4 *	38 ± 2	38 ± 1
	LPS-MV	58 ± 1	57 ± 2	43 ± 3	43 ± 4
pO_2	placebo-SP	149 ± 16	122 ± 10	113 ± 10	110 ± 9
	LPS-SP	142 ± 10	126 ± 12	117 ± 10	108 ± 8
	placebo-MV	161 ± 10	134 ± 7	126 ± 7	122 ± 6
	LPS-MV	139 ± 11	141 ± 9	130 ± 9	113 ± 5

*LPS/placebo administered at T=-1, MV started at T=0. Data are represented as mean ± SEM of 8-9 animals per group. * indicates $p<0.05$, one-way ANOVA with Bonferroni post-hoc test.*

At T=0 and T=2 (1 and 3 hours post-administration), both spontaneously breathing and ventilated rats treated with LPS displayed a significantly lower blood pressure compared with placebo-treated ventilated animals (Table 2). At T=4, blood pressure was restored in LPS-treated spontaneously breathing rats, but still significantly decreased in LPS-MV rats. pH was significantly lower and pCO_2 significantly higher in spontaneously breathing LPS-treated rats compared with the other groups. Compared with placebo-treated ventilated animals, oxygenation was significantly lower in LPS-treated spontaneously breathing animals. However, no difference in oxygenation between LPS-treated ventilated animals and placebo-treated ventilated animals was found.

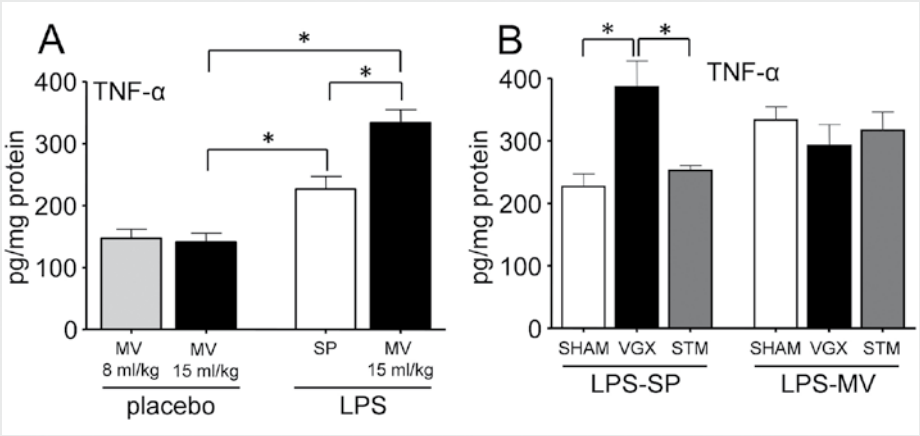

Figure 4 *A: Pulmonary concentrations of TNF-α in placebo- and LPS-treated spontaneously breathing and mechanically ventilated (MV, 8 or 15 mL/kg) rats. B: Pulmonary concentrations of TNF-α in LPS-treated spontaneously breathing and mechanically ventilated (MV, 15 mL/kg) rats that underwent a sham operation, bilateral vagotomy (VGX) or vagus nerve stimulation (STM). Pulmonary cytokine levels are normalized to the total amount of protein in each lung homogenate. Data are represented as mean ± SEM of 6-8 animals per group. * indicates p<0.05, one-way ANOVA with Bonferroni post-hoc test.*

Table 2 *Cardiorespiratory parameters in placebo and LPS-treated (10 mg/kg) sham-operated rats ventilated with 15 mL/kg or left breathing spontaneously.*

Parameter	group	T=-1	T=0	T=2	T=4
Mean arterial pressure	placebo-MV	96 ± 6	113 ± 5	128 ± 6	113 ± 5
	LPS-SP	102 ± 0	92 ± 6 *	102 ± 5 *	102 ± 5 *
	LPS-MV	102 ± 2	90 ± 4 *	92 ± 4 *	81 ± 6
pH	placebo-MV	7.35 ± 0.03	7.32 ± 0.03	7.58 ± 0.01	7.55 ± 0.02
	LPS-SP	7.37 ± 0.01	7.33 ± 0.02	7.34 ± 0.01 *	7.40 ± 0.02 *
	LPS-MV	7.34 ± 0.01	7.32 ± 0.01	7.54 ± 0.03 *	7.53 ± 0.03 *
pCO_2	placebo-MV	63 ± 5	68 ± 6	30 ± 1	32 ± 2
	LPS-SP	61 ± 3	60 ± 3	57 ± 3 *	51 ± 3 *
	LPS-MV	65 ± 3	58 ± 3	30 ± 8 *	30 ± 2 *
pO_2	placebo-MV	144 ± 22	139 ± 15	198 ± 3	199 ± 5
	LPS-SP	133 ± 17	164 ± 3	143 ± 4 *	135 ± 3 *
	LPS-MV	182 ± 20	162 ± 13	172 ± 21	166 ± 22

*LPS/placebo administered at T=-1, MV started at T=0. Data are represented as mean ± SEM of 6 animals per group. * indicates p<0.05, one-way ANOVA with Bonferroni post-hoc test.*

Vagotomy enhances pulmonary inflammation and impairs oxygenation in spontaneously breathing, but not in ventilated rats

Vagotomy resulted in significantly increased pulmonary levels of all proinflammatory cytokines in LPS-treated spontaneously breathing animals compared with sham operated or vagus nerve stimulated rats (TNF-α depicted in Figure 4B). IL-10 was not affected by vagotomy (data not shown). Furthermore, vagotomy exerted no effects in ventilated rats (Figure 4B). Plasma cytokine levels were not affected by vagotomy in spontaneously breathing or ventilated LPS-treated animals. In ventilated animals, no effects of either vagotomy or vagus nerve stimulation on blood pressure or blood gas parameters were found (data not shown). However, in spontaneously breathing rats, vagotomy resulted in significantly lower blood pressure at T=0, and lower pO_2 levels at T=0 and T=2 compared with sham-operated animals (Table 3). Vagus nerve stimulation affected neither pulmonary or plasma cytokine levels, nor blood gas parameters in both spontaneously breathing and ventilated (15 mL/kg) LPS-treated animals.

Table 3 *Cardiorespiratory parameters in placebo and LPS-treated (10 mg/kg) spontaneously breathing rats.*

Parameter	group	T=-1	T=0	T=2	T=4
Mean arterial pressure	SHAM	102 ± 0	92 ± 6 } *	102 ± 5	102 ± 5
	VGX	101 ± 6	74 ± 2 } *	103 ± 5	99 ± 8
	STM	96 ± 4	87 ± 5	99 ± 7	91 ± 9
pH	SHAM	7.37 ± 0.01	7.33 ± 0.02	7.34 ± 0.01	7.39 ± 0.02
	VGX	7.38 ± 0.03	7.33 ± 0.02	7.36 ± 0.02	7.38 ± 0.01
	STM	7.37 ± 0.02	7.32 ± 0.02	7.33 ± 0.03	7.40 ± 0.02
pCO_2	SHAM	61 ± 3	60 ± 3	57 ± 3	51 ± 3
	VGX	57 ± 6	54 ± 3	48 ± 1	48 ± 2
	STM	59 ± 4	59 ± 3	58 ± 4	49 ± 4
pO_2	SHAM	133 ± 17	164 ± 3 } *	143 ± 4 } *	135 ± 3
	VGX	124 ± 20	116 ± 13 } *	93 ± 11 } *	105 ± 4
	STM	124 ± 22	147 ± 14	128 ± 11	118 ± 13

*LPS/placebo administered at T=-1. VGX: vagotomy, STM: vagus nerve stimulation. Data are represented as mean ± SEM of 6 animals per group. * indicates $p<0.05$, one-way ANOVA with Bonferroni post-hoc test.*

Discussion

In this study we show that mechanical ventilation with moderate, but not with low tidal volumes potentiates the pulmonary inflammatory response elicited by systemic LPS administration. Vagotomy applied after LPS administration resulted in amplification of the LPS-induced pulmonary inflammatory response and reduced oxygenation in spontaneously breathing animals. However it did not affect inflammatory or respiratory parameters in ventilated rats. Vagus nerve stimulation performed after LPS administration had no effects in either ventilated or spontaneously breathing rats.

In the absence of LPS, MV with low (8 mL/kg) or moderate (15 mL/kg) tidal volumes did not result in a pulmonary or systemic inflammatory response. In contrast, we[14, 18] and others[19] have recently shown that 4-5 hours of MV with 8 mL/kg in mice results in increased levels of inflammatory cytokines in both lungs and plasma. It appears that rats are less susceptible to MV-induced pulmonary inflammation. For instance, 4-hour ventilation with 8 mL/kg did not result in increased levels of pulmonary cytokines or neutrophils[6]. Even MV with 24 mL/kg did not lead to increased wet/dry ratios or impaired oxygenation, while only moderate increases in pulmonary inflammation were found[6]. This probably accounts for the common use of very high tidal volumes (>30 mL/kg) in rat VILI models, which hinders interpretation with regard to the human situation[20-22]. These findings signify that there are large interspecies differences with regard to the inflammatory response to MV. With respect to humans, a recent study demonstrated that, in ARDS patients, lowering of tidal volumes from 6.3 to 4.2 mL/kg accompanied by extracorporeal carbon dioxide removal was associated with a significant decrease in inflammatory mediators in bronchoalveolar lavage fluid[23], suggesting that there is no clear cutoff value for safe tidal volumes in humans.

The second hit MV potentiated the first hit induced by systemic LPS administration with regard to pulmonary inflammation, but only when a moderately high tidal volume of 15 mL/kg was used. This tidal volume might still be clinically relevant in light of the partially collapsed or fluid-filled 'baby lungs'[24] observed in septic patients with lung injury, of which only a portion is ventilated[15]. We chose to start MV one hour after LPS administration. At this time point, high plasma concentrations of TNF-α, an important cytokine in the pathophysiology of VILI[25], are found. Using this approach, we attempted to simulate a patient with systemic hyperinflammation admitted to the ICU for initiation of mechanical ventilation. While we did find increased levels of all proinflammatory cytokines in lungs of LPS-treated ventilated animals compared with LPS-treated spontaneously breathing rats, we could not

demonstrate a statistically significant impaired gas exchange. In this respect, our two-hit model does not represent a true lung injury model. The direct interaction of systemic LPS administration and MV has been sparsely studied. The most commonly used two-hit models employ intratracheal LPS administration or haemorrhagic shock followed by MV[15, 26-28]. Both of these treatments are expected to have a more severe effect on lung function compared with systemic LPS administration, which might explain the lack of an effect on gas exchange in our study. In a rat study where LPS was also i.v. administered one hour before the start of 4-hour MV, increased lung inflammation and injury was found in LPS-treated ventilated rats compared with non-ventilated non-LPS-treated rats[29]. Unfortunately, no spontaneously breathing LPS-treated group or placebo-treated MV group was studied for comparison[29]. Two similar studies in rabbits have investigated direct interactions between systemic LPS and MV using moderate tidal volumes (10-15 mL/kg)[30, 31]. However, in these studies, LPS was administered 2 hours after the start of a 6-8 hour MV period. Nevertheless, the authors did find impaired oxygenation, altered histopathological lung alterations, and reduced compliance in LPS-treated MV animals compared with a spontaneously breathing LPS-treated group or placebo-treated MV group. In line with our results, these studies also demonstrated that MV potentiates the pulmonary, but not systemic, inflammatory response to i.v. LPS administration[30, 31].

Our result partly corroborate previously described findings of increased pulmonary inflammation after vagotomy[15]. In spontaneously breathing rats, vagotomy enhanced LPS-induced pulmonary inflammatory cytokine levels and was associated with impaired oxygenation. However, no effects were observed in LPS-treated MV rats. We do not have a clear explanation for this discrepancy but it could be speculated that the MV-induced potentiation of the pulmonary inflammatory response elicited by LPS somehow overrides the vagotomy-associated amplification of the inflammatory response. Furthermore, plasma cytokine levels were not affected by vagotomy in any of the groups. Several other groups have reported elevated plasma cytokine levels in vagotomized rats[11, 16, 32, 33]. This disparity could be due to timing, because in the other studies, vagotomy was performed prior to the inflammatory insult while we transected the vagus nerve in between two inflammatory 'hits', representing a more clinically relevant time point.

It was recently demonstrated that vagus nerve stimulation attenuates the pulmonary inflammatory response and improves oxygenation in mice ventilated for 3 hours with injurious tidal volumes (20 mL/kg)[15]. Similar effects were found in rats that were

subjected to haemorrhagic shock and subsequent MV with 12 mL/kg for 4 hours[15]. In contrast, we found no effects of vagus nerve stimulation on any of the studied parameters. This could be due to a number of factors. First, we used a different first hit, namely LPS administration compared with haemorrhagic shock. Second, one might argue that we have stimulated the nerve for a too short period (3 minutes). This appears not to be a plausible explanation however, as in mice, the anti-inflammatory effects of vagus nerve stimulation were equal whether it was performed for 0.5, 2 or 20 minutes[17]. Finally and most likely, the lack of an effect could be due to the timing of the intervention. In the aforementioned study, vagus nerve stimulation was performed prior to the induction of haemorrhagic shock, hence before the 'first hit'[15]. We stimulated the vagus nerve following the first hit but before initiation of MV; in our view a more clinically relevant time point. The inflammatory cascade induced by LPS administration might have been too far advanced for vagus nerve stimulation to limit it. However, this does not explain why stimulation did not attenuate the MV-induced increase in pulmonary inflammation elicited by LPS. Nevertheless, almost all studies that reported beneficial results of vagus nerve stimulation on inflammation have performed stimulation before or simultaneous to the inflammatory stimulus/insult[10-13, 15-17, 34-36]. Only two investigations assessed delayed stimulation of the cholinergic anti-inflammatory pathway, and they have yielded conflicting results[37, 38]. Both studies used a murine cecal ligation and puncture (CLP) model, which is widely used to study polymicrobial sepsis. The acetylcholinesterase inhibitor physostigmine, which reinforces the cholinergic anti-inflammatory pathway by increasing ACh availability, attenuated inflammation and septic shock when administered before CLP induction, but not when administered 6 hours afterwards[37]. In contrast, another study reported beneficial effects of administration of the α7nAChR agonist nicotine 24 hours after induction of CLP[38].

In conclusion, this study demonstrates no beneficial effects of vagus nerve stimulation in a two-hit model in rats, where stimulation was performed at a clinically relevant time point: following the initial hit but before initiation of MV. This questions the clinical applicability of stimulation of the cholinergic anti-inflammatory pathway in systemically inflamed patients admitted to the ICU where MV is initiated.

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Chapter 6

GTS-21 inhibits proinflammatory cytokine release independent of the Toll-like receptor stimulated via a transcriptional mechanism involving JAK2 activation

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Abstract

Background The vagus nerve can limit inflammation via the α7 nicotinic acetylcholine receptor (α7nAChR). Selective pharmacological stimulation of the α7nAChR may have therapeutic potential for the treatment of inflammatory conditions.

Methods We determined the anti-inflammatory potential of GTS-21, an α7nAChR-selective partial agonist, on primary human leukocytes and compared it with nicotine, the nAChR agonist widely used for research into the anti-inflammatory effects of α7nAChR stimulation. Furthermore, we investigated whether the effects of both nicotinic agonists were restricted to specific Toll-like receptors (TLRs) stimulated and explored the mechanism behind the anti-inflammatory effect of GTS-21.

Results GTS-21 and nicotine inhibited the release of proinflammatory cytokines in peripheral blood mononuclear cells (PBMCs), monocytes, and whole blood independent of the TLR stimulated, with higher potency/efficacy for GTS-21 compared with nicotine. The anti-inflammatory cytokine IL-10 was relatively unaffected by both nicotinic agonists. The effects of GTS-21 and nicotine could not be reversed by nAChR antagonists, while the JAK2 inhibitor AG490 abolished the anti-inflammatory effects. GTS-21 downregulated monocyte cell-surface expression of TLR2, TLR4, and CD14. qPCR analysis demonstrated that the anti-inflammatory effect of GTS-21 is mediated at the transcriptional level and involves JAK2-STAT3 activation.

Conclusions GTS-21 has a profound anti-inflammatory effect in human leukocytes and GTS-21 is more potent/efficacious than nicotine. The absence of a blocking effect of nAChR antagonists in human leukocytes might indicate different pharmacological properties of the α7nAChR in human leukocytes compared with other cell types. GTS-21 may be promising from a therapeutic perspective because of its suitability for human use.

Introduction

In the past few years, a novel link between the vagus nerve and the inflammatory response has been established. In addition to 'sensing' focal inflammation in the periphery and relaying it to the brain via afferent fibers[1-3], recent work has demonstrated that the efferent vagus nerve can modulate the inflammatory response in a reflex-like fashion, termed 'the cholinergic anti-inflammatory pathway'[4]. It has become clear that the α7 nicotinic acetylcholine receptor (α7nAChR), expressed in various cell types including human leukocytes[5], is an essential regulator of this anti-inflammatory effect of the vagus nerve[6]. Consequently, more specific agonists of this receptor were identified or developed and used in various studies[7-9]. To date, one of the most effective α7-selective partial agonists for modulating inflammatory responses is 3-(2,4-dimethoxybenzylidene)-anabaseine (GTS-21) which has proven to be effective in attenuating the immune response and improving outcome in animal models of pancreatitis[9], endotoxemia[7, 8], sepsis[7], acute lung injury[10, 11], and ischemia-reperfusion injury[12]. However, its anti-inflammatory potential in human inflammatory cells has never been evaluated. This is of particular interest because GTS-21, which has been primarily developed for the treatment of Alzheimer's disease, has been administered to human volunteers and patients, and is well tolerated without clinically significant safety findings[13]. Therefore, GTS-21 may have therapeutic potential for the treatment of inflammatory conditions and is preferred above nicotine which lacks pharmacologic specificity and has toxic side effects and the potential to produce physical dependence (addiction).

Although comparisons between GTS-21 and non-selective nAChR agonists have been reported at α7nAChR receptor activation level in electrophysiological studies using heterologous expression in *Xenopus laevis* oocytes[14, 15], the immunomodulating effects of selective and non-selective agonists of the α7nAChR have not been compared. Furthermore, *in vitro* studies investigating the cholinergic anti-inflammatory pathway almost exclusively used the principal Toll-like receptor 4 (TLR4) agonist LPS as a trigger for inflammation. It is unknown whether the cholinergic anti-inflammatory pathway is restricted to certain TLRs stimulated or is a general mechanism not constrained to a specific stimulus. Finally, the mechanism by which α7nAChR stimulation attenuates proinflammatory cytokine production has not been fully elucidated and sparsely studied in human cells. A role for the JAK2-STAT3 pathway[16-19] as well as suppression of NFκB transcriptional activity [20, 21] is implied. However, the cholinergic anti-inflammatory pathway is believed to be regulated at a posttranscriptional level[4, 16]. Mechanistic studies regarding the anti-inflammatory effect of GTS-21 are limited to one study reporting decreased NFκB activity in a murine cell line[7].

In this study, we investigated for the first time the anti-inflammatory potential of GTS-21 on primary human leukocytes and compared it with nicotine. Furthermore, we investigated whether the effects of both nicotinic agonists were restricted to specific TLRs stimulated and whether they affected cell-surface expression of receptors involved in the innate immune response. Finally, we studied whether the anti-inflammatory effects of GTS-21 and nicotine are regulated at the transcriptional level, and determined the involvement of the JAK-STAT signal transduction pathway.

Materials and methods

General reagents

RPMI culture medium (RPMI 1640 Dutch modification, ICN Biomedicals; Costa Mesa, CA, USA) was supplemented with gentamicin 10 μg/mL, L-glutamine 10 mM, and pyruvate 10 mM. GTS-21 was obtained from the University of Florida (a kind gift of Prof. Dr. Roger L. Papke) and from Comentis inc. (South San Francisco, CA, USA). No differences in potency/efficacy between GTS-21 from the University of Florida and from Comentis inc. were observed (data not shown). Nicotine (liquid, naturally occurring isomer), mecamylamine, α-bungarotoxin, methyllycaconitine (MLA), tyrphostin AG490, d-tubocurarine, and *Escherichia coli* lipopolysaccharide (LPS, serotype O55:B5) were obtained from Sigma-Aldrich (St Louis, MO, USA). LPS was further purified as described previously[22]. LPS concentration was 1 ng/mL in all experiments. Pam3Cys was purchased from EMC Micro-collections (Tübingen, Germany). Flagellin and polyI:C were obtained from InvivoGen (San Diego, CA, USA). All stimuli except AG490, which was dissolved in ethanol, were dissolved in RPMI.

Peripheral blood mononuclear cells, monocyte and whole blood stimulation

After obtaining informed consent, venous blood was drawn from the cubital vein of healthy non-smoking male volunteers into EDTA or lithium heparin tubes (Vacutainer System, BD Biosciences, Plymouth, UK). All volunteers refrained from caffeine-containing beverages/food for at least 12 hours before blood collection. Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA anticoagulated blood by density gradient centrifugation over Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden), washed three times in ice-cold sterile phosphate-buffered saline (B Braun Melsungen AG, Melsungen, Germany), and resuspended in RPMI 1640 culture medium supplemented with 10% autologous serum obtained by centrifugation (6000 rpm, 10 min) of lithium-heparin anticoagulated blood. Cells were counted in a Bürker hemocytometer and viability was assessed using trypan blue (viability

was >95%). The number of cells was adjusted to 2.5 x 10^6/mL and 5 x 10^5 cells per well (200 μL) were seeded in duplicate in 96-well flat bottom plates (Greiner, Alphen a/d Rijn, The Netherlands) and stimulated for 22 hours (37 °C, 95% O_2, 5% CO_2) with various compounds. Incubation with RPMI alone served as a negative control. Nicotine or GTS-21 were added 30 minutes before TLR agonists. Nicotinic antagonists or the JAK2 inhibitor AG-490 were added 30 minutes before nicotine or GTS-21.

Primary monocytes were obtained from PBMCs using magnetic bead negative depletion (Monocyte Isolation kit II and LS columns, Miltenyi Biotec, Utrecht, The Netherlands). This procedure yields untouched monocytes suitable for short-term stimulation experiments where magnetic beads attached to cell surface epitopes are unwanted. Monocyte purity was evaluated using flow cytometry and was 85-90%. 1 x 10^5 cells per well (200 μL) in duplicate were stimulated in the presence of 10% autologous serum for 22 hours as described above.

After stimulation, PBMC/monocyte well plates were centrifuged (1700 rpm, 8 min) and supernatants were stored at -80 °C until assayed.

For whole blood stimulation experiments, venous blood was drawn into 2 mL lithium-heparin containing vacutainers (Vacutainer System, BD Biosciences). Whole blood was diluted 1:5 in RPMI and stimulated for 24 hours as described above. After stimulation, whole blood cultures were centrifuged (14000 rpm, 5 min) after which supernatants were stored at -80 °C until assayed.

Cytokine measurements

Cytokines in supernatants of whole blood cultures were determined using a simultaneous Luminex Assay according to the manufacturer's instructions (Bio-plex cytokine assay, BioRad, Hercules, CA, USA). Cytokines in supernatants of PBMC and monocytes cultures were determined using enzyme-linked immunosorbent assays. TNF-α was determined by a specific ELISA using four antibodies as described previously[23]. IL-1β, IL-6, and IL-10 were measured by commercial ELISA kits (IL-1β: R&D systems, Minneapolis, MN, USA; IL-6 and IL-10: Pelikine Compact, Sanquin, Amsterdam, The Netherlands, according to the manufacturer's instructions).

Viability assays

Monocyte viability was assessed using the *in vitro* toxicology assay kit, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) based (Sigma-Aldrich). Monocytes were stimulated as described above (22 hours). After stimulation, plates were centrifuged (1700 rpm, 8 min), 100 μL of supernatant was stored at

-80 °C for cytokine analysis, and 10 µL of MTT stock solution (5 mg/mL) was added to the wells. Monocytes were incubated at 37 °C for an additional 4 hours after which formazan crystals were solubilized and absorbance at 570 nm was measured in a plate reader. Additionally, lactate dehydrogenase (LDH) was determined in supernatants of PBMCs stimulated for 22 hours as described above.

Flow cytometry

Flow cytometry was performed on PBMCs stimulated as described in section 2.2 (22 hours), After stimulation, plates were centrifuged (1700 rpm, 8 min), supernatants were stored at -80 °C for cytokine analysis and adherent cells were detached by adding 200 µL of ice-cold FACS buffer (PBS with 0.5 % BSA) and incubating the plate for 15 min on ice. Subsequently, cells were collected by vigorous resuspending and scraping of the bottom of the wells and washed twice in ice-cold FACS buffer. TLR2, TLR4, and CD14 expression was determined with the following directly conjugated antibodies: CD282 PE (mouse IgG2a, TLR 2.1 clone, eBioscience, San Diego, CA, USA), CD284 PE-Cy7 (TLR4, mouse IgG2a, HTA125 clone eBioscience), CD14 ECD (mouse IgG2a, RMO52 clone Immunotech, Beckman Coulter, Mijdrecht, The Netherlands). Expression of α7nAChR on monocytes was determined with a primary antibody against α7nAChR (rat IgG1 monoclonal, 319 clone, Abcam, Cambridge, UK) and CD14 ECD followed by a FITC labeled secondary antibody (donkey anti-rat FITC, Beckman Coulter). After antibody incubation, cells were washed with FACS buffer, resuspended, and analyzed on a Beckman Coulter FC500 flow cytometer. TLR2, TLR4, CD14, and α7nAChR expression were analyzed within CD14 positive monocytes.

Quantitative PCR and JAK-STAT signaling arrays

Untouched primary monocytes were isolated as described in section 2.2 and seeded in duplicate at a density of 1 x 10^6/well in 24-well plates in the presence and absence of LPS and nicotinic agonists. After 4 hours, plates were centrifuged (1700 rpm, 8 min) and supernatants were aspirated. Subsequently, RNA was isolated using RNeasy plus mini kits (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Per experiment, equal amounts of RNA (150-400 ng) were used for cDNA synthesis using iScript cDNA synthesis kits (Bio-Rad). Negative control reactions were performed in the absence of reverse transcriptase.

The sequences of the primer pairs used were:

TLR2	forward:	5'-GAATCCTCCAATCAGGCTTCTCT-3'
	reverse:	5'-GCCCTGAGGGAATGGAGTTTA-3
TLR4	forward:	5'-GGCATGCCTGTGCTGAGTT-3'
	reverse:	5'-CTGCTACAACAGATACTACAAGCACACT-3'
TNF-α	forward:	5'-TGGCCCAGGCAGTCAGA-3'
	reverse:	5'-GGTTTGCTACAACATGGGCTACA-3'
IL-1β	forward:	5'-CAGCTACGAATCTCCGACCAC-3'
	reverse:	5'-GGCAGGGAACCAGCATCTTC-3'
IL-6	forward:	5'-AATTCGGTACATCCTCGACGG-3'
	reverse:	5'-GGTTGTTTTCTGCCAGTGCCT-3'
IL-10	forward:	5'-CAACCTGCCTAACATGCTTCG-3'
	reverse:	5'-TCATCTCAGACAAGGCTTGGC-3'
CD14	forward:	5'-ACGCCAGAACCTTGTGAGC-3'
	reverse:	5'-GCATGGATCTCCACCTCTACTG-3'
α7nAChR	forward:	5'-AAACTCACAGATGGGCAAGG-3'
	reverse:	5'- AGGGAACACTGGAGTTGTGG-3'
B2M	forward:	5'-ATGAGTATGCCTGCCGTGTG-3'
	reverse:	5'-CCAAATGCGGCATCTTCAAAC-3'

Primers were obtained from Biolegio (Nijmegen, The Netherlands). The quantitative PCR (qPCR) reaction was performed using Power SybrGreen master mix (Applied Biosystems, Nieuwekerk a/d Ijssel, The Netherlands) on an ABI Prism 7300 Real Time PCR system (Applied Biosystems). Negative control reactions were cycled alongside test samples to ensure the absence of contaminating genomic DNA. The amplification of a single product was ensured by melt-curve analysis for each primer pair. A standard curve constructed from 1:5 serial dilutions of a mixture of cDNA of the samples in the same run was included for each primer pair in every run to perform relative quantification of mRNA expression. To investigate the involvement of the JAK-STAT signaling pathway we used RT^2 Profiler JAK-STAT qPCR arrays (PAHS-039, SABiosciences, Frederick, MD, USA) according to the manufacturer's instructions. For these experiments, isolated monocytes of three different donors were stimulated and mRNA was isolated as described above. Four housekeeping genes, hypoxanthine phosphoribosyltransferase (HPRT1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB) and β2 microglobulin (B2M) present on the PCR array were used for normalization.

Calculations

Data are expressed as mean±SEM except for whole blood stimulation data which are expressed as median±interquartile range as they were not normally distributed. Statistical significance in the dose-response curves of nicotine and GTS-21 was evaluated using repeated measures one-way analysis of variance with Bonferroni post-hoc test. Elsewhere, the paired student's t-test was used to test for statistical significance except for the whole blood data which was analyzed using the Wilcoxon matched pairs test and the qPCR JAK-STAT array data for which the analysis procedure is described below. A p-value below 0.05 (except for qPCR JAK-STAT array data, $p<0.01$) was considered statistically significant.

For whole blood experiments, % inhibition/stimulation was calculated as follows: 100x (TLR-agonist-induced cytokine release in the presence of GTS-21 or nicotine / TLR-agonist-induced cytokine release in the absence of GTS-21 or nicotine) – 100. If more than 50% of the subjects had a cytokine response in response to a TLR-agonist in the absence of a nicotinic agonist that was lower than 4 times the detection limit, no inhibition/stimulation calculations for this TLR-agonist-cytokine combination were performed.

In the experiments where nAChR antagonists were used, the % blocking effect of the nAChR antagonists was calculated by subtracting the % inhibition of the LPS-response by GTS-21 or nicotine in presence of the nAChR antagonist from the % inhibition of the LPS-response by GTS-21 or nicotine in the absence of the nAChR antagonist.

qPCR data was analyzed using ABI Prism software and calculations were performed as follows: per sample, the relative quantity of mRNA of the gene of interest (e.g. TNF-α) was divided by the relative quantity of the housekeeping gene B2M. To calculate fold induction compared with the control sample, we divided the TNF-α/B2M ratio by the TNF-α/B2M ratio of the unstimulated control (RPMI) sample. If fold induction was <1, it was represented as -(1/fold induction).

For the JAK-STAT qPCR array experiments each replicate cycle threshold (CT) was normalized to the average CT of four housekeeping genes on a per plate basis. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative quantification of gene expression in stimulated samples compared with RPMI samples. A gene was considered not detectable when CT >35. CT was defined as 35 for the $2^{-\Delta\Delta CT}$ calculation when the signal was below detectable limits. If for a specific gene both the stimulated and control sample expression was below detectable limits the sample was excluded from analysis. Genes were considered differentially expressed if mean up- or downregulation was equal or greater than 2-fold and $p<0.01$ (permutation test, 100 permutations).

Statistical calculations were performed using Graphpad Prism V4.03 (Graphpad software) except for the permutation tests in the JAK-STAT qPCR array experiments which were analyzed with Multi-experiment Viewer V4.3 (TM4 software suite).

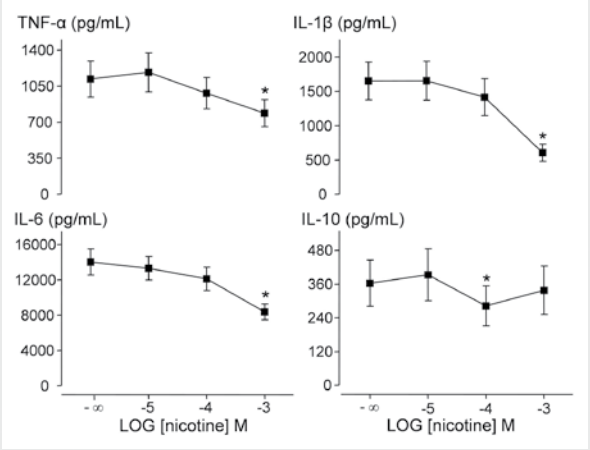
Results

Nicotine and GTS-21 dose-dependently inhibit LPS-induced cytokine production in PBMCs

To determine the effect of nAChR agonists on LPS-induced cytokine release we incubated human PBMCs with 1 ng/mL LPS in combination with nicotine or GTS-21 for 22 hours. The classic nAChR agonist nicotine dose-dependently inhibited production of the proinflammatory cytokines TNF- α, IL-1β, and IL-6 (Figure 1) with a maximum inhibition of 30±4%, 65±2%, and 36±5% respectively at the highest dose of nicotine used (1 mM). The anti-inflammatory cytokine IL-10 was relatively unaffected, with a small but significant inhibition at a submaximal concentration of nicotine (21±3% at 100 μM).

Figure 1

*Dose-response curves of the effect of nicotine on cytokine production in human PBMC stimulated with 1 ng/mL LPS for 22 hours. Data are represented as mean ± SEM (8 different donors, two independent experiments). * indicates p<0.05 compared with the LPS response in the absence of nicotine (repeated measures ANOVA with Bonferroni post-hoc test).*



The selective α7nAChR agonist GTS-21 also dose-dependently inhibited TNF-α and IL-1β (Figure 2), but was more potent and efficacious than nicotine with a maximum inhibition of 87±2% (IC_{50}: 8.9 μM) and 89±3% (IC_{50}:17.9 μM) respectively at the highest dose of GTS-21 used (100 μM). Production of IL-6 was not affected by GTS-21. IL-10 was only inhibited by the highest dose of GTS-21 used (42±20% at 100 μM) but not at 10 μM GTS-21, a dose which strongly inhibited release of TNF-α and IL-1β.

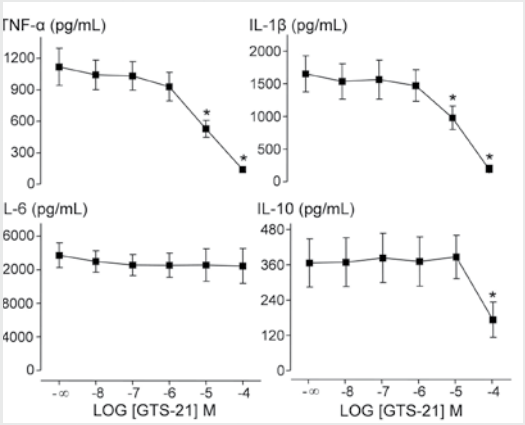


Figure 2
*Dose-response curves of the effect of GTS-21 on cytokine production in human PBMC stimulated with 1 ng/mL LPS for 22 hours. Data are represented as mean ± SEM (8 different donors, two independent experiments). * indicates $p<0.05$ compared with the LPS response in the absence of GTS-21 (repeated measures ANOVA with Bonferroni post-hoc test).*

Inhibition of proinflammatory cytokine production by nicotine and GTS-21 is mediated by monocytes and does not involve cell death

To investigate which cell type is involved in the inhibition of proinflammatory cytokine production by nAChR agonists we performed stimulation experiments on isolated primary human monocytes using the same experimental conditions as in PBMCs. GTS-21 and nicotine both significantly inhibited TNF-α production to a similar extent as in PBMCs with GTS-21 showing a distinctively more pronounced effect than nicotine (84±3% and 39±8% respectively, Figure 3A). To exclude the possibility that loss of viability or cell death was responsible for the observed inhibition of cytokine production we performed MTT viability assays on the stimulated monocytes which revealed no loss of cell viability with any of the stimuli used (Figure 3B). Moreover, lactate dehydogenase levels in supernatants of stimulated PBMCs were not elevated compared with control (RPMI) in any of the samples (n=4; RPMI 75.3±6.2 U/L, LPS 72.0±5.8 U/L, GTS-21 100 μM 57.5±4.1 U/L, nicotine 1 mM 53.5±4.9 U/L, LPS+GTS-21 100 μM 76.5±6.1 U/L, LPS+nicotine 1 mM 66.0±3.4 U/L).

Inhibition of proinflammatory cytokine production by nicotine and GTS-21 is not restricted to a specific TLR stimulated

Pattern recognition of the diverse classes of microbial products causing infection involves various TLRs that modulate the subsequent immune response and cytokine profiles[24]. To explore whether the anti-inflammatory effects of nicotine and GTS-21 are dependent on a specific TLR stimulated we incubated human whole blood cultures with TLR2 (Pam3Cys, 1 μg/mL), TLR3 (PolyI:C, 50 μg/mL), TLR4 (LPS, 1 ng/mL), and TLR5 (flagellin, 1 μg/mL) agonists in combination with nicotine and GTS-21. Incubation with TLR2, TLR4, and TLR5 agonists resulted in production of

pro- (TNF-α, IL-1β, IL-6, and IFN-γ) as well as anti-inflammatory (IL-10) cytokines (data not shown). The TLR3 agonist PolyI:C only evoked a robust IL-6 release while TNF-α and IFN-γ were secreted in very small quantities in whole blood cultures of some but not all of the subjects (and therefore were excluded from inhibition/ stimulation calculations). PolyI:C did not result in release of IL-1β and IL-10 in whole blood of any of the subjects.

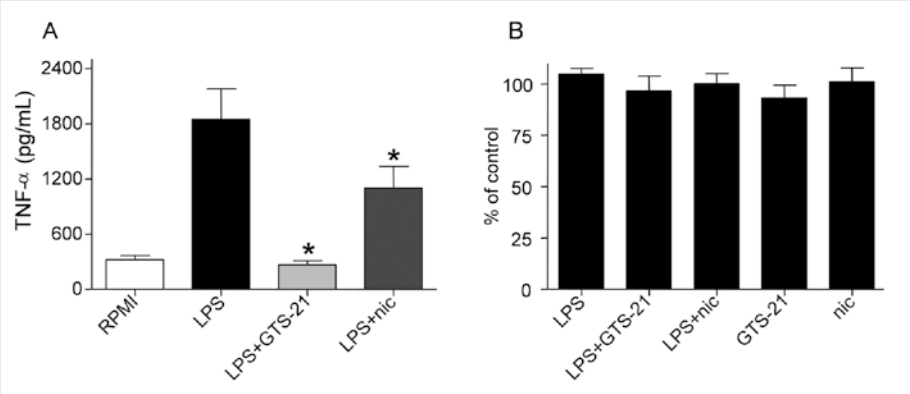


Figure 3 *A) Effects of nicotine and GTS-21 on TNF-α production in isolated primary human monocytes stimulated with LPS (1 ng/mL) for 22 hours. Both GTS-21(100 μM) and nicotine (1 mM) attenuate TNF-α production in isolated monocytes stimulated with LPS. Data are represented as mean ± SEM (6 different donors, two independent experiments). * indicates $p<0.05$ compared with the LPS alone (paired Student's t-test). B) MTT viability assay on isolated primary human monocytes stimulated with LPS, nicotine and GTS-21. Percentage viability was calculated relative to viability in the control (RPMI) samples. Data are represented as mean ± SEM (6 different donors, two independent experiments).*

Coincubation with 100 μM of nicotine inhibited TLR-agonist induced proinflammatory cytokine release in a mild fashion (data not shown) but 1 mM of nicotine inhibited cytokine release up to 100%, independent on the TLR stimulated (Figure 4, upper panel). The anti-inflammatory cytokine IL-10 was not significantly inhibited by either 100 μM or 1 mM of nicotine.

The selective α7nAChR agonist GTS-21 (100 μM) also significantly inhibited nearly all TLR-agonist induced proinflammatory cytokines (up to 95% inhibition, Figure 4, lower panel). As with nicotine, GTS-21 did not attenuate the anti-inflammatory cytokine IL-10 release to a great extent, only TLR5-induced IL-10 production was significantly inhibited.

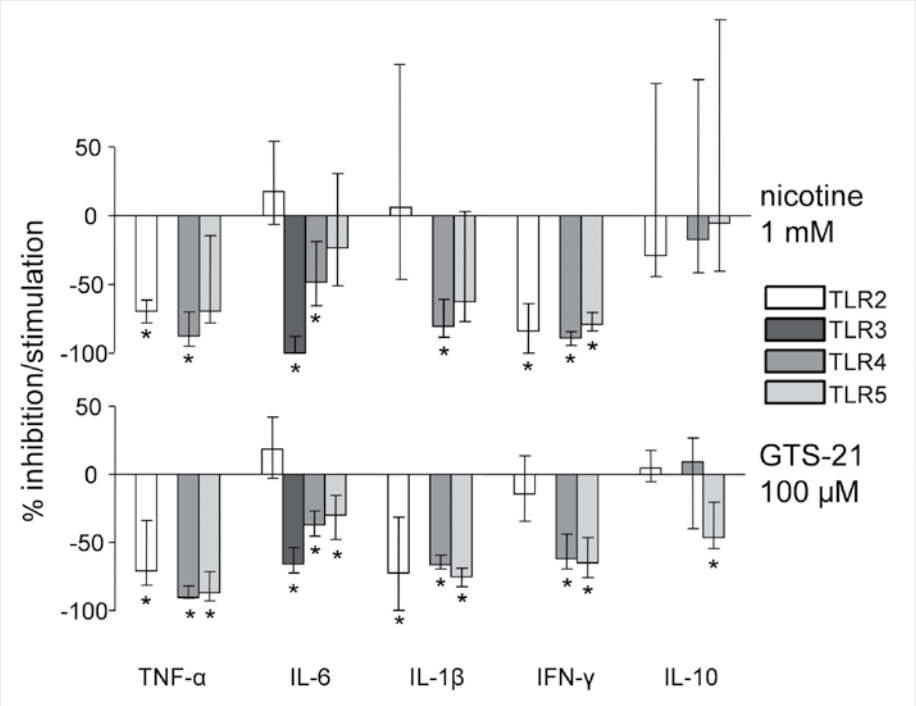


Figure 4 *Percentage inhibition/stimulation of TLR-agonist induced cytokine production in human 1:5 diluted whole blood by 1 mM nicotine and 100 µM GTS-21(24-hour stimulation). TLR agonists used were TLR1+2: Pam3Cys (1 µg/mL); TLR3: PolyI:C (50 µg/ml); TLR4: LPS (1 ng/mL); TLR5: flagellin (1 µg/mL). Data are represented as median and interquartile range (6 different donors). * indicates p<0.05, Wilcoxon Matched Pairs test. Concentrations of cytokines in absence of nicotine or GTS-21 after stimulation with TLR2, TLR3, TLR4 and TLR5 agonists in pg/mL (interquartile range) were; TNF-α: 154 (41-592), 6 (2-15), 3150 (2020-3927), 2054 (692-4309). IL-6: 1374 (482-4406), 89 (61-134), 6979 (4678-9309), 8341 (4864-11842). IL-1β: 19 (3-80), 0 (0-2), 1637 (922-1963), 1502 (740-2126). IFN-γ 106 (33- 349), 9 (0-27), 734 (521- 954), 802 (593-1072). IL-10: 43 (6-120), 0 (0-0), 76 (56-116), 221 (99-447).*

Nicotinic antagonists do not restore cytokine production

As the α7nAChR has been proposed as the receptor responsible for the anti-inflammatory effect of nicotinic agonists[6], we attempted to reverse the effects of nicotine and GTS-21 with nAChR antagonists. However, coincubation with both α7nAChR-specific antagonists α-bungarotoxin (10 nM – 1 µM) and methyllycaconitine (10 µM) as well as non-specific nAChR antagonists mecamylamine (10 nM-100 µM) and d-tubocurarine (10-100 µM) could not reverse the anti-inflammatory effects of any of the concentrations of nicotine and GTS-21. Inhibition of the GTS-21 (100

μM) induced attenuation of the cytokine response by the highest concentration of the nAChR blockers was: α-bungarotoxin 1.9 ± 1.3% (n=6), methyllycaconitine -2.9 ± 1.0% (n=4), mecamylamine -1.8 ± 3.2% (n=6), d-tubocurarine 2.8 ± 2.8% (n=4). % inhibition of the nicotine (1 mM) induced cytokine attenuation by the highest concentration of the various nAChR blockers was: α-bungarotoxin 5.7 ± 3.4% (n=6), methyllycaconitine 0.6 ± 6.9% (n=4), mecamylamine 2.3 ± 4.1% (n=6), d-tubocurarine -10.3 ± 8.1% (n=4). Coincubation of other concentrations of GTS-21/nicotine with the various concentrations of the nAChR blockers mentioned above was also ineffective in blocking anti-inflammatory effects (data not shown).

GTS-21 downregulates cell surface receptors on monocytes

To obtain more insight into the mechanisms by which nicotine and GTS-21 exhibit their anti-inflammatory effects, we stimulated human PBMCs with LPS in combination with nicotine and GTS-21 and determined cell surface expression of TLR2, TLR4, α7nAChR, and CD14 on the monocyte fraction by flow cytometry. We chose the 22-hour incubation based on similar experiments where LPS induced significant upregulation of TLR2 and TLR4 after 24 hours of incubation[25]. As depicted in Figure 5, LPS incubation upregulated monocyte cell-surface expression of TLR2 and TLR4 compared with control (RPMI). CD14 expression was also upregulated by LPS (2990±237.8 vs. 2173±227.3, $p<0.05$). GTS-21 inhibited LPS-induced upregulation of TLR2 and CD14 (2467±218.9, $p<0.05$ compared with LPS) and abolished LPS-induced TLR4 upregulation. Moreover, in the absence of LPS, GTS-21 lowered TLR4 expression below control levels. LPS had no effect on α7nAChR expression, while GTS-21 in the absence of LPS inhibited α7nAChR expression below control level (387±50.6 vs. 713.3±116.3, $p<0.05$). Nicotine had no effects on expression of any of the measured receptors (data not shown).

GTS-21, but not nicotine, regulates cytokine production at the transcriptional level

To investigate whether the anti-inflammatory effects of GTS-21 and nicotine are transcriptionally regulated we assessed mRNA levels of pro- and anti-inflammatory cytokines and receptors using quantitative PCR on isolated monocytes stimulated for 4 hours. As expected, incubation with LPS significantly upregulated TNF-α, IL-1β, IL-6, and IL-10 mRNA expression (respectively 5.4, 14.7, 1715, and 9.7-fold compared with RPMI, Figure 6). GTS-21 significantly attenuated LPS-induced upregulation of TNF-α and IL-1β (-2.1 and 4.1-fold compared with RPMI respectively). Moreover, GTS-21 in the absence of LPS significantly decreased TNF-α mRNA levels (-9.6-fold compared with RPMI) while there was a trend towards IL-1β downregulation (-11.9-fold

compared with RPMI). There was also a trend towards inhibition of LPS-induced upregulation of IL-6 and IL-10 expression (637.8 and 5-fold compared with RPMI respectively) by GTS-21 as well as downregulation of IL-10 in the absence of LPS (-4.5 compared with RPMI). TLR2 and TLR4 mRNA expression was not altered by LPS. GTS-21 significantly downregulated TLR2 in the absence of LPS (-3.2-fold compared with RPMI) but had no effect on TLR4 mRNA levels. LPS significantly downregulated CD14 (data not shown, -5.6 fold compared with RPMI), this was inhibited by GTS-21 (-3.5 fold compared with RPMI). Nicotine did not alter expression of any of the cytokine genes assessed (data not shown). α7nAChR gene expression was not altered by any stimulus (data not shown).

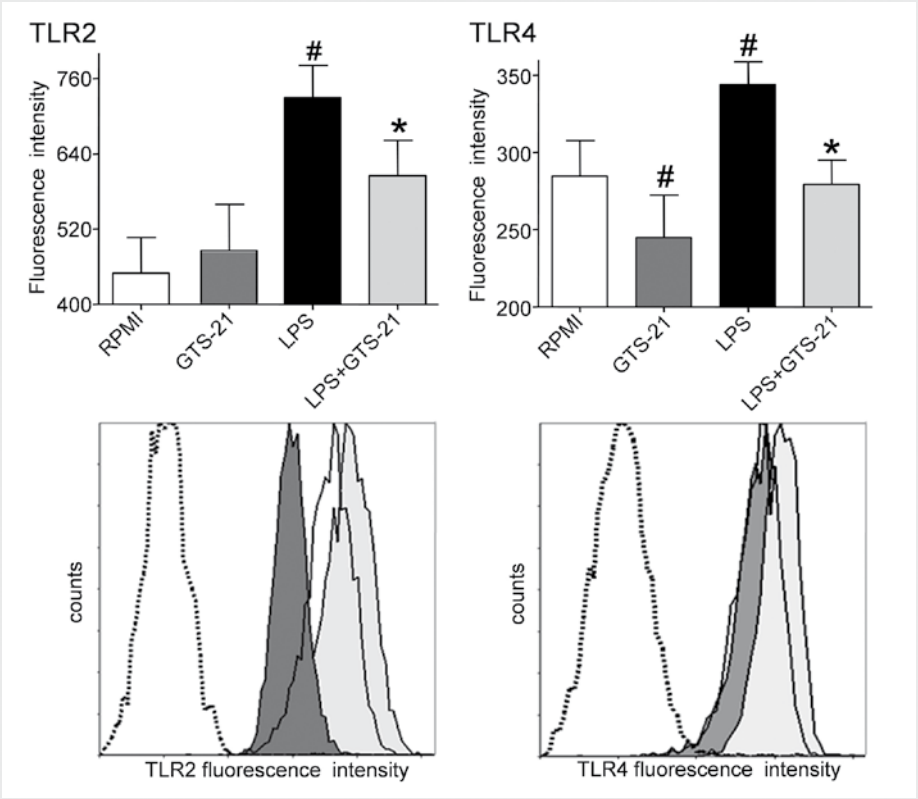


Figure 5 *Flow cytometric analysis of the effects of LPS (1 ng/mL) and GTS-21 (100 μM) on cell-surface expression of TLR2 and TLR4 on primary human monocytes incubated for 22 hours. In the lower panels overlays of histograms depicting TLR2 and TLR4 expression on incubated monocytes of a typical subject are displayed. The dashed line represent isotype control, dark grey represents RPMI, light grey represents LPS and white represents LPS+GTS-21. Data are represented as mean ± SEM (8 different donors, two independent experiments). # indicates p<0.05 compared with RPMI, * indicates p<0.05 compared with LPS (paired Student's t-test).*

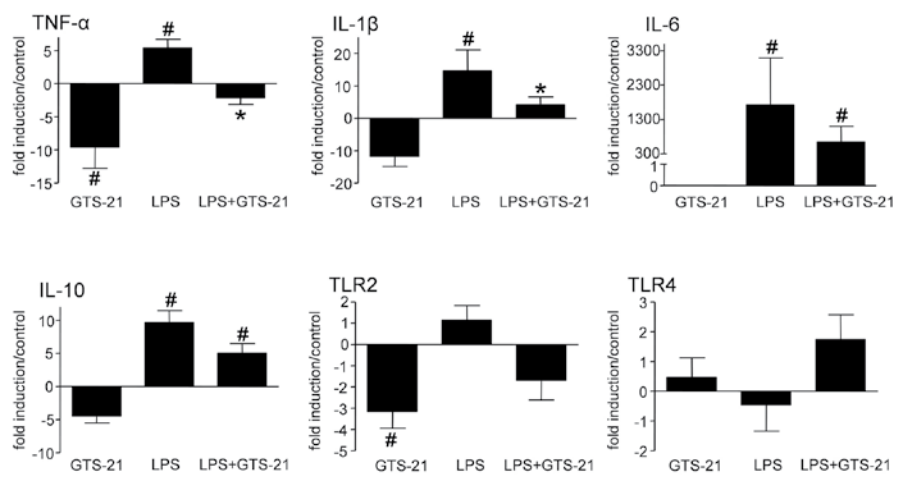


Figure 6 *mRNA expression of TNF-α, IL-1β, IL-6, IL-10, TLR2, and TLR4 in isolated primary human monocytes stimulated with LPS (1 ng/mL) and GTS-21 (100 μM) for 4 hours. Data are represented as mean ± SEM fold expression of the gene of interest relative to control (expression in monocytes incubated with medium alone). Data from 5 different donors, three independent experiments are shown. # indicates p<0.05 compared with medium, * indicates p<0.05 compared with LPS (paired Student's t-test).*

The anti-inflammatory effect of GTS-21 and nicotine are dependent on JAK2 phosphorylation

To evaluate the involvement of the JAK-STAT pathway in the anti-inflammatory effects of nicotine and GTS-21 we incubated PBMCs with LPS, nicotine, and GTS-21 in combination with AG490, a selective inhibitor of JAK2 phosphorylation[16, 17, 26]. 100 μM AG490 inhibited the anti-inflammatory effect of 100 μM GTS-21 while completely restoring the attenuated TNF-α production by 10 μM GTS-21 and 1 mM nicotine (Figure 7). AG490 alone or vehicle (1% etOH) had no significant effects on LPS-induced TNF-α release (LPS+AG490 2719± 379 pg/mL; LPS+1% etOH 1666±333 pg/mL).

GTS-21 effects are likely mediated by JAK2/STAT3 signaling

To further explore the role of the JAK-STAT signaling pathway in the anti-inflammatory effects of GTS-21 we used RT2 profiler qPCR arrays which contain a panel of 84 genes related to JAK-STAT-mediated signaling on isolated monocytes. Genes that were significantly and more than twofold up- or downregulated by 4-hour incubations with GTS-21, LPS and LPS+GTS-21 are shown in Table 1. GTS-21 clearly inhibited LPS-induced expression of IFN-γ and IFN-γ-inducible genes such as CXCL9, GBP1, and OAS1[27-29]. In concordance with the implicated pivotal role for the JAK2-STAT3 pathway in α7nAChR signaling, GTS-21 upregulated STAT3

expression in the presence of LPS. Furthermore, LPS-induced downregulation of F2 (thrombin), an activator of JAK2/STAT3 pathway and SH2B1, a JAK2 activator, were abolished by GTS-21 whereas GTS-21 upregulated OSM which is involved in STAT3 phosphorylation and PRLR, a receptor which upon ligand binding activates JAK2[30-33]. In the presence of LPS, GTS-21 induced upregulation of PTPN1, a protein tyrosine phosphatase which dephosphorylates JAK2, which may represent a negative feedback mechanism preventing excessive JAK2 activation[34]. STAT5A was upregulated by GTS-21 in the presence of LPS while LPS-induced downregulation of STAT5B was blocked by GTS-21 indicating activation of the JAK2-STAT5 pathway. The transcription of NFκB and SOCS3 was upregulated by LPS but not affected by co-incubation with GTS-21.

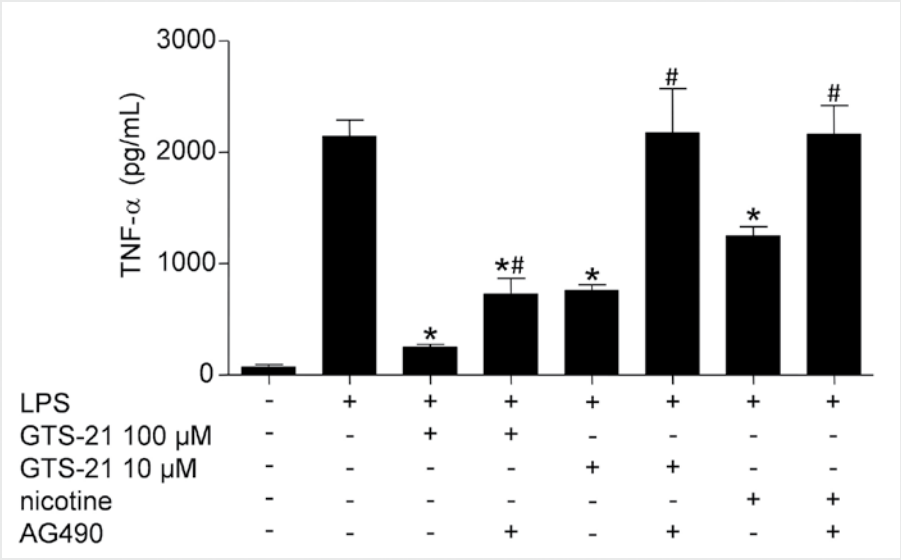


Figure 7 *Effects of the JAK2 inhibitor AG490 (100 µM) on TNF-α production in human PBMCs stimulated with LPS (1 ng/mL), GTS-21 (10 and 100 µM) and nicotine (1 mM) for 22 hours. Data are represented as mean ± SEM (5-7 different donors, two independent experiments). * indicates p<0.05 compared with LPS, # indicates p<0.05 compared with TNF-α response with the same (concentration of) nicotinic agonist in the absence of AG490 (paired Student's t-test).*

Table 1 *up/down regulation of JAK-STAT related genes compared with RPMI. Only genes which are significantly and more than twofold up/downregulated are shown*

GTS-21		LPS		LPS+GTS-21	
SOCS2	8,30	IL2RA	176,11	IL2RA	376,84
INSR	3,11	SOCS1	48,56	STAT4	34,75
EPOR	2,55	ISG15	35,88	SOCS1	24,44
OSM	2,36	CXCL9	24,82	ISG15	16,12
PRLR	2,28	GBP1	18,96	SOCS3	12,92
SMAD1	2,24	SOCS2	18,69	GBP1	9,01
IRF9	-2,32	SOCS3	17,31	NFKB1	3,22
IRF1	-2,50	STAT4	17,29	STAT5A	3,03
GBP1	-2,68	IL20	8,52	SMAD1	2,75
A2M	-2,90	IFNG	6,53	STAT2	2,58
ISG15	-2,97	OAS1	5,34	STAT1	2,45
GATA3	-3,30	STAT1	4,70	IL4R	2,30
CCND1	-3,38	NFKB1	3,09	PTPN1	2,11
IFNAR1	-4,05	STAT2	3,04	STAT3	2,09
OAS1	-4,99	NR3C1	2,35	FCGR1A	-2,25
MYC	-10,96	CDKN1A	2,29	SMAD2	-2,26
		IL6ST	2,14	TYK2	-2,33
		TYK2	-2,38	USF1	-2,66
		USF1	-2,38	IFNGR1	-3,08
		STAT5B	-2,45	EGFR	-3.67
		SMAD3	-2,52	SMAD3	-3,92
		IFNGR1	-2,94	HMGA1	-4,39
		SH2B1	-2,95	FCER1A	-4,75
		SOCS5	-2,97	NOS2A	-4,92
		NOS2A	-4,15	MYC	-5,43
		F2	-5,22	SIT1	-7,36
		FCER1A	-5,43	A2M	-8,83
		EPOR	-5,48	CSF1R	-13,08
		CSF1R	-8,46		
		INSR	-9,77		
		SMAD5	-30,56		

Discussion

The cholinergic anti-inflammatory pathway may represent new treatment options for inflammatory conditions such as sepsis, acute lung injury and autoimmune diseases. Vagus nerve stimulation in humans is a very invasive procedure and is not feasible in acute situations. Therefore, pharmacological stimulation of the cholinergic anti-inflammatory pathway via the α7nAChR is a more practical approach. The nonspecific nAChR agonist nicotine has little therapeutic potential because of its toxicity and other unwanted side effects. We are the first to show that GTS-21, a compound acting specifically at the α7nAChR, is a strong inhibitor of proinflammatory cytokine release in primary human leukocytes while leaving anti-inflammatory cytokine production relatively unaffected. Nicotine has similar effects, but much less pronounced. Therefore, GTS-21 causes a profound shift in the pro-/anti-inflammatory balance towards an anti-inflammatory phenotype. While earlier reports state that the anti-inflammatory effect of α7nAChR stimulation relies on a post-transcriptional mechanism[4, 16], we present evidence that GTS-21 attenuates the inflammatory response at the transcriptional level.

In our study, GTS-21 and nicotine exerted their anti-inflammatory effects equally on PBMCs and isolated monocytes suggesting that these effects are mainly monocyte-mediated. However, because the monocyte isolation method we employed does not yield completely pure monocytes, effects of the nicotinic agonists on lymphocytes or other cell types present in PBMCs cannot be ruled out. In this respect, it is of note that in whole blood experiments the T-cell/NK-cell cytokine IFN-γ was significantly inhibited by both nicotinic agonists. Non-activated lymphocytes are generally not noted for TLR-responses but NK cells have been reported to produce IFN-γ after TLR-stimulation[35]. Whether the observed inhibition of IFN-γ production represents a direct inhibitory effect on NK-cells or an indirect effect via attenuation of cytokines that stimulate TLR-induced IFN-γ production by these cells, such as IL-12 and TNF-α, has to be determined. Nonetheless, while we have focused on the effects of GTS-21 and nicotine on innate immune responses, an effect on the adaptive immune system by these compounds is anticipated because of alterations in costimulatory factors, cell interactions, and interplay between cytokines. Moreover, because primary human lymphocytes express the α7nAChR, a direct effect of α7nAChR agonists on these cells and the adaptive immune response should not be neglected[36, 37] The lack of an inhibitory effect on IL-6 release by GTS-21 is in line with a study in mice which demonstrates a rather selective effect of GTS-21 on TNF-α release[8, 37]. There was a trend towards lower IL-6 mRNA levels in GTS-21-treated monocytes, but it did not reach statistical significance.

We further demonstrate, using a whole blood stimulation assay, that the anti-inflammatory effects of both nicotine and GTS-21 are not specific for the TLR stimulated. This is important because pattern recognition of the diverse classes of microbial products involves various TLRs that modulate the subsequent immune response[24, 38]. As a consequence, cytokine release profiles evoked by Gram-positive bacteria, Gram-negative bacteria, viruses, and various endogenous agonists differ. Previous work of our group has shown that the effect of immunomodulating compounds can differ depending on the TLR stimulated[39]. The data in this paper illustrate that both nicotinic compounds exhibit a generalized anti-inflammatory effect not dependent on the inflammatory stimulus and therefore not confined to a specific sort of infection or endogenous stimulus. Additionally, we affirm the anti-inflammatory potential of GTS-21 and nicotine in whole blood containing all cell types and humoral factors present, which is more reflective of *in vivo* conditions than an isolated cell model.

While the α7nAChR has been identified as the pivotal receptor in the cholinergic anti-inflammatory pathway[6], we could not reverse the actions of nicotine or GTS-21 with antagonists of this receptor (α-bungarotoxin and MLA), or with non-specific nAChR antagonists (mecamylamine and d-tubocurarine) in human immune cells. In accordance, for nicotine, blockade of the anti-inflammatory effect by α-bungarotoxin has been described by some[16, 40] but not all[41]. To date, no attempts have been made to reverse the anti-inflammatory effect of GTS-21 by nAChR antagonists. There are a number of possible reasons to explain the failure to block GTS-21 and nicotine effects by α7 antagonists. First of all, the possibility remains that some of the nicotine or GTS-21 effects in human leukocytes are mediated by a non-α7nAChR-related mechanism which remains to be elucidated. Another explanation might rely on differences in the α7nAChR between cell types. In excitable neuronal cells, α7nAChRs are ligand-gated ion channels composed of 5 α7 subunits which upon activation cause depolarization of the cell membrane and influx of Ca2+ via voltage-operated Ca2+ channels. However, leukocytes do not possess these Ca2+ channels and it was demonstrated that in PBMCs, α7nAChRs stimulation by nicotine or acetylcholine does not result in detectable membrane currents, while it does in neuronal cells[42]. Others have demonstrated that, despite the fact that T-cells express an essentially identical transcript for the α7nAChR subunit as neuronal cells, they do not form functional ligand-gated ion channels[37]. Furthermore, it was shown that leukocytes do not express the normal α7 subunit, but an α7 duplicate nicotinic acetylcholine receptor-related protein (dupα7) which lacks the α-bungarotoxin binding site and

has most likely different pharmacological properties[42]. This is supported by the aforementioned study in T-cells, where the α7nAChR specific antagonist MLA and α-bungarotoxin did not inhibit nicotine-induced effects[37]. Different pharmacological properties may also explain why the partial α7nAChR agonist GTS-21 has a much more potent effect on cytokine release in primary human leukocytes than the full agonist nicotine, while in classic α7nAChRs, the opposite is true[43]. Interestingly, bone marrow and brain cells were positive for both the normal α7 and the dupα7 subunit[42]. This indicates that the presence of the normal α7nAChR or the dupα7 might vary between different cell types, which could explicate why nicotinic blockers antagonize α7nAChR in some cell types but not in others.

LPS-induced increases in monocyte cell-surface expression of TLR2 and TLR4 have been described previously[25]. We confirm these findings and demonstrate that LPS also increases cell surface expression of CD14 which is essential in LPS-induced cytokine production[44]. GTS-21, but not nicotine, inhibits TLR and CD14 upregulation and this could play a role in the diminished proinflammatory cytokine production in GTS-21 treated leukocytes, because modulation of cell-surface expression of TLR4 has been linked to the degree of cytokine production[45]. The observed LPS-induced upregulation of TLRs could also be mediated by cytokine production, so-called 'priming', which would explain why the non-TLR2 agonist LPS increases TLR2 expression[46]. In this respect inhibition of cell-surface expression of the TLRs by GTS-21 might be a result of inhibition of cytokines rather than a cause. mRNA levels of TLR4 and TLR2 were not affected by LPS and only TLR2 mRNA expression was modestly inhibited by GTS-21. The discrepancies between TLR protein and mRNA expression might result from the different incubation period used in the protein expression experiments compared with the mRNA experiments (22 hours vs. 4 hours) but can also indicate that the modulation of TLR cell-surface expression relies mainly on post-transcriptional mechanisms. An earlier report demonstrates that nicotine downregulates cell-surface expression of TLR4 and CD14 on human monocytes[47]. This discrepancy with our study might be explained by the modest anti-inflammatory effect of nicotine in our experiments.

De Jonge et al. have demonstrated that the anti-inflammatory effect of nicotine in murine macrophages acts through the recruitment of JAK2 to the α7nAChR and subsequent phosphorylation of JAK2, thereby initiating the anti-inflammatory STAT3 cascade[16]. We show that in primary human leukocytes, both GTS-21 and nicotine effects could be inhibited by AG490, a JAK2 phosphorylation inhibitor, confirming a pivotal role for JAK2 activation. We further explored activation of the JAK-STAT

pathway using quantitative PCR arrays. Although these arrays do not provide data on phosphorylation of JAK-STAT family members, it evaluates expression of known activators/inhibitors of this signaling cascade at the transcriptional level. Our results indicate that GTS-21 strongly downregulates IFN-γ-inducible genes related to STAT1 activation in monocytes[48]. Therefore, as stated before, GTS-21 may have a pronounced inhibitory effect on the adaptive immune response which warrants future research. JAK2/STAT3 signaling appeared to be activated by GTS-21 reflected by increased expression of STAT3, receptors activating JAK2 (PRLR) and activators of STAT3 (OSM). Furthermore, GTS-21 blocked LPS-induced downregulation of JAK2/STAT3 activators (F2, SH2B1). The upregulation of STAT5A and the blockade of LPS-induced downregulation of STAT5B by GTS-21 further indicate JAK2 activity as STAT5 is also activated by JAK2 in monocytes[49]. There is much debate regarding the pro- and anti-inflammatory roles of STAT3 which appear to be highly cell- and stimulation-specific. A number of studies implicate proinflammatory actions of STAT3, such as a recent paper describing the critical role of STAT3 activation in IL-1β and IL-6 production in a RAW 264.7 macrophage cell line[50]. However, there is also a large body of evidence associating STAT3 activity with anti-inflammatory effects. For instance, STAT3 is essential for responsiveness to IL-10 which is known to deactivate macrophages[51, 52] and STAT3 deficient macrophages are constitutively activated and secrete large amounts of proinflammatory mediators[52]. Blocking STAT3 in tumor cells increases expression of proinflammatory cytokines and mice lacking STAT3 in macrophages and neutrophils are highly susceptible to endotoxic shock[52, 53]. Moreover, mice bearing a STAT3 deletion in bone marrow cells display higher levels of circulating TNF-α and IFN-γ compared with control mice in the absence of an inflammatory process[54]. The mechanism by which GTS-21 (and probably nicotine) inhibits cytokine release may rely on enhanced IL-10 signaling or actions mimicking IL-10 signaling, resulting in STAT3 activation. Because IL-10 downregulates its own production, excessive IL-10 signaling could account for the inhibited production of IL-10 observed with the highest dose of GTS-21 used (100 μM)[51]. Similar to what has been reported for nicotine[16], SOCS3 appears not to play a role in the anti-inflammatory effects of GTS-21, as the LPS-induced upregulation of SOCS3 was not further enhanced by GTS-21. Our quantitative PCR experiments illustrate that GTS-21 inhibits LPS-induced upregulation of proinflammatory cytokines at the transcriptional level. While others argue that the anti-inflammatory effects of α7nAChR stimulation predominantly rely on post-transcriptional modulation[4, 16], our data are in accordance with IL-10 (-like) signaling which inhibits TNF-α production at both the transcriptional and the translational level[55]. Moreover, GTS-21 and nicotine

both have been reported to inhibit LPS-induced NFκB activation which implies transcriptional modulation[7, 20, 21]. While our JAK-STAT array results demonstrate that the transcription of NFκB is not downregulated by GTS-21 compared with LPS, this does not exclude the possibility that GTS-21 modulates NFκB activity/nuclear translocation, as this is not dependent on transcription or translation[56]. Nicotine did not affect mRNA levels of any of the genes assessed. Whether this is due to the relatively low anti-inflammatory potential that nicotine displayed in our experiments or to other factors remains to be elucidated.

The proposed mechanisms of GTS-21 signaling in primary human monocytes is presented in Figure 8.

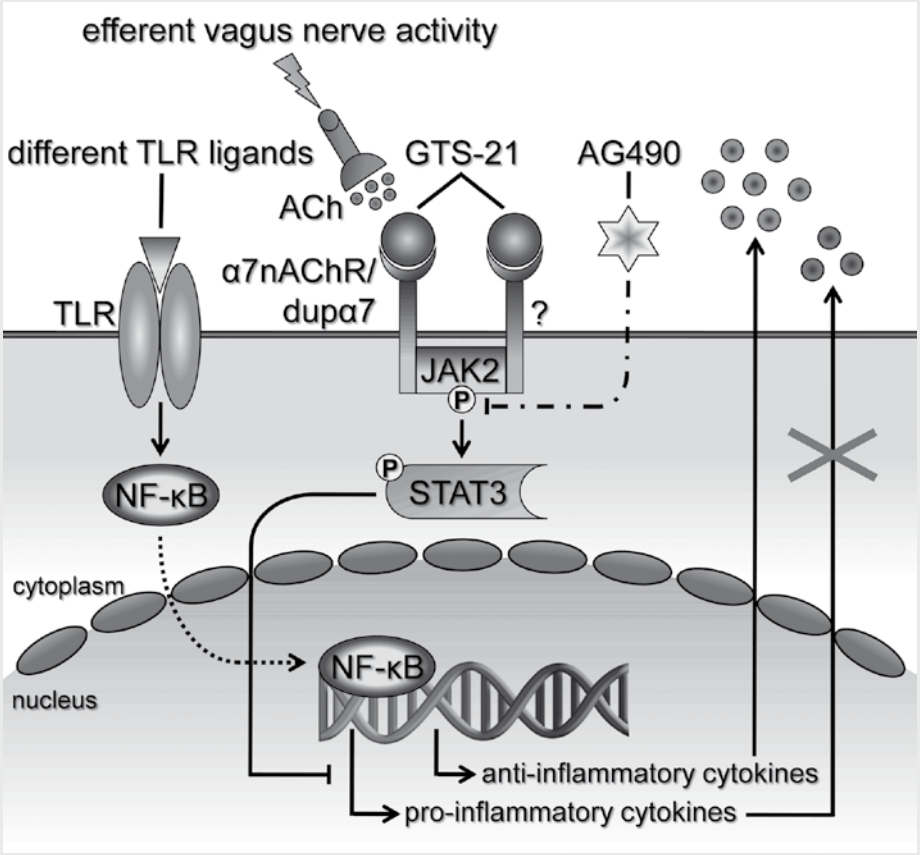


Figure 8 *Mechanisms of GTS-21 signaling in primary human monocytes*

In conclusion, GTS-21 represents novel opportunities for human research into the cholinergic anti-inflammatory pathway and possibly for future therapeutic applications. It is more effective than nicotine in modulating the immune response in human leukocytes and its suitability for human use makes it a candidate for human *in vivo* trials to further explore the cholinergic anti-inflammatory pathway.

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Chapter 7

α7nAChR agonist GTS-21 attenuates ventilator-induced TNF-α production and lung injury

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Abstract

Background Mechanical ventilation (MV) induces an inflammatory response that can lead to lung injury. The vagus nerve can limit the inflammatory response through the cholinergic anti-inflammatory pathway. We evaluated the effects of stimulation of the cholinergic anti-inflammatory pathway with the selective partial α7nAChR agonist GTS-21 on inflammation and lung injury induced by MV using clinically relevant ventilator settings. Furthermore, we investigated whether altering endogenous cholinergic signalling, by administration of the nonspecific nicotinic acetylcholine receptor antagonist mecamylamine and the peripherally acting acetylcholinesterase inhibitor neostigmine, modulates the MV-induced inflammatory response.

Methods C57BL6 mice were injected i.p. with either the selective α7nAChR agonist GTS-21 (8 mg/kg), acetylcholinesterase inhibitor neostigmine (80 μg/kg), nAChR antagonist mecamylamine (1 mg/kg), or placebo; followed by 4 hours of MV (8 mL/kg, 1.5 cmH_2O PEEP).

Results MV resulted in release of cytokines in plasma and lungs compared with unventilated mice. Lung and plasma levels of TNF-α, but not of IL-10, were lower in GTS-21-treated animals compared with placebo ($p<0.05$). In addition, GTS-21 lowered the alveolar-arterial gradient indicating improved lung function ($p=0.04$). Neither neostigmine nor mecamylamine had an effect on MV-induced inflammation or lung function.

Conclusions MV with clinically relevant ventilator settings results in pulmonary and systemic inflammation. Stimulation of the cholinergic anti-inflammatory pathway with GTS-21 attenuates MV-induced release of TNF-α, which was associated with reduced lung injury. Modulation of endogenous cholinergic signalling did not affect the MV-induced inflammatory response. Selective stimulation of the cholinergic anti-inflammatory pathway may represent new treatment options for MV-induced lung injury.

Introduction

Mechanical ventilation (MV) is a lifesaving intervention but can also lead to, or exacerbate pre-existing lung injury, a condition called ventilator-induced lung injury (VILI)[1]. This inflammatory response, reflected by release of inflammatory cytokines, recruitment of neutrophils and lung oedema, impairs lung function[2-4] and is proposed to play a pivotal role in the pathogenesis of VILI[1, 5]. In addition, inflammation of the lungs can spread systemically and cause multiple organ dysfunction syndrome (MODS)[6]. Patients with an underlying inflammatory process are particularly susceptible to MODS[7, 8]. We and others have shown that not only ventilation with high tidal volumes but so-called 'protective ventilation' leads to the release of proinflammatory mediators and lung oedema[9, 10]. These data suggest that limiting the inflammatory response during MV may provide new therapeutic options to reduce lung injury and subsequent multi-organ failure.

Recently, it was discovered that stimulation of the efferent vagus nerve attenuates the innate immune response[11]. This so-called 'cholinergic anti-inflammatory pathway' is based on binding of the vagal neurotransmitter acetylcholine (ACh) to the α7 nicotinic acetylcholine receptor (α7nAChR) present on resident macrophages and other immune cells resulting in inhibition of proinflammatory cytokine release[12]. In several animal studies, 3-(2,4-dimethoxybenzylidene)-anabaseine (GTS-21), a selective partial α7nAChR agonist, has proven to be effective in attenuating the immune response and improving outcome[13-15]. GTS-21, which has been primarily developed for the treatment of Alzheimer's disease and schizophrenia, has been administered to human volunteers and patients and is well tolerated with no clinically significant safety findings[16, 17]. Therefore, GTS-21 might represent a new clinically relevant treatment option for inflammatory conditions.

The cholinergic anti-inflammatory pathway is regarded as a regulatory system, a reflex-type response to limit excessive inflammation[18]. Endogenous activation of the cholinergic anti-inflammatory pathway results in increased ACh release by the vagus nerve. Recently, it was shown that cholinesterase inhibition attenuates the cytokine response and lowers mortality in experimental sepsis, suggesting that cholinesterase inhibition reinforces the cholinergic anti-inflammatory response by increasing ACh availability[19]. Therefore, cholinesterase inhibition might represent another clinically relevant means of limiting excessive inflammation.

In the present study, we investigated the effects of direct stimulation of the cholinergic anti-inflammatory pathway by GTS-21 on inflammation and lung injury induced by MV using clinically relevant ventilator settings in mice. To determine whether alterations of endogenous cholinergic signalling modulate the MV-induced inflammatory response, we studied the effects of the acetylcholinesterase inhibitor neostigmine and the non-specific nAChR antagonist mecamylamine.

Materials and methods

Reagents

3-(2,4-dimethoxybenzylidene)-anabaseine (GTS-21) was a kind gift from Comentis Inc (San Francisco, CA, USA). Neostigmine methylsulphate was purchased from Centrafarm (Etten-Leur, The Netherlands), lipopolysaccharide (LPS, derived from *E. coli* serotype O55:B5) and mecamylamine hydrochloride from Sigma-Aldrich (St Louis, MO, USA), ketamine from Eurovet (Bladel, The Netherlands), dexmedetomidine from Pfizer (Berlin, Germany) and atropine from Pharmachemie (Haarlem, The Netherlands). LPS was further purified as described previously[20]. RPMI culture medium (RPMI 1640 Dutch modification, ICN Biomedicals; Costa Mesa, CA, USA) was supplemented with 10% fetal calf serum, 10 μg/mL gentamicin, 10 mM L-glutamine, and 10 mM pyruvate.

Animals

All procedures described were in accordance with the requirements of the Dutch Experiments on Animals Act, the EC Directive 86/609 and approved by the Animal Ethics Committee of the Radboud University Nijmegen Medical Centre. Male C57BL6 mice (Charles River, Sutzfield, Germany) aged 10 to 12 weeks and weighing 23-29 g were used in all experiments.

Experimental protocols

Non-ventilated control group:

A group of 8 mice that were immediately killed after anaesthesia induction were used as non-ventilated controls in all following protocols (NV group).

GTS-21 protocol:

Mice received either an i.p. injection of phosphate buffered saline (PBS, 200 μL) 30 minutes before the start of ventilation (placebo-MV group, n=18) or an i.p. injection of GTS-21 (8 mg/kg in 200 μL PBS) 30 minutes before the start of ventilation (GTS21-MV group, n=16). Earlier reports have demonstrated that the anti-inflammatory effects of GTS-21 in mice are dose-dependent and that 4 mg/kg

significantly attenuates cytokine production[14, 21]. To maximize its effects, we used 8 mg/kg, which is still a non-toxic dose according to the manufacturer. One mouse in the GTS21-MV group died shortly after the start of MV.

neostigmine/mecamylamine protocol:

Mice received either an i.p. injection with 200 μL PBS 30 minutes prior to the start of ventilation (placebo-MV group, n=15) or of neostigmine (80 μg/kg i.p. in 200 μL PBS) 30 minutes before the start of ventilation (neostigmine-MV group, n=14), or of mecamylamine (Sigma-Aldrich, 1 mg/kg dissolved in 200 μL PBS) 30 minutes before the start of ventilation (mecamylamine-MV group, n=15). Neostigmine and mecamylamine doses were based on previous studies[15, 22]. Three mice died before the start of MV (two in the neostigmine-MV group and one the placebo-MV group).

Instrumentation and MV

With the exception of the NV group, all mice were mechanically ventilated for 4 hours in an identical fashion. Before intubation, mice were anaesthetized with an i.p. injection of 126 mg/kg ketamine, 0.4 mg/kg dexmedetomidine, and 0.5 mg/kg atropine. Atropine is a vagolytic drug, but it predominantly antagonizes muscarinergic ACh receptors (mAChRs); it is therefore not expected to interfere with the anti-inflammatory effects of the vagus nerve to a great extent[11]. Mice were intubated with an tracheal tube and the carotid artery was cannulated to administer Ringer's lactate with heparin (4 IU/mL) and to monitor arterial pressure. Mice were ventilated (MiniVent® type 845; Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) with clinically relevant settings: a tidal volume (Vt) of 8 mL/kg, a breathing frequency of 160-170 per minute, a PEEP of 1.5 cm H_2O and a fraction of inspired oxygen (FiO_2) of 45%. Anaesthesia was maintained by administration of 18 mg/kg ketamine, 20 μg/kg dexmedetomidine, and 37.5 μg/kg atropine as a bolus every 30 minutes via an intraperitoneally placed catheter. Furthermore, depth of anaesthesia was checked regularly by elicitation of the pedal withdrawal reflex. When mice reacted to the stimulus by pedal withdrawal, an increase in heart rate or blood pressure (a sign of discomfort), or both, an additional dose of maintenance anaesthesia was administered. Rectal temperature was monitored throughout the experiment and was kept between 36.5° C and 37.5° C using a heating pad and blankets.

Material harvesting

After four hours of ventilation, mice were killed by exsanguination. We chose this specific time-point based on previous work of our group[9], where a transient increase in inflammatory mediators was demonstrated with nearly all mediators peaking after four hours of MV. Blood gas analysis was performed using an i-STAT Blood Gas

Analyzer (Abbot, Hoofddorp, The Netherlands) and plasma was stored at -80° C until analysis. Immediately after exsanguination, the lungs were carefully removed. The middle lobe of the right lung was fixed for light microscopic histological examination. The left lung (cytokine & myeloperoxidase [MPO] determination] and one lobe of the right lung (mRNA analysis) were snap-frozen in nitrogen and stored at -80° C until analysis.

Isolation and stimulation of alveolar macrophages

Alveolar macrophages from 3 healthy, non-ventilated mice were isolated by bronchoalveolar lavage. Under anaesthesia, the trachea was isolated, incised, and a 20g endotracheal catheter (Angiocath, BD medical, Sandy, UT, USA) was inserted. Lungs were lavaged 10 times with 1 mL of sterile PBS. Lavage fluid was centrifuged at 300g to pellet alveolar macrophages and cells were resuspended and cultured in 12-well plates at a concentration of 3 X 10^5 cells per well in 1 mL RPMI at 37 °C, 95% O_2, 5% CO_2. After 2 hours of incubation, nonadherent cells were discarded and the culture medium refreshed. Cells were then incubated with medium, LPS (100 ng/mL) or LPS + GTS-21 (10 μM) for 4 hours. GTS-21 was added 30 minutes prior to LPS. The supernatants were stored at -20° C until analysis by ELISA. cells were lysed by addition of 350 μL RLT buffer to each well and RNA was purified according to the RNeasy plus mini kit protocol (RNeasy plus mini kit, Qiagen, Venlo, The Netherlands).

Histological examination

Lung tissue was fixed for 24 hours in Unifix (Klinipath, Duiven, The Netherlands), dehydrated and embedded in paraffin wax. Sections of 4-μm thickness were used. Pulmonary neutrophil influx was visualized using chloracetatesterase (Leder) staining. Neutrophils were counted manually in a blinded fashion (20 fields per mouse, 8-14 mice per group), and after automated correction for air/tissue ratio, the number of neutrophils/μm^2 was calculated.

Cytokine and MPO assays

A simultaneous Luminex assay was used to determine plasma cytokine levels (IL-6, IL-10, and keratinocyte-derived chemokine [KC]: Bio-Plex cytokine assay, BioRad, Hercules, CA, USA; TNF-α: Fluorokine MAP, R&D systems, Minneapolis, USA).

For lung cytokine determination, snap-frozen tissue was placed in 500 μL lysisbuffer containing PBS, 0.5% triton X-100 and protease inhibitor (complete EDTA-free tablets, Roche, Woerden, The Netherlands), homogenized using a

polytron and subjected to two rapid freeze-thaw cycles using liquid nitrogen. Finally, homogenates were centrifuged (10 minutes, 14000 rpm, 4 °C) and the supernatant was stored at -80° C until analysis. In lung homogenates and alveolar macrophage culture supernatants, cytokines were determined by enzyme-linked immunosorbent assays (ELISA; IL-6 and IL-10: CytoSet, Invitrogen, Breda, The Netherlands; KC and TNF-α: ELISA DuoSet, R&D Systems). Total protein concentration was determined using the BCA protein assay (Thermo Fisher Scientific, Etten-Leur, The Netherlands). MPO was quantitated using ELISA (HK210, Hycult biotechnology, Uden, The Netherlands).

Quantitative PCR analysis

Snap frozen lung tissue was placed in ceramic bead tubes (MagNAlyser Green Beads, Roche) containing RLT Buffer (RNeasy plus mini kit, Qiagen, Venlo, The Netherlands) and was lysed in a MagNAlyser instrument (3X20 sec, 6500 rpm). RNA was purified according to the RNeasy plus mini kit protocol and RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). 1 μg of RNA (lung) or 30 ng of RNA (alveolar macrophage cultures) was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). The quantitative PCR (qPCR) reaction was performed using Power SybrGreen master mix (Applied Biosystems, Nieuwekerk a/d Ijssel, The Netherlands) on an ABI Prism 7300 Real Time PCR system (Applied Biosystems). Negative control reactions were performed in the absence of reverse transcriptase alongside test samples to ensure the absence of contaminating genomic DNA. The amplification of a single product was ensured by melt-curve analysis for each primer pair. A standard curve constructed from 1:5 serial dilutions of a mixture of cDNA of the samples in the same run was included for each primer pair in every run to perform relative quantification of expression. The housekeeping gene β-actin was used for normalization: the relative quantity of gene of interest was divided by the relative quantity of beta-actin.
The sequences of the primer pairs used were:

TNF-α	forward:	5'-CAGACCCTCACACTCAGATCATCT-3'
	reverse:	5'-CCTCCACTTGGTGGTTTGCTA-3'
IL-6	forward:	5'-CAAGTCGGAGGCTTAATTACACATG-3'
	reverse:	5'-ATTGCCATTGCACAACTCTTTTCT-3'
IL-10	forward:	5'-CCAGGGAGATCCTTTGATGA-3'
	reverse:	5'-AACTGGCCACAGTTTTCAGG-3'
β-actin	forward:	5'-GGCTGTATTCCCCTCCATCG-3'
	reverse:	5'-CCAGTTCGTAACAATGCCATGT-3'

Statistical Analysis

Data were normally distributed (calculated by the Kolmogorov-Smirnov test). Because there was a considerable variation in the LPS response between alveolar macrophage cultures obtained from 3 different mice, the effect of GTS-21 was expressed as % of the LPS-induced TNF-α production in the absence of GTS-21. Alveolar-arterial gradient was calculated using the formula: FiO_2*(Atmospheric pressure-H_2O pressure) - ($PaCO_2$/0.8) - PaO_2. The Grubbs test (extreme studentized deviate method) was performed and significant outliers were excluded from analysis (maximum of one exclusion per dataset). Statistical differences between the groups were detected by one-way analysis of variance (ANOVA, with a Bonferroni post-hoc test to check for significant differences between ventilated groups) or unpaired two-sided Student's t-tests as appropriate. Pearson correlation analysis was used to investigate the relation between TNF-α, IL-6, and KC concentrations. A p-value less than 0.05 was considered statistically significant. All tests were performed with Graphpad Prism 5 (Graphpad Software, La Jolla, USA).

Results

Treatment with the selective partial a7nAChR agonist GTS-21 attenuates MV-induced TNF-α production at the transcriptional level

In ventilated placebo-treated mice both proinflammatory (TNF-α, IL-6, and KC) and anti-inflammatory (IL-10) mediators were increased in plasma compared with non-ventilated controls (Figure 1A). Similarly, in lung homogenates, TNF-α, IL-6, and KC levels were elevated in ventilated animals compared with the NV group (Figure 1B). Pretreatment with GTS-21 significantly attenuated MV-induced TNF-α levels in plasma and lung homogenates. Although plasma and lung concentrations of TNF-α correlated with levels of the other proinflammatory cytokines IL-6 and IL-10 in our model (supplementary Figure 1), GTS-21 pretreatment did not affect these cytokines or the anti-inflammatory mediator IL-10.

Four hours of MV upregulated TNF-α and IL-10, but not IL-6 mRNA expression in lung tissue, measured by quantitative PCR analysis (Figure 2). GTS-21 pretreatment abrogated the MV-induced TNF-α upregulation while not significantly affecting IL-10 expression, indicating that GTS-21 inhibits TNF-α production at the transcriptional level.

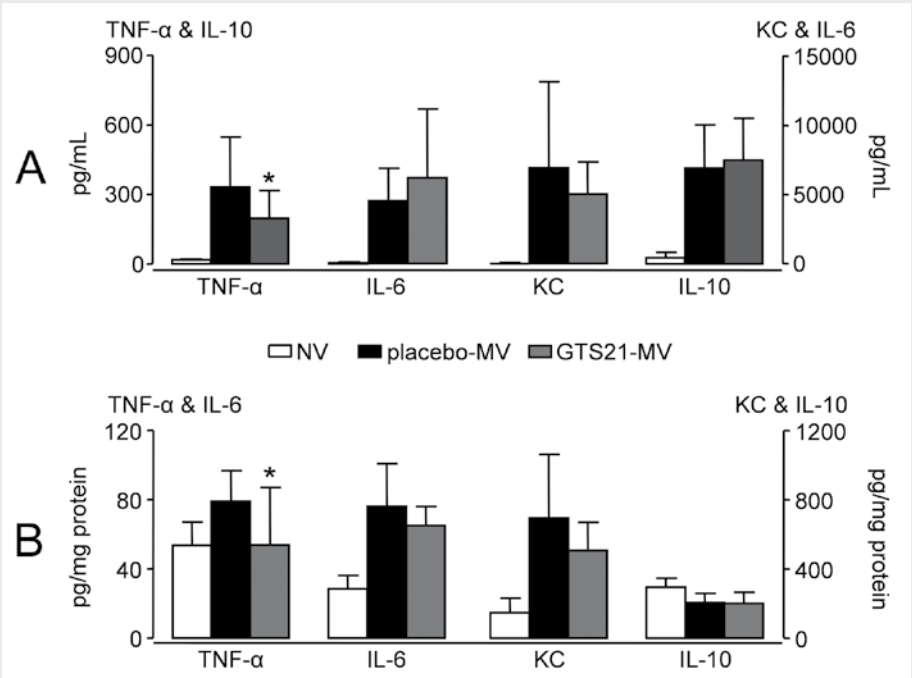

Figure 1 *Inflammatory cytokine levels in plasma (A) and lung homogenate (B) of non-ventilated (NV, n=8), placebo-treated & ventilated (placebo-MV, n=18) and GTS-21 treated & ventilated (GTS21-MV, n=16) mice. Lung homogenate cytokine levels are normalized to the total amount of protein in each homogenate. ANOVA p-values: plasma: TNF-α p=0.0002, IL-6 p=0.0008, KC p=0.0035, IL-10 p<0.0001. Lung: TNF-α p=0.034, IL-6 p<0.0001, KC p=0.0007, IL-10 p=0.0048. Data are represented as mean+SD. * indicates p<0.05 compared with placebo-MV (Bonferroni post-hoc test of placebo-MV vs. GTS21-MV).*

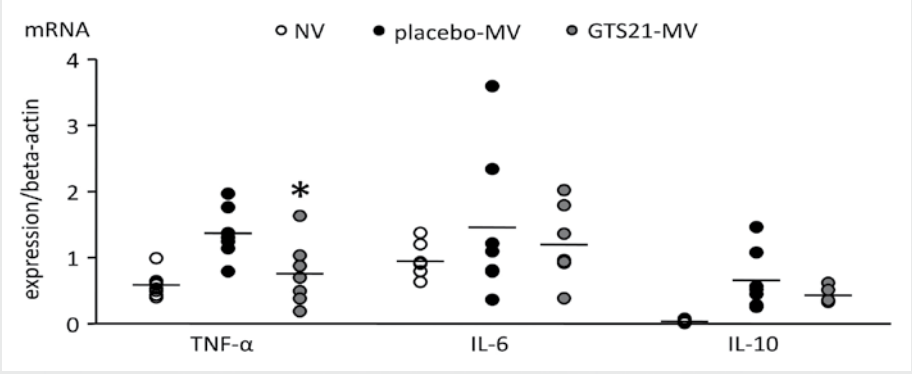

Figure 2 *mRNA expression (relative to the housekeeping gene beta-actin) of inflammatory cytokines in lung tissue of non-ventilated (NV, n=7), placebo-treated & ventilated (placebo-MV, n=7), and GTS-21 treated & ventilated (GTS21-MV, n=7). ANOVA p-values: TNF-α p=0.0017, IL-6 p=0.45, IL-10 p=0.0013. Horizontal line indicates mean. * indicates p<0.05 compared with placebo-MV (Bonferroni post-hoc test of placebo-MV vs. GTS21-MV).*

Because alveolar macrophages are important cytokine-producing cells in the lung, we wanted to confirm whether GTS-21 inhibited TNF-α transcription and production in these cells. Therefore, we isolated murine alveolar macrophages and stimulated them with LPS in the presence and absence of GTS-21 and found that GTS-21 inhibited LPS-induced TNF-α production (Figure 3A) and TNF-α gene expression (Figure 3B).

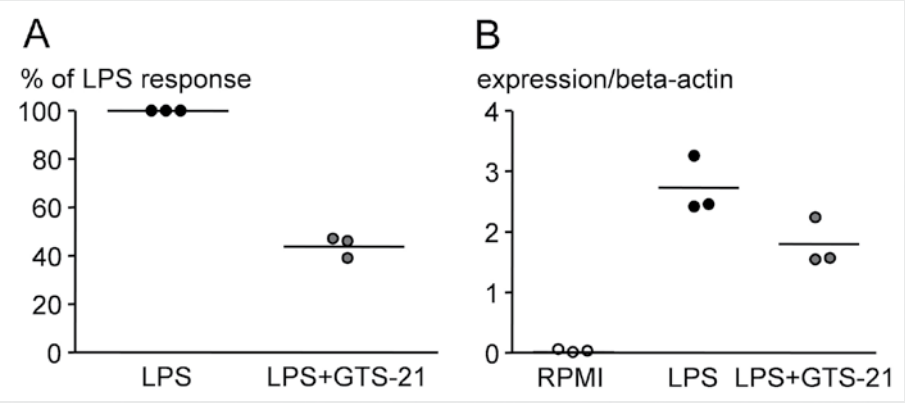

Figure 3 *TNF-α production (A, represented as % of LPS-induced production) and mRNA expression (B, relative to the housekeeping gene beta-actin) in isolated alveolar macrophages stimulated with LPS (100 ng mL^{-1}) for four hours in the presence and absence of GTS-21 (100 μM). TNF-α production in the absence of LPS is below the detection limit (32 pg mL^{-1}); mean LPS-induced TNF-α production is 2503 ± 1462 pg mL^{-1}. Data are represented as individual data points of 3 macrophage cultures obtained from 3 different mice. Horizontal lines indicate mean.*

GTS-21 does not influence MV-induced increases in pulmonary neutrophil influx or lung MPO levels

As depicted in supplementary Figure 2, four hours of MV resulted in a increase in pulmonary neutrophils compared with non-ventilated animals but GTS-21 pretreatment did not have an effect on neutrophil influx (NV: 4.3 ± 1.5 X 10^{-4}, placebo-MV: 8.6 ± 3.0 X 10^{-4}, GTS21-MV: 9.6 ± 3.1 X 10^{-4} neutrophils/μm^2; ANOVA p-value: p=0.0012; post-hoc test placebo-MV vs. GTS21-MV: p=NS). Similarly, lung homogenate MPO levels were not affected by pretreatment with GTS-21 (placebo-MV: 45.6 ± 7.0 ng/mg protein, GTS21-MV: 44.9 ± 9.2 ng/mg protein; p=0.86).

GTS-21 lowers the arterial-alveolar gradient

Both placebo- and GTS-21-treated animals exhibited stable haemodynamic parameters throughout the experiment (Table 1). Mean arterial pressure (MAP) decreased from approximately 100 mmHg at the beginning of the ventilation period to 80 mmHg

after 4 hours in both groups. Lung function after 4 hours of MV was better in GTS-21 pretreated mice compared with placebo, reflected by a trend towards a higher PaO_2, and a significant reduction in the Alveolar-arterial (A-a) gradient.

Table 1 *Cardiorespiratory parameters after four hours of MV in placebo- and GTS-21-treated ventilated animals.*

	placebo-MV (n=18)	GTS21-MV (n=16)	p-value
MAP 0h (mmHg)	104 ± 11	104 ± 12	0.95
MAP 4h (mmHg)	81 ± 11	80 ± 10	0.90
pH	7.36 ± 0.05	7.38 ± 0.06	0.26
PaO2 (mmHg)	128 ± 21	145 ± 28	0.07
PaCO2 (mmHg)	29 ± 5	28 ± 5	0.63
HCO3- (mmol/L)	16.3 ± 1.7	16.4 ± 2.3	0.88
BE	-9 ± 3	-9 ± 2	0.99
lactate (mmol/L)	1.5 ± 0.4	1.5 ± 0.8	0.67
A-a gradient (mmHg)	156 ± 18	140 ± 23	**0.04**

Data are represented as mean ± SD. p-values are t-test. Values in bold indicate significant p-value.

Table 2 *Cardiorespiratory parameters after four hours of MV in placebo-, neostigmine, and mecamylamine-treated animals.*

	placebo-MV (n=15)	neostigmine-MV (n=14)	mecamylamine-MV (n=15)	p-value
MAP 0h (mmHg)	105 ± 10	110 ± 12	108 ± 9	0.47
MAP 4h (mmHg)	81 ± 8	84 ± 7	83 ± 7	0.43
pH	7.36 ± 0.05	7.37 ± 0.05	7.38 ± 0.05	0.65
PaO2 (mmHg)	143 ± 18	152 ± 23	143 ± 38	0.57
PaCO2 (mmHg)	30 ± 4	31 ± 3	32 ± 8	0.64
HCO3- (mmol/L)	17.1 ± 2.1	17.9 ± 1.7	18.9 ± 3.1	0.17
BE	-8 ± 3	-7 ± 2	-6 ± 3	0.12
lactate (mmol/L)	2.0 ± 0.9	2.1 ± 0.7	2.2 ± 0.8	0.89
A-a gradient (mmHg)	140 ± 16	129 ± 20	137 ± 29	0.42

Data are represented as mean ± SD. p-values are one-way ANOVA.

Neither the acetylcholinesterase inhibitor neostigmine nor the nAChR antagonist mecamylamine affects the MV-induced inflammatory response or change in lung function

To investigate whether alterations of endogenous cholinergic signalling modulate the MV-induced inflammatory response, we determined the effects of acetylcholinesterase inhibition by neostigmine and nAChR blockade by mecamylamine. As shown in Table 2 and Figure 4, pretreatment with neostigmine or mecamylamine had no effect on MV-induced changes in inflammatory mediators, haemodynamic parameters, or blood gas measures.

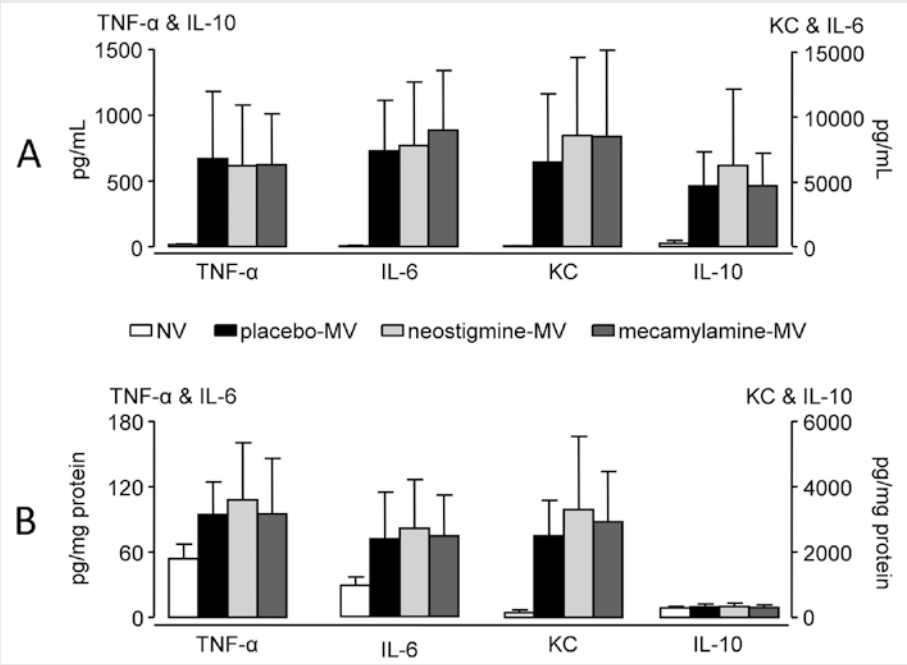

Figure 4 *Inflammatory cytokine levels in plasma (A) and lung homogenate (B) of non-ventilated (NV, n=8), placebo-treated & ventilated (placebo-MV, n=15), neostigmine treated & ventilated (neostigmine-MV, n=14), and mecamylamine-treated & ventilated (mecamylamine-MV, n=15) mice. Lung homogenate cytokine levels are normalized to the total amount of protein in each homogenate. ANOVA p-values: plasma: TNF-α p=0.0046, IL-6 p<0.0001, KC p=0.0051, IL-10 p=0.0060. Lung: TNF-α p=0.0462, IL-6 p=0.0250, KC p=0.0003, IL-10 p=0.5006. Data are represented as mean+SD.*

Discussion

In the present study, we demonstrate that MV with clinically relevant settings elicits a local and systemic inflammatory response. Pretreatment with the selective partial α7nAChR agonist GTS-21 attenuates MV-induced TNF-α production at the transcriptional level and improves lung function.

Recently, it was demonstrated that administration of a centrally acting vagus-mimetic drug attenuates VILI induced by high tidal volumes (20 mL/kg)[24]. In the present study, we demonstrate that selective pharmacological stimulation of the peripheral branch of the cholinergic anti-inflammatory pathway attenuates VILI elicited by MV with clinically relevant settings as well. This study confirms earlier findings from our group and others that MV using clinically relevant settings results in local and systemic cytokine release[9, 10]. In addition to these studies, we show upregulation of cytokine mRNA (TNF-α and IL-10) in lung tissue, suggesting that the MV-induced elevation of pulmonary cytokine levels is the result of local production and not merely of systemic spillover. As we have shown earlier, using electron microscopy, alveolar integrity is preserved with a tidal volume of 8 mL/kg in this model[9], indicating that the observed inflammatory response is a result of biotrauma[1, 3, 25]. We used the same protocol in the present study and demonstrate that GTS-21 attenuates the MV-induced proinflammatory response (TNF-α) at the transcriptional level, but does not dampen anti-inflammation (IL-10). These effects are associated with an improved lung function. How TNF-α affects lung function is largely unknown, but a critical role for this cytokine in the pathophysiology of VILI has been underlined by others, as in a rabbit VILI model, intratracheal administration of TNF-α antibody improved oxygenation and respiratory compliance[26]. In the absence of a reduction in alveolar inflammatory cell infiltration, the beneficial effects of GTS-21 on oxygenation in our model might be mediated by effects on alveolar capillary membrane integrity, which was previously shown to be compromised in our model[9]. However, because we did not measure wet/dry weight ratios in this study, this remains speculative. Another possible explanation for the improvement in lung function by GTS-21 might rely on regulation of p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) phosphorylation. These signalling molecules have been implicated in the proinflammatory response related to oxygenation in VILI[27-31], and cholinergic stimulation has been shown to mitigate hypoxia-induced MAPK and JNK phosphorylation[32].

Our data are in accordance with recent *in vitro* studies of our group and others demonstrating that GTS-21 limits TNF-α mRNA expression and production in response to various Toll-like receptor agonists in isolated primary human monocytes[33, 34]. Similar to these *in vitro* studies, GTS-21 did not attenuate MV-induced IL-6 release in our model. i.p. GTS-21 also limits plasma TNF-α, but not IL-6, in a murine peritonitis model[13] and GTS-21 administered locally in the airways inhibits TNF-α, but not IL-6 release, in the mouse lung after intratracheal LPS delivery[21]. The anti-inflammatory effects of GTS-21 in our VILI model are probably attributable to its effect on alveolar macrophages, because these are the most likely target for GTS-21[35, 36], given that it inhibits LPS-induced TNF-α transcription and production in these cells.

Despite limiting pulmonary TNF-α production, lung neutrophil influx was not inhibited by GTS-21 in our study. This may be explained by the fact that although lower TNF-α levels correlated with lower KC concentrations, GTS-21 did not significantly reduce KC in our model. KC (the murine homolog of human IL-8) is an important chemokine that plays a major role in attracting neutrophils[36, 37]. These findings are in accordance with those of the intratracheal LPS instillation model, where GTS-21 did not influence pulmonary neutrophil influx[21]. In contrast, in the peritonitis study[13], GTS-21 did attenuate neutrophil influx into the pancreas, indicating that the effect of GTS-21 on neutrophil tissue infiltration may be dependent on other factors such as tissue type, dose, and timing of drug delivery[21]. The lack of effects of TNF-α reduction on neutrophil influx is of interest in view of the failure of anti-TNF-α therapy in changing outcomes in clinical trials.

In our model, the peripheral acetylcholinesterase inhibitor neostigmine had no anti-inflammatory effects. We chose to administer the same dose (80 μg/kg) which was shown by Hofer and colleagues to have remarkable anti-inflammatory effects in a murine caecal ligation and puncture (CLP) sepsis model[19]. In contrast, neostigmine in a comparable dose was not protective against endotoxin-induced histopathological organ injury in mice[38]. There are several possible explanations for the lack of an effect in our study. First, the cholinergic anti-inflammatory pathway is regarded as a reflex-type response to control excessive inflammation[18], and peripheral acetylcholinesterase inhibition can merely reinforce this endogenous pathway trough increasing ACh availability at the α7nAChR. The relatively mild MV-induced inflammatory response in our model (especially compared with the fulminant sepsis caused by CLP) may not be severe enough to activate this pathway. Along these lines, there would be no vagus-

borne ACh release into the tissues, hence no pronounced anti-inflammatory effect of inhibition of ACh breakdown. The lack of endogenous activation of the cholinergic anti-inflammatory pathway might also explain why, in our study, nAChR blockade with mecamylamine did not worsen the MV-induced inflammatory response, while in murine pancreatitis[15], mecamylamine treatment led to an amplified inflammatory response and enhanced severity. However, mecamylamine is not selective for the α7nAChR and might therefore exert various other, yet unknown, effects. Future experiments with α7nAChR deficient mice in our model could give a definitive answer whether the cholinergic anti-inflammatory pathway is activated in VILI. Another explanation for the ineffectiveness of neostigmine in our study might depend on the brain's impermeability to neostigmine. Although Hofer and colleagues demonstrated that neostigmine was equally effective as physostigmine (an acetylcholinesterase inhibitor that crosses the blood-brain barrier) in attenuating the inflammatory response in murine sepsis, other studies suggest otherwise. For instance, it was demonstrated that anti-inflammatory effects of other acetylcholinesterase inhibitors that cross the blood brain barrier are blocked by atropine and vagotomy, but not by atropine methyl nitrate, which does not enter the brain[39]. These data suggest that acetylcholinesterase inhibitors dampen inflammation through increasing brain acetylcholine levels leading to enhanced activation of mAChRs and subsequent increased vagus nerve activity. In accordance, direct central mAChR stimulation results in activation of the cholinergic anti-inflammatory pathway[40].

A potential limitation of this study is the use of atropine as part of the anaesthetic regime. While atropine is a classic mAChR agonist, several studies have demonstrated that atropine can also (partially) block nicotinic acetylcholine receptor responses in *Xenopus laevis* oocytes and bovine adrenal chromaffin cells[41, 42]. Very recently, atropine was shown to reduce the mitigating effects of acetylcholine on hypoxia–induced TNF-α expression in a cardiomyocyte cell line[32], and interestingly, it also blocked vasodilatory effects of acetylcholinesterase inhibitors, including neostigmine, in porcine arterial rings[43]. This could represent another explanation for the lack of an effect of neostigmine in our model. However, atropine did not block anti-inflammatory effects of acetylcholine in human macrophages[11], and the fact that GTS-21 limits inflammation in our model suggests that α7nAChR signalling is still functional in the presence of atropine.

In conclusion, our data show that stimulation of the cholinergic anti-inflammatory pathway by the selective partial α7nAChR agonist GTS-21 attenuates the inflammatory

response induced by four hours of MV using clinically relevant ventilator settings and improves lung function. Modulation of endogenous cholinergic signalling by either peripheral cholinesterase inhibition or nAChR blockade did not affect MV-induced inflammation or lung function. GTS-21 might be a promising compound because of its suitability for human use, and therefore, further research regarding its effects on VILI is warranted.

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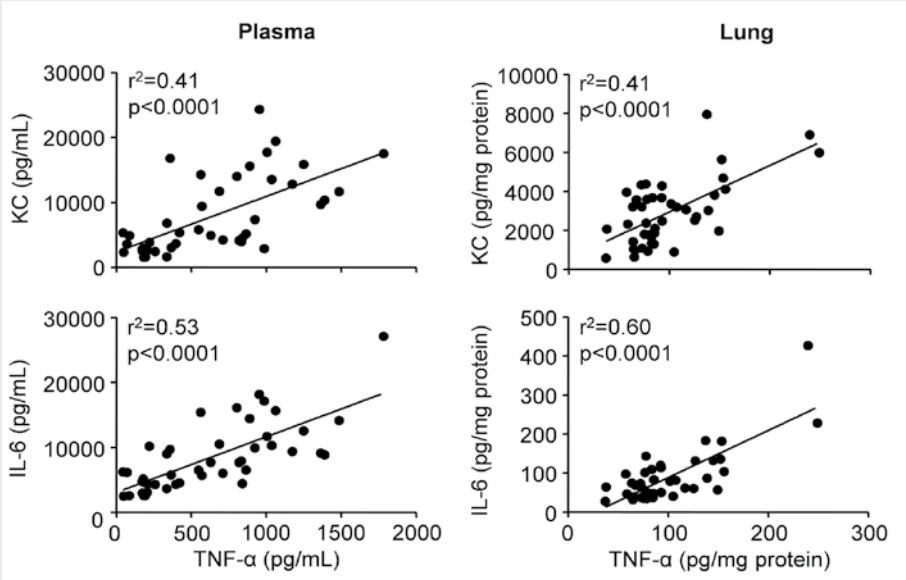
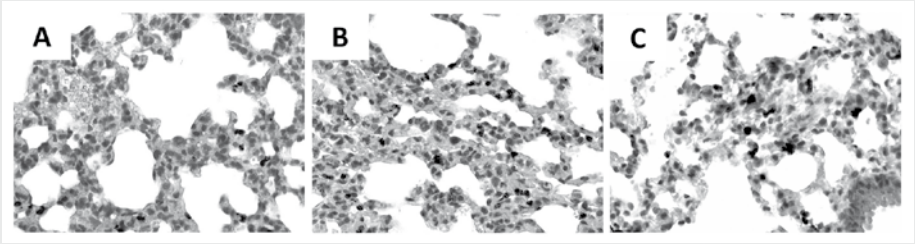
Supplementary material



Supplementary figure 1 *Scatterplots of the association between plasma/lung levels of TNF-α and KC (upper panels) and IL-6 (lower panels). Data depicted were obtained from the neostigmine/mecamylamine protocol (all three ventilated groups, n=44). Pearson correlation coefficients and p-values are indicated in the plots.*



Supplementary figure 2 *Histochemical analysis of lung tissue in non-ventilated (A), placebo-treated & ventilated (B) and GTS-21-treated & ventilated mice (C). Neutrophils are stained black (chloracetatesterase staining).*

Chapter 8

Effects of the α7nAChR agonist GTS-21 on the innate immune response in humans

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Abstract

Background The vagus nerve can reflexively attenuate the innate immune response via binding of the vagal neurotransmitter acetylcholine to the α7 nicotinic acetylcholine receptor (α7nAChR). We recently reported potent anti-inflammatory effects of the a7nAChR agonist GTS-21 in human leukocytes. In the present work, we investigated the anti-inflammatory effects of GTS-21 on the innate immune response during experimental human endotoxemia.

Methods We performed a double-blind placebo-controlled pilot study in 14 healthy nonsmoking male volunteers. Subjects received 150 mg GTS-21 (n=7) or placebo (n=7) orally three times per day during 3 days before endotoxin administration and on the day of the human endotoxemia experiment. This GTS-21 dosage scheme is the highest reported to be safe in humans. Subsequently, subjects were intravenously administered 2 ng/kg endotoxin (LPS derived from *E coli* O:113). Serial blood withdrawals were performed to determine GTS-21 plasma concentrations and inflammatory mediators.

Results Plasma concentrations of GTS-21 and its active metabolite 4-OH-GTS-21 were highly variable between subjects. LPS administration resulted in a transient inflammatory response. There were no differences in the LPS-induced cytokine response between the GTS-21- and placebo-treated groups. However, within the GTS-21-treated group, higher GTS-21 plasma concentrations correlated with lower levels of TNF-α ($r=-0.78$, $p=0.03$), IL-6 ($r=-0.76$, $p=0.04$) and IL-1RA ($r=-0.86$, $p=0.01$), but not IL-10 ($r=-0.35$, $p=0.25$).

Conclusions Although higher GTS-21 plasma concentrations significantly correlated with lower cytokine levels, the highest dose tested to be safe in humans did not result in significant differences in inflammatory mediators between the GTS-21- and placebo-treated groups.

Introduction

The innate immune system is crucial to host defense because it embodies the initial inflammatory response to invading pathogens[1]. However, an excessive inflammatory response, reflected by high levels of inflammatory mediators such as TNF-α, can lead to tissue damage and organ injury[2]. These detrimental effects are often observed in critically ill patients, for instance in the syndrome of sepsis or after major trauma, but also in autoimmune disorders such as rheumatoid arthritis and inflammatory bowel disease[3]. As such, modulation of the innate immune system could prove to be beneficial.

In the past two decades, the interplay between the vagus nerve and the immune system has been firmly established. Inflammatory mediators in the periphery are sensed by the afferent vagus nerve, and this information is relayed to the brain, resulting in fever generation and activation of the hypothalamic-pituitary-adrenal axis[4, 5]. More recently, it was demonstrated in animal models that the vagus-immune link is bidirectional; efferent vagus nerve activity can dampen inflammation in a reflex-like fashion via the so-called cholinergic anti-inflammatory pathway[6]. The anti-inflammatory effect of the vagus nerve is mediated via binding of the vagal neurotransmitter acetylcholine to the α7 nicotinic achetylcholine receptor (α7nAChR) expressed on macrophages and other cytokine-producing cells[7].

Human data regarding the cholinergic anti-inflammatory pathway is scarce. To date, only one study has reported the effects of stimulation of the cholinergic anti-inflammatory pathway on the inflammatory response in man. Transdermal administration of the nonselective nAChR agonist nicotine during human endotoxemia did not affect proinflammatory cytokine levels, however, it did increase plasma concentrations of the anti-inflammatory cytokine IL-10[8].

GTS-21 (3-(2,4-dimethoxybenzylidene) anabaseine, also known as DMXBA, is an α7nAChR agonist that enhances cholinergic transmission in the brain. Studies using human α7nAChR expressed in *Xenopus* oocytes have demonstrated that specific α7nAChR antagonists block GTS-21 receptor activation, and that GTS-21 has only very weak agonist activity at other nAChRs[9]. Therefore, GTS-21 is widely regarded as a selective α7nAChR agonist[10-17]. GTS-21 is suitable for human use and underwent phase I/II trials in healthy subjects and for the treatment of schizophrenia[18-20]. Apart from neuronal effects, *in vitro* experiments in cell lines and *in vivo* animal studies have investigated the effects of GTS-21 on inflammation. These studies demonstrated that

GTS-21 inhibits TNF-α and high-mobility group box protein 1 (HMGB1) release and improves survival during endotoxemia and severe sepsis in mice[12, 15]. Recently, we and others demonstrated that stimulation of the α7nAChR by GTS-21 in primary human monocytes, peripheral blood mononuclear cells, and whole blood leads to a dose-dependent inhibition of proinflammatory, but not anti-inflammatory cytokine release induced by stimulation of various Toll-like receptors[16, 21]. These results suggest that GTS-21 might be effective in limiting the inflammatory response in humans. So far, no data are available on the immunomodulatory effects of GTS-21 in humans *in vivo*.

In this proof-of-principle pilot study, our primary aim was to investigate the effects of oral administration of GTS-21 (in the maximum dose reported to be safe in humans) on the innate immune response using the human endotoxemia model. Second, we wanted to study whether higher GTS-21 plasma concentrations were associated with lower LPS-induced cytokine levels, both *in vivo* and *ex vivo*.

Materials and methods

Subjects & medication

We performed a prospective double-blind, placebo-controlled, randomized pilot human endotoxemia study (Clinical Trial Register number NCT00783068) in 14 healthy young male subjects (range 18-28 years). Furthermore, we assessed the effects of oral treatment with GTS-21 on the cytokine response *ex vivo* in 6 healthy male volunteers (range 19-25 years).

After approval by the local ethics committee of the Radboud University Nijmegen Medical Centre, all healthy volunteers signed written informed consent. Screening of the subjects within 14 days before the test revealed no abnormalities in medical history or physical examination. Exclusion criteria were use of any medication, smoking, history of cardiovascular disease, (family) history of cerebrovascular disease, previous vagal collapse, hypertension (RR systolic > 160 or RR diastolic > 90 mmHg), hypotension (RR systolic < 100 or RR diastolic < 50 mmHg), renal impairment (plasma creatinine > 120 μmol/l), liver enzyme abnormalities, and positive hepatitis or HIV serology. Subjects refrained from food 12 hours before the start of the experiment, and caffeine or alcohol containing substances 24 hours before the start of the experiment. GTS-21 and placebo capsules were a kind gift from CoMentis Inc (South San Francisco, CA, USA).

Study protocols

Endotoxemia protocol

Subjects ingested capsules of 150 mg GTS-21 (n=7) or placebo (n=7) three times per day starting 3 days before the endotoxemia experiment (day -3; 10 am, 6 pm, and 11 pm), and a single oral dose of 150 mg of GTS-21 or placebo on the endotoxin infusion (experimental) day before 7 am. Subjects then received an oral dose of 150 mg GTS-21 or placebo at 8 am and another oral dose of 150 mg GTS-21 or placebo at 1 hour before endotoxin infusion. The last dose of GTS-21 was administered 1 hour before endotoxin administration. All experiments were performed at the research unit of the intensive care department, with subjects in supine position. After local anesthesia (lidocaine HCl 20 mg/ml) the radial artery was cannulated using a 20-Gauge arterial catheter (Angiocath; Becton Dickinson, Sandy UT, USA) and connected to an arterial pressure monitoring set (Edwards Lifesciences LLC, Irvine CA, USA), connected to a Phillips IntelliVue MP70 monitor (Philips Medical Systems, Eindhoven, The Netherlands). The arterial line was used for continuous monitoring of blood pressure and blood sampling. A cannula was placed in the antecubital vein to permit infusion of 2,5% glucose/0,45% saline solution; subjects received 1,5 L during one hour starting one hour before endotoxin infusion (prehydration), followed by 150 ml/h until 6 hours after endotoxin infusion and 75 ml/h until the end of the experiment. Body temperature was measured every 30 minutes using an infrared tympanic thermometer (FirstTemp Genius, Sherwood Medical, Crawley/Sussex, UK). Heart rate was continuously monitored using a 3-lead ECG. Subjects received U.S. Reference *Escherichia coli* endotoxin (LPS derived from *Escherichia coli* O:113, Clinical Center Reference Endotoxin; National Institute of Health, Bethesda, MD). Endotoxin, supplied as a lyophilized powder, was reconstituted in 5 ml saline 0.9% for injection and vortex-mixed for at least 10 minutes after reconstitution. The endotoxin solution was administered as an intravenous bolus injection at a dose of 2 ng/kg of body weight. Blood withdrawal time points for GTS-21/4-OH-GTS-21 plasma concentration and cytokine measurements are indicated in the corresponding graphs.

***Ex vivo* protocol**

Subjects ingested capsules of 150 mg GTS-21 three times per day starting 1 day before the experimental day (day -1; 10 am, 6 pm, and 11 pm), and two 150 mg doses on the experimental day (the first before 7 am, the second at 10 am, which was set at T=0). The rest of the protocol was identical to the endotoxemia protocol, with the exception of LPS administration. Blood was drawn from the arterial line for GTS-21 plasma concentration measurement and *ex vivo* whole-blood stimulation at day -1, T=0, T=1, T=1.5, T=2, T=4, and T=6.

GTS-21 and 4-OH-GTS-21 plasma concentration

Blood samples to determine GTS-21 and its active metabolite 4-OH-GTS-21 concentrations were collected in lithium-heparin-containing tubes (Vacutainer System, Becton Dickinson; Rutherford, NJ, USA), centrifuged for 10 minutes at 2450 g at 4°C after which plasma was collected and stored at -80 °C until analysis. Pharmacokinetic (PK) analysis was performed by Absorption Systems (Exton, PA, USA) using a high-performance liquid chromatography assay. Blood tests on day -1 & -3 were performed on venous blood samples; on the experimental days, tests were performed on blood samples drawn from the arterial cannula.

Ex vivo whole-blood stimulation

Blood was drawn into 4 mL lithium-heparin-containing vacutainers (Vacutainer System, BD Biosciences). Whole blood was diluted 1:5 in culture medium (RPMI 1640 Dutch modification; ICN Biomedicals, Costa Mesa, CA, USA; supplemented with gentamicin 10 μg/mL, L-glutamine 10 mM and pyruvate 10 mM) and placed in 24-well plates (1 mL/well) for 24 hours in the presence and absence of 100 pg/mL *E. coli* LPS (serotype O55:B5, Sigma-Aldrich, St Louis, MO, USA; further purified as described previously[22]). After incubation for 24 hours at 37 °C, 95% O_2 and 5% CO_2, whole-blood cultures were centrifuged (14000 rpm, 5 min) after which supernatants were stored at -80 °C until assayed.

Cytokine measurements

Plasma concentrations of TNF-α, IL-6, IL-10 and IL-1RA were measured using a simultaneous Luminex Assay according to the manufacturer's instructions (Bio-plex cytokine assay, BioRad, Hercules, CA, USA). Plasma concentrations of HMGB1 were measured using the HMGB1 ELISA kit II according to the manufacturer's instructions (Shino-Test corporation, Kanagawa, Japan). Apart from the acute endotoxemic phase, HMGB1 levels were also assessed 24 hours after LPS administration, because HMGB1 levels peak 16 to 32 hrs after endotoxin exposure in mice[23]. IL-6 in *ex vivo* stimulated whole-blood supernatants was determined by a commercial ELISA according to the manufacturer's instructions (Pelikine Compact, Sanquin, Amsterdam, The Netherlands). TNF-α in supernatants was determined by a specific ELISA using four antibodies as previously described[24].

Data analysis, calculations, and statistics

Because the *in vivo* immunomodulatory potency of GTS-21 was unknown, a formal power calculation was not possible. Therefore we decided to perform a pilot study of

7 subjects per group and based on the data of these 14 subjects, a power calculation using the Areas Under Curve (AUCs) of the LPS-induced cytokine response was performed to decide whether a larger study was necessary or feasible. In view of the relatively small group size (n=7), the normality of distribution was not assumed. Data are expressed as median (interquartile range) unless specified otherwise. Mann-Whitney U tests and Friedman tests were used as indicated (two-sided p-values). To increase the sensitivity of this pilot study, the AUCs of GTS-21 and 4-OH-GTS-21 plasma concentrations obtained in GTS-21-treated subjects were correlated with their AUC cytokine responses. Because GTS-21 plasma concentrations were frequently measured during endotoxemia, an accurate PK profile for AUC calculation was obtained. Pearson correlation was used for regression analysis of log-transformed variables. Because we investigated whether increasing plasma concentrations of GTS-21 were associated with a lower cytokine response, both *in vivo* and *ex vivo*, one-sided p-values were calculated in the correlation analysis. A p-value <0.05 was considered significant. Statistical analyses were performed using Graphpad Prism 5.0 (Graphpad Software, San Diego, CA, USA).

Results

Baseline characteristics and safety

Demographic data are shown in Table 1. No relevant differences in baseline characteristics between both endotoxemia groups were present. No serious adverse events occurred and no abnormal laboratory findings were found during the trial. Two subjects had minor abdominal discomfort after ingestion of GTS-21, without vomiting or pain.

Table 1 *Demographic characteristics*

	endotoxemia protocol		*ex vivo* protocol
	Placebo	*GTS-21*	
Age (yrs)	21.7 ± 0.8	22.0 ± 1.3	21.2 ± 1.0
Height (cm)	186 ± 2	184 ± 3	183 ± 3
Weight (kg)	78 ± 3	77 ± 5	83 ± 4
BMI (kg/m^2)	22.5 ± 0.4	22.6 ± 1.2	24.9 ± 1.2
HR (bpm)	60 ± 2	68 ± 3	69 ± 2
MAP (mmHg)	83 ± 3	85 ± 5	89 ± 4

BMI, body mass index; HR, heart rate; MAP, mean arterial blood pressure. Data are presented as mean ± SEM of 7 (endotoxemia protocol) and 6 (ex vivo protocol) subjects per group.

GTS-21 and 4-OH-GTS-21 PK during endotoxemia

GTS-21 plasma concentrations were not detectable in any of the subjects of the placebo group at any time point or in the GTS-21 group before the start of treatment (data not shown). Median plasma concentrations of GTS-21 and its metabolite 4-OH-GTS-21 on the endotoxin infusion day are shown in Figure 1. GTS-21 and 4-OH-GTS-21 levels were highly variable between subjects. 4-OH-GTS-21 levels were consistently higher than GTS-21 levels, but displayed a virtually identical PK profile, as depicted in Figure 1 and also per subject (data not shown). Peak concentrations of both GTS-21 and its metabolite were observed on T=-1 hrs (approximately one hour after the penultimate GTS-21 dose) and decreased at T=0 hrs (one hour after the last GTS-21 dose), probably as a result of prehydration of the subjects before the LPS administration. At T=0 hrs (time of LPS administration) plasma concentration of GTS-21 ranged from 3 to 89 ng/mL (median 13 ng/mL, equivalent to 34 nM). The highest concentration of GTS-21 measured in a subject was 199 ng/mL (524 nM at T=1 hr).

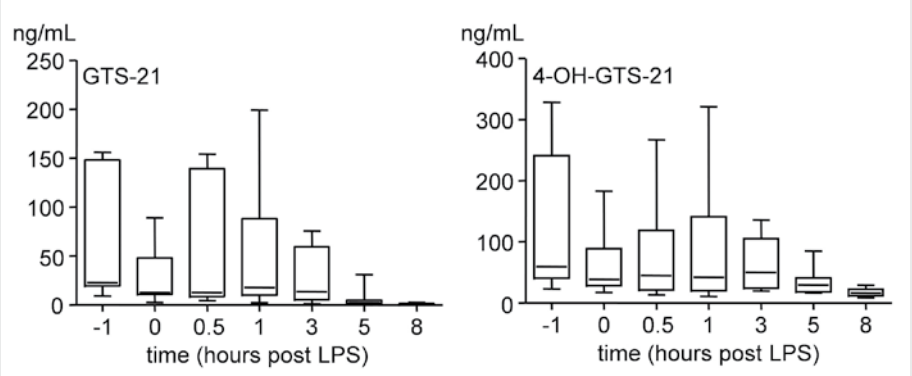

Figure 1 *Box-and-whisker plots of plasma concentration of GTS-21 and its metabolite 4-OH GTS-21 in GTS-21-treated subjects. Whiskers indicate maximum/minimum. n=7 subjects.*

Hemodynamic, hematological, and clinical parameters during endotoxemia

As summarized in Table 2, LPS administration resulted in typical changes in hemodynamic, hematological and, clinical parameters in both groups. All LPS-induced changes were statistically significant in both groups, but there were no differences between the placebo- and the GTS-21-treated groups.

Table 2 *LPS-induced changes in hematological and clinical parameters*

	Baseline	LPS-induced maximal/minimal value		p-value within group	p-value of between groups
MAP, mmHg					
Placebo	92 (84-94)	74 (72-77)		p<0.0001	p=0.73
GTS-21	92 (88-94)	72 (69-78)		p=0.0015	
HR, bpm					
Placebo	61 (54-67)	88 (77-92)		p=0.0001	p=0.23
GTS-21	70 (67-79)	90 (84-95)		p=0.0024	
TEMP, °C					
Placebo	35.6 (35.3-35-7)	36.9 (36.7-37.5)		p<0.0001	p=0.38
GTS-21	35.2 (34.9-35.5)	37.0 (36.7-37.3)		p<0.0001	
WBC, 10^9/L					
Placebo	3.9 (3.5-4.9)	2.1 (1.8-2.8)	10.0 (8.8-12.0)	p=0.0002	p=0.23
GTS-21	5.3 (4.2-7)	3.1 (1.5-4.7)	10.7 (8.3-15.1)	p<0.0001	

MAP, mean arterial pressure (minimum value at T=7); HR, heart rate (maximum value at T=4); TEMP, temperature (minimum value at T=3.5); WBC, white blood cell count (minimum value at T=1, maximum value at T=8). Data are presented as median (interquartile range) of 7 subjects per group. p-values within groups were calculated by Friedman's test (entire curve, all time points); p-values between groups were calculated by Mann-Whitney U-test of AUCs of the entire curve.

Plasma cytokines during endotoxemia

LPS administration resulted in a significant transient increase of inflammatory cytokines (TNF-α, IL-6, IL-10 and IL-1RA) in both groups (Figure 2). There were no significant differences in cytokine response between the GTS-21- and placebo-treated group. Plasma HMGB1 levels were not increased during endotoxemia, neither in the acute (4 and 8 hours post-LPS), nor the late phase (24 hours post-LPS). Moreover, there were no differences between placebo- and GTS-21-treated subjects. Data of the whole group (n=14) are reported: baseline (T=0): 0.85 (0.93) ng/mL vs. T=24: 1.02 (0.94) ng/mL; p=0.19.

Correlation between GTS-21 plasma concentrations and cytokine response during endotoxemia

One GTS-21-treated subject had barely detectable plasma concentrations of GTS-21; this subject was excluded from the correlation analysis. As depicted in Figure 3, higher GTS-21 plasma concentrations were strongly associated with lower levels of TNF-α, IL-6, and IL-1RA, but not IL-10. Correlation coefficients between 4-OH-GTS-21 and cytokines were TNF-α: r=-0.58 (p=0.11); IL-6: r=-0.73 (p=0.05); IL-10: r=0.01 (p=0.49) and IL-1RA: r=-0.89 (p=0.01).

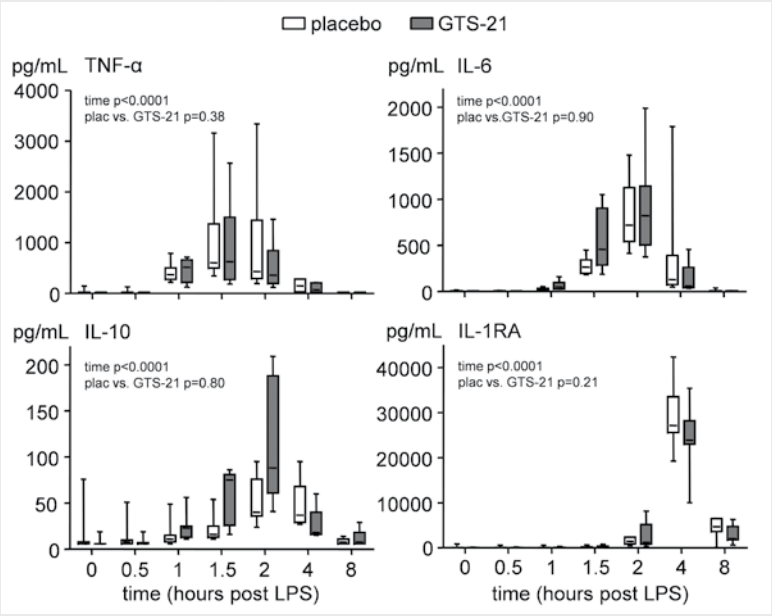

Figure 2 *Box-and-whisker plots of plasma concentrations of inflammatory cytokines after LPS administration. Whiskers indicate maximum/minimum. n=7 subjects per group. Time p-value indicates the cytokine response in time within both groups (Friedman test); Plac vs. GTS-21 p-value represents comparison of Area Under Curves (AUC) between both groups (Mann-Whitney U test).*

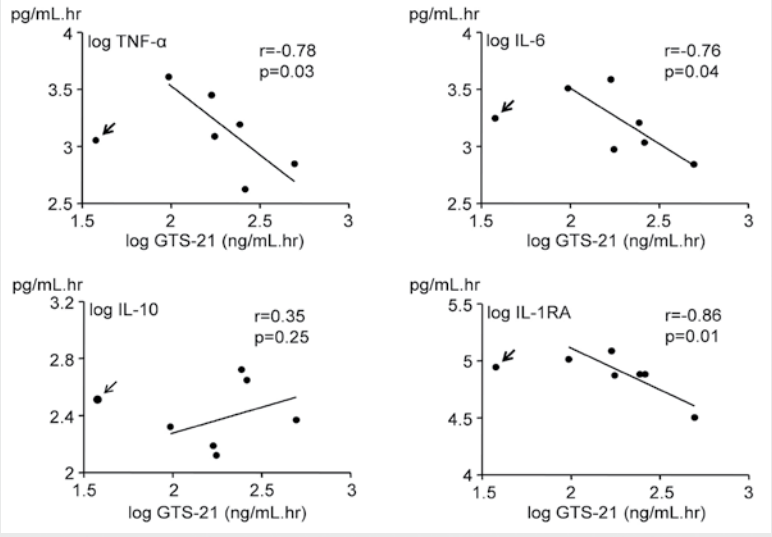

Figure 3 *Correlation between log Area Under Curve (AUC) of the plasma concentrations of GTS-21 and the log AUC of the TNF-α, IL-6, IL-10 and IL-1RA plasma concentrations per subject. Pearson correlation coefficient (r) and significance (p) are indicated in the plots. n=7 subjects. Arrows indicate excluded subject.*

Power calculation using the obtained results from the endotoxemia protocol
To determine how many subjects would be needed to detect a statistically significant difference, we performed a power calculation on the obtained TNF-α AUC data. With a mean±SD AUC TNF-α of 2518±2166 in the placebo group and 1703±1295 in the GTS-21 group, we determined that in order to achieve a power of 80%, 61 subjects per group would be needed.

Cytokine production in ex vivo LPS-stimulated whole blood obtained from GTS-21-treated subjects
In a separate set of healthy male volunteers (n=6), we studied the cytokine production in *ex vivo* LPS-stimulated whole blood obtained before and at various time points after GTS-21 administration. GTS-21 plasma concentrations were again highly variable between subjects and somewhat higher compared with those enrolled in the endotoxin protocol (22.0 (103.5) vs. 12.7 (47.0) ng/mL 1 hour after the last GTS-21 dose and 43.8 (105.8) vs. 12.6 (130.6) ng/mL 1.5 hours after the last GTS-21 dose, data of 6 and 7 subjects, respectively). *Ex vivo* stimulation with LPS led to robust release of TNF-α and IL-6 compared with incubation with medium alone (TNF-α: 413 (254) vs. 48 (79) pg/mL; IL-6: 2062 (2490) vs. 237 (441) pg/mL, data of 6 subjects measured at 7 time points). However, neither TNF-α nor IL-6 production was influenced by GTS-21 treatment; production was similar in whole blood obtained before GTS-21 treatment compared with blood obtained at any of the time-points following GTS-21 administration (data not shown). We also correlated plasma concentrations of GTS-21 to cytokine production of blood obtained at the same time point, but no significant correlations were found (log GTS-21 – log TNF-α: r=0.11, p=0.27; log GTS-21 – log IL-6: r=0.18, p=0.15).

Discussion

We and others have demonstrated that GTS-21 potently attenuates the LPS-induced inflammatory response in human leukocytes[16, 21]. Furthermore, GTS-21 has been shown to limit the LPS-induced inflammatory response in animals *in vivo*[12, 13, 15]. Because GTS-21 is suitable for human use, herein we investigated whether GTS-21 modulates the LPS-induced innate immune response in humans *in vivo*. We used the human endotoxemia model, a standardized model to study the systemic inflammatory response *in vivo* in man[25]. Subjects were administered GTS-21 at the maximum dose that has been tested safe in humans[19]. We found large interindividual variations of obtained GTS-21 plasma concentrations and cytokine responses and did not find differences in LPS-induced plasma cytokine levels between the GTS-21- and placebo-treated groups. However, within the group of GTS-21-treated subjects, higher plasma

concentrations of GTS-21 correlated with lower cytokine levels. GTS-21 treatment did not exert anti-inflammatory effects in *ex vivo* LPS-stimulated whole blood.

An explanation for the lack of differences in cytokine response between the GTS-21- and placebo-treated group could be the highly variable (between subjects) and relatively low median plasma GTS-21 concentrations observed in our study. We have shown earlier that GTS-21 is effective in attenuating LPS-induced cytokine release from primary human leukocytes in a dose-dependent manner with concentrations ranging from 1 μM to 100 μM[21]. In accordance, animal *in vivo* studies have shown that the effects of GTS-21 are highly dose dependent[13, 15]. The highest concentration of GTS-21 found in a subject in our study (approximately 0.5 μM) was still below the concentration found to be effective *in vitro*. To our knowledge, Kitagawa *et al.* performed the only PK study on GTS-21[19]. To maximize GTS-21 effects, we dosed the subjects with the highest dose per day that has been reported in man (150 mg three timers per day. in the aforementioned pharmacokinetics study[19]); this dose was found to be well-tolerated without significant side effects in healthy volunteers. In addition, to limit intersubject variability in plasma concentrations, which has been shown to decrease with continued dosing[19], and to maximize tissue penetration and receptor activation we started treatment 3 days before LPS administration. We administered the last dose of GTS-21 one hour before LPS administration because the Tmax of GTS-21 is approximately 1 hour[19]. The distinct dip in the plasma concentration of GTS-21 one hour after ingestion of the last dose was probably a result of prehydration of the subjects with 1.5 L of fluid administered before LPS administration, which might have reduced the effectiveness of GTS-21 in our study. The prehydration is part of our standard human endotoxemia protocol to attenuate the chances of a vagal collapse[26]. In accordance with our study, Kitagawa *et al.* also found a large between-subject variability with maximum plasma concentrations ranging from 9 to 147 ng/mL using a similar dosing scheme (5 days, 150 mg three times per day)[19]. Plasma levels of the GTS-21 metabolite 4-OH-GTS-21 were also determined because this metabolite displays higher efficacy than GTS-21 for stimulating human α7nAChR[27]. Concentrations of 4-OH-GTS-21 were higher than those of GTS-21, probably as a result of accumulation of the metabolite during the previous 3 days of GTS-21 dosing, but displayed a virtually identical pharmacokinetic profile.

Because of the relatively low median plasma concentrations and high interindividual variation in GTS-21/4-OH-GTS-21 plasma concentrations, we aimed to improve the sensitivity of our study by examination of a correlation of plasma drug

concentrations and cytokine concentrations per subject. Significantly lower levels of TNF-α, IL-1RA and IL-6 were observed with increasing GTS-21 concentrations. Plasma IL-10 did not correlate with drug levels. In concordance, GTS-21 strongly attenuates endotoxin-induced TNF-α production *in vitro* while IL-10 production is not affected at doses of 1-10 μM[21]. While IL-1RA is generally regarded as an anti-inflammatory mediator, its production is induced by TNF-α and IL-6[28, 29]. In addition, established anti-inflammatory compounds such as epinephrine or glucocorticoids also downregulate IL-1RA (and TNF-α and IL-6) plasma levels, whereas IL-10 production is enhanced[30-33]. As such, our findings suggests an anti-inflammatory effect of GTS-21 at higher plasma concentrations. After our pilot study, we performed a power calculation with the obtained results and found that to achieve a power of 80%, 61 subjects per group would be needed. It was not feasible to conduct a study of this size. Moreover, in light of the highly variable and relatively low GTS-21 plasma concentrations, higher dosage schemes or another route of administration for GTS-21 should first be tested for suitability/safety. Therefore, we decided not to extend this pilot-study.

Our previous data show that GTS-21 potently inhibits LPS-induced cytokines in whole blood, peripheral blood mononuclear cells, and isolated monocytes *in vitro*[21]. Anti-inflammatory effects between the three cell systems were very similar, indicating that, in short-term stimulation experiments (up to 24 hours), GTS-21 effects are mainly mediated by monocytes in these cell systems. However, during human endotoxemia, most of the cytokines are produced by tissue-resident macrophages, not circulating monocytes. We wanted to exclude the possibility that the absence of an anti-inflammatory effect of GTS-21 at the group level was caused by the difference in cell populations responsible for cytokine production between the *in vivo* and *in vitro* situation. Apart from the lower plasma concentrations of GTS-21 obtained *in vivo* compared with the *in vitro* experiments, this could represent another explanation of the observed results. To this end, we studied the cytokine production in *ex vivo* LPS-stimulated whole blood obtained before and at various time-points after GTS-21 administration (in subjects *in vivo*) in a separate set of healthy volunteers and observed no anti-inflammatory effects of GTS-21. These findings indicate that not the difference in cell populations, but the relatively low plasma concentrations account for the difference between the *in vivo* and *in vitro* experiments. The fact that significant correlations between higher plasma levels of GTS-21 and lower cytokine levels were observed during human endotoxemia but not in the *ex vivo* protocol might rely on tissue accumulation of GTS-21 and hence more pronounced effects on tissue-resident macrophages.

Human data regarding the cholinergic anti-inflammatory pathway are very limited. A number of association studies investigated the relationship between heart rate variability (HRV), as a measure of cardiac vagal function, and inflammatory mediators such as C-reactive protein and IL-6 in healthy adults. It was found that higher cardiac vagal tone correlates with lower levels of inflammatory markers[34, 35]. Recently, the correlation between HRV measures and the inflammatory response was investigated in the human endotoxemia model. Surprisingly, this study did not reveal a correlation between baseline vagal HRV measures and cytokine release[36].

Our study is the first to investigate selective stimulation of the cholinergic anti-inflammatory pathway in humans. In the only other study that investigated stimulation of the cholinergic anti-inflammatory pathway in humans, Wittebole *et al.* evaluated the effectiveness of the nonselective nAChR agonist nicotine using a similar human endotoxemia study design[8]. Healthy volunteers received a cutaneously applied nicotine or placebo patch after which they were injected with 2 ng/kg LPS. This study failed to show an effect of nicotine on proinflammatory cytokine release. There was a slight, but statistically significant, increase in the anti-inflammatory cytokine IL-10 in the nicotine-treated group. However, because nicotine is nonselective, its effects might be mediated via other pathways than α7nAChR stimulation. In accordance, the authors speculate that the increase in IL-10 might result from increased cortisol levels found in the nicotine-treated group rather than a direct effect of α7nAChR stimulation, because glucocorticosteroids have been shown to enhance IL-10 production during human endotoxemia[31, 32]. Possibly, the lack of an effect of nicotine on proinflammatory cytokine production in this study was also caused by low plasma concentrations of nicotine. *In vivo*, nicotine is rapidly metabolized into cotinine; as such nicotine concentrations were not determined in this study[8]. Mean plasma cotinine concentration was 52 ng/mL[8]. Because plasma levels of cotinine are higher than those of nicotine[37], it is highly unlikely that plasma nicotine concentrations were achieved that have been shown to modulate the inflammatory response in human leukocytes *in vitro* (100 μM – 1 mM, equivalent to 16.2-162 μg/mL)[16, 21].

In conclusion, oral administration of GTS-21 in the maximum dose tested to be safe in humans does not appear to modulate the inflammatory response *in vivo* in man. This is likely caused by the high intersubject variability in GTS-21 plasma concentrations and relatively low median GTS-21 plasma concentrations achieved, because we did find correlations between higher plasma concentrations and lower inflammatory cytokine levels within the group of GTS-21 treated subjects. Large groups would be needed to

definitively establish the anti-inflammatory effects of GTS-21 in man using this dosage scheme. In view of these findings, similar studies using higher doses or another route of administration (e.g. intravenous) should be undertaken to determine the definitive potential of GTS-21 to modulate the innate immune response in humans. Studies exploring the potential of other selective α7nAChR agonists to modulate the innate immune response in humans are also warranted. Compounds such as AR-R17779 and TC-7020 have shown promise in animal models of inflammation, but have not been administered to humans up to now[38-40]. These drugs might have better PK properties and potency/efficacy compared with GTS-21, resulting in higher plasma concentrations and more pronounced α7nAChR stimulation.

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Chapter 9

Lipid- and protein-enriched enteral nutrition limits inflammation during experimental human endotoxemia

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Submitted

Abstract

Background A dysregulated inflammatory response is an important cause of morbidity and mortality in critically ill patients. Enteral administration of lipid-enriched nutrition was previously shown to attenuate inflammation and organ damage via a cholecystokinin-mediated vagovagal reflex in animal studies. The current proof-of-principle study investigates the immunomodulatory potential of enteral lipid- and protein-enriched (enriched) nutrition during experimental human endotoxemia.

Methods After an overnight fast, 18 healthy male subjects received an intravenous bolus of *Escherichia coli* lipopolysaccharide (LPS; 2 ng/kg). Subjects in the fasted group (n = 6) were deprived of food throughout the study, while subjects in the intervention groups were fed either enriched (n = 6) or isocaloric control nutrition (n = 6) via a nasojejunal tube, starting 1 hour prior to LPS administration until 6 hours afterwards.

Results LPS administration resulted in a marked inflammatory response. Continuous postpyloric administration of nutrition increased plasma cholecystokinin levels. Enriched nutrition attenuated circulating levels of the proinflammatory cytokines TNF-α and IL-6, and IL-1 receptor antagonist compared with control nutrition (all: $p<0.01$) and fasted subjects (all: $p<0.05$). Additionally, enriched nutrition augmented the anti-inflammatory response, reflected by increased IL-10 release compared with fasted subjects ($p<0.0001$).

Conclusions The current study establishes the anti-inflammatory potential of enriched nutrition in humans. The instant anti-inflammatory effect of enriched nutrition suggests that the beneficial effects are mediated via a cholecystokinin-dependent vagovagal reflex. Enteral administration of enriched nutrition is a promising intervention to modulate the immune response in the early course of systemic inflammation.

Introduction

Despite diagnostic and therapeutic advances in medical care, a dysregulated systemic inflammatory response remains a major complication in surgical, trauma and critically ill patients, which is associated with increased morbidity and mortality[1, 2]. Modulation of the early excessive inflammatory response represents a potential therapeutic option to improve outcome[3]. Although experimental studies demonstrated promising results of interventions aimed at inhibition of single proinflammatory mediators, clinical implementation has failed to be successful[4]. Enhanced insight in disease pathology and development of novel treatment modalities which broadly affect the inflammatory response are warranted to reduce morbidity and mortality[5-7]. Recently, enteral administration of nutrients was shown to improve immune competence and clinical outcome in surgical and critically ill patients[8-10]. Although nutritional interventions are promising, the exact mechanisms behind the beneficial effects remain to be elucidated.

In the past two decades, the autonomic nervous system has been identified as a potent endogenous modulator of the immune response[7]. Both microbial toxins and inflammatory cytokines are sensed by afferent vagal fibers, resulting in fever and a humoral anti-inflammatory response via the hypothalamic-pituitary-adrenal axis[11]. In turn, parasympathetic outflow suppresses release of proinflammatory cytokines and enhances phagocytosis through binding of acetylcholine to nicotinic acetylcholine receptors on inflammatory cells[12, 13]. Selective pharmacologic or electric stimulation of this neuroimmune axis, called the cholinergic anti-inflammatory pathway, improves outcome in various inflammatory models[14, 15]. Our group demonstrated that this endogenous neuroimmune axis can be activated by short-term administration of enteral lipid-enriched nutrition[16-18]. The luminal presence of lipid-enriched nutrition results in cholecystokinin (CCK) release, which activates CCK-receptors located on afferent vagal fibers[19]. Activation of these receptors triggers a vagovagal reflex, that reduces systemic and organ-specific inflammation, and decreases intestinal damage via activation of peripheral nicotinic receptors[17, 18].

A well-timed nutritional stimulation of this potent gut-brain-immune axis could be a promising intervention to treat inflammatory conditions in the clinical setting. Therefore, the aim of the current proof-of-principle study was to investigate the anti-inflammatory potential of a nutritional intervention, designed to result in a marked and prolonged CCK-release, in man. Based on observations that predominantly enteral lipids and proteins trigger CCK release[20], continuous postpyloric administration of a

lipid- and protein-enriched (enriched) nutrition was compared with an isocaloric low-lipid and low-protein control nutrition and to fasting. The effect of enriched nutrition on acute inflammation was studied utilizing the experimental human endotoxemia model[21]. Furthermore, the influence of enriched nutrition on endotoxin-induced subclinical intestinal damage was investigated.

Materials and methods

Subjects

This study was registered at ClinicalTrail.gov (NCT01100996). After approval of the ethics committee of the Radboud University Nijmegen Medical Centre, 12 healthy male subjects gave written informed consent to participate in the experiments in accordance with the Declaration of Helsinki. Samples of fasted subjects (n = 6) were obtained from the placebo-group that participated in another double-blind LPS study (NCT00513110). There were no differences in subject characteristics (Table 1). All subjects tested negative for HIV and hepatitis B. The subjects did not have any febrile illness or used any medications in the two weeks preceding the study.

Table 1 *Subject characteristics*

	Fasted	Enriched	Control	*P* value between groups
Age (y)	24 ± 1	23 ± 1	25 ± 2	.30
BMI (kg/cm²)	22.0 ± 0.7	23.0 ± 0.6	23.1 ± 0.9	.60
TER (kcal)	2822 ± 44	2845 ± 79	3020 ± 137	.22
Rate of infusion (kcal/min)	NA	2.0 ± 0.1	2.1 ± 0.1	.31

Data are represented as mean ± SEM; BMI, body mass index; TER, total energy requirement; NA, not applicable.

Experimental human endotoxemia

Subjects were prehydrated with 1.5 L glucose 2.5%/NaCl 0.45% after which they received an intravenous bolus of 2 ng/kg body weight U.S. reference *E. coli* endotoxin (*Escherichia coli* O:113, Clinical Center Reference Endotoxin, National Institute of Health, Bethesda, MD) within one minute[22]. LPS-induced symptoms were rated using grades ranging from 0 (no symptoms) to 5 (most severe ever experienced), resulting in a cumulative sickness score[23]. Blood was drawn before the start of postpyloric feeding and serially thereafter up to 24 hours after LPS administration (Figure 1). Routine hematology parameters were determined using flow cytometry (Sysmex XE-2100; Goffin Meyvis, Etten-Leur, the Netherlands).

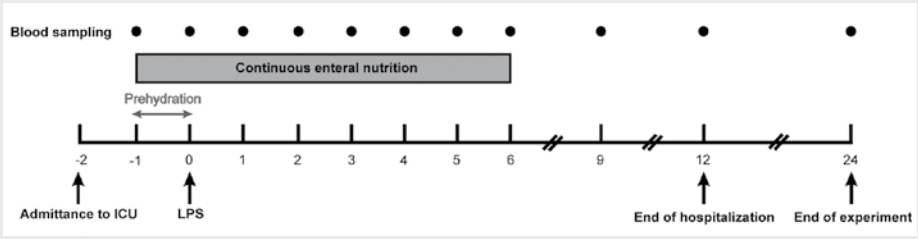

Figure 1 *Experimental design. Subjects are admitted to the intensive care unit (ICU) after an overnight fast. One hour prior to LPS administration, subjects are prehydrated and the continuous administration of enteral nutrition is started in the nutritional intervention groups, lasting until six hours after LPS administration. Blood is withdrawn at indicated time points during the experiment. Subjects leave the hospital 12 hours after intravenous administration of LPS and return the day after for final blood sampling.*

Postpyloric feeding

On the experimental day, two groups received a nutritional intervention in a double-blind randomized fashion, while one group was fasted during the entire experiment (all groups: n=6). The nutritional intervention groups received continuous postpyloric infusion with a liquid, enriched or an isocaloric control enteral nutrition for 7 hours via a self-advancing nasojejunal feeding tube (Tiger 2, Cook Medical, Bloomington, IN). No feeding was aspirated via nasogastric tube or regurgitated throughout the study. The rate of feeding for each subject was based on their total energy requirement (TER). TER was calculated by multiplying basal metabolic rate of each subject with their activity level (1.55 times for all subjects) using the Harris-Benedict equation (Table 1).

Feeding composition and CCK measurement

The enriched nutrition contained 44 energy percent (en%) fat, 25en% protein and 31en% carbohydrates. The protein fraction consisted of intact casein, whey protein and soy protein hydrolysate. The control nutrition contained 20en% fat, 16en% protein and 64en% carbohydrates. Both the enriched and control nutrition provided 1 kcal/ml. Systemic CCK levels were determined in plasma using a CCK-radioimmunoassay (Eurodiagnostica, Malmö, Sweden).

Determination of plasma cytokines and subclinical intestinal damage

TNF-α, IL-6, IL-10, and IL-1 receptor antagonist (IL-1RA) were measured batchwise using a multiplex Luminex Assay (Millipore, Billerica, MA). Intestinal-fatty acid binding protein (i-FABP) was determined in plasma using an in-house developed ELISA.

Statistical analysis

All values are depicted as mean ± SEM. Two-way analysis of variance was used to detect differences between groups for serial data. Differences in serial data within groups were analyzed by one-way ANOVA with Bonferroni's post-hoc test. Data were excluded from the analysis after being identified as significant outlier using the Grubb's test (extreme studentized deviate method). A p-value less than 0.05 was considered statistically significant.

Results

Hematologic and clinical response

As summarized in Table 2, administration of endotoxin resulted in changes in hematologic and clinical parameters in the fasted and nutritional intervention groups. In all groups, mean arterial blood pressure decreased from 90 minutes after LPS administration onwards ($p < 0.001$), while a compensatory rise in heart rate was observed ($p < 0.001$). Also, endotoxemia resulted in a rise in core body temperature ($p < 0.001$) and an initial fall followed by a rise in white blood cell count ($p < 0.001$) in both the fasted and nutritional intervention groups. The LPS-induced changes in hemodynamic parameters, body temperature and white blood cell count were not affected by enteral nutrition. Administration of endotoxin resulted in flu-like symptoms such as headache, nausea, vomiting, backache, shivering and myalgia, which were expressed as sickness score. The sickness score of all subjects peaked at 90 minutes following LPS administration. Administration of enriched or control nutrition did not affect the sickness score compared with fasted subjects ($p = 0.43$ and $p = 0.28$, respectively).

Enteral feeding with enriched nutrition modulates the cytokine profile during experimental human endotoxemia

Intravenous administration of LPS resulted in a marked proinflammatory response. The TNF-α values of one subject in the enriched nutrition group were removed from the analysis after being identified as significant outliers. Treatment with enriched nutrition significantly attenuated TNF-α levels compared with fasted ($p < 0.0001$) and control nutrition ($p < 0.05$; Figure 2A). Enriched nutrition lowered peak TNF-α levels with 40 ± 8% compared with fasted subjects and 29 ± 10% compared with control nutrition. The control nutrition demonstrated a trend towards lower TNF-α plasma levels compared with fasted subjects ($p = 0.06$). Enriched nutrition significantly reduced IL-6 plasma concentrations during the endotoxemia protocol compared with control nutrition ($p < 0.001$) and fasting ($p < 0.05$. Figure 2B), whereas the control nutrition did not affect IL-6 compared with fasted subjects ($p = 0.63$). Administration

of enriched nutrition attenuated peak levels of IL-6 with 41 ± 9% compared with fasted subjects and 54 ± 7 % compared with control nutrition. Intravenous injection of LPS is known to trigger a complex compensatory anti-inflammatory response. The specific IL-1 receptor antagonist, IL-1RA is released during inflammation and is thought to control the immune-modulating effects of IL-1. Enriched nutrition decreased circulating IL-1RA during the experiment compared with control nutrition ($p < 0.0001$) and fasted ($p < 0.0001$; Figure 2C). Peak levels of IL-1RA were 37 ± 8% lower in the enriched nutrition group compared with fasted subjects, and 25 ± 6% lower compared with control nutrition. The control nutrition did not affect IL-1RA levels compared with fasted. Continuous postpyloric infusion of enriched nutrition resulted in elevated plasma concentrations of IL-10 over time compared with fasted ($p < 0.0001$), whereas the control nutrition demonstrated a trend towards higher IL-10 levels ($p = 0.07$; Figure 2D). Enriched nutrition enhanced peak levels of IL-10 with 231 ± 19% compared with fasted subjects and 130 ± 12% with control nutrition.

Table 2 *Hemodynamic parameters, blood leukocyte count and sickness score during human endotoxemia*

		T = 0	*T = 1*	*T = 2*	*T = 3*	*T= 4*	*T = 8*	*T = 24*	*p value*
MAP, mm Hg	Fasted	96 ± 3	94 ± 5	85 ± 5	81 ± 4	81 ± 3	88 ± 3	ND	
	Enriched	104 ± 5	95 ± 4	91 ± 5	89 ± 3	83 ± 2	85 ± 1	ND	.66
	Control	100 ± 2	98 ± 3	94 ± 5	89 ± 3	82 ± 3	87 ± 2	ND	
HR, beats/min	Fasted	68 ± 2	77 ± 4	78 ± 4	87 ± 3	85 ± 4	79 ± 2	ND	
	Enriched	63 ± 3	66 ± 3	75 ± 3	91 ± 2	82 ± 3	77 ± 4	ND	.08
	Control	68 ± 6	81 ± 5	84 ± 6	90 ± 5	90 ± 6	79 ± 5	ND	
Temp, °C	Fasted	36.7 ±0.2	37.0 ± 0.2	38.0 ± 0.3	38.5 ± 0.4	38.3 ± 0.3	37.5 ± 0.1	ND	
	Enriched	36.7 ± 0.2	37.1 ± 0.2	37.7 ± 0.2	38.3 ± 0.3	38.1 ± 0.2	37.5 ± 0.1	ND	.72
	Control	36.7 ± 0.1	37.0 ± 0.1	38.0 ± 0.3	38.3 ± 0.2	38.3 ± 0.2	37.4 ± 0.2	ND	
Leukocytes, x10*9/L	Fasted	5.7 ± 1.2	3.1 ± 0.9	5.2 ± 0.7	ND	8.7 ± 0.2	11.7 ± 0.7	7.5 ± 0.9	
	Enriched	5.9 ± 0.6	2.7 ± 0.7	5.2 ± 0.4	ND	9.7 ± 0.8	12.2 ± 0.9	7.1 ± 0.5	.81
	Control	5.2 ± 0.4	3.1 ± 0.6	5.6 ± 0.6	ND	9.7 ± 0.9	13.2 ± 1.3	7.9 ± 0.6	
Sickness score	Fasted	0.3 ± 0.3	1.3 ± 0.8	2.3 ± 0.6	1.8 ± 0.6	0.5 ± 0.3	0.5 ± 0.3	ND	
	Enriched	0 ± 0	1.7 ± 1.3	4.0 ± 1.4	3.2 ± 1.4	1.3 ± 0.6	0.7 ± 0.5	ND	.12
	Control	0.3 ± 0.3	3.5 ± 0.9	1.7 ± 0.3	1.7 ± 0.4	0.8 ± 0.6	0.3 ± 0.2	ND	

T, time expressed in hours after lipopolysaccharide administration; MAP, mean arterial pressure; HR, heart rate; ND, not determined. Data expressed as mean ± SEM. p values are comparisons between groups over time and were determined by two-way repeated measures-analyses of variance.

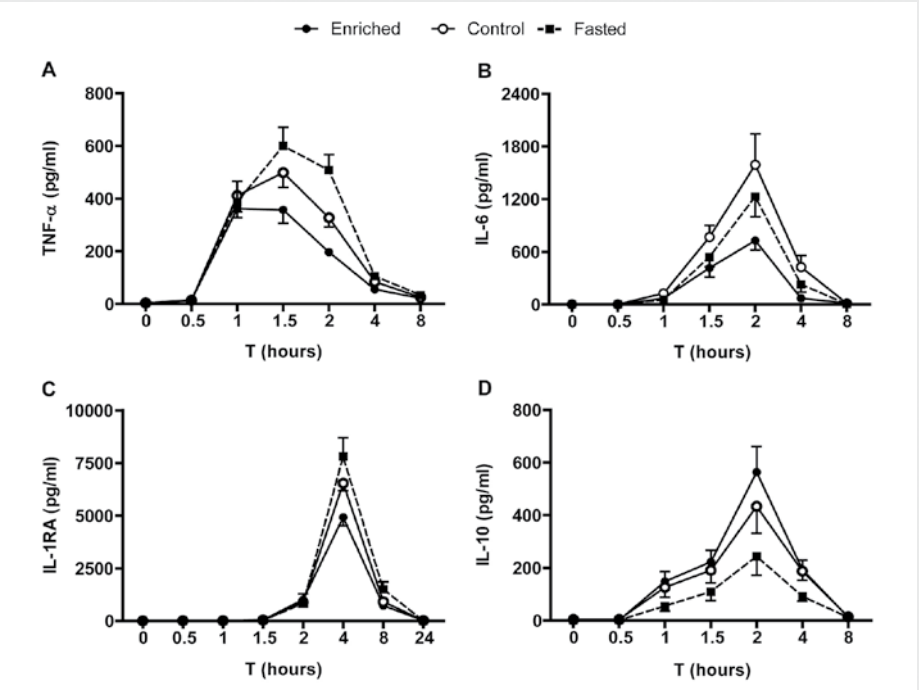

Figure 2 *Enriched nutrition modulates the inflammatory response during endotoxemia. Mean plasma concentrations of TNF-α (A), IL-6 (B), IL-1RA (C), and IL-10 (D) following intravenous LPS administration. Enriched nutrition attenuates TNF-α, IL-6 and IL-1RA levels compared with control nutrition ($p < 0.05$) and fasting ($p < 0.0001$). Administration of the control product displays a trend towards lower TNF-α levels ($p = 0.06$). The enriched nutrition enhances IL-10 release ($p < 0.0001$ vs fasted), while a trend is observed with control nutrition ($p = 0.07$ vs fasted).*

Enterocyte damage remains unaffected by enteral nutrition

In all subjects, administration of LPS resulted in a gradual increase in i-FABP plasma levels until 4 hours post-LPS, representing the occurrence of enterocyte damage (Figure 3A). From 4 hours post-LPS to 8 hours, levels of i-FABP in all groups returned to baseline. Fasted subjects and subjects receiving control nutrition displayed a more prominent increase in i-FABP levels during the experiment compared with subjects fed with enriched nutrition. Total i-FABP release tended to be lower for the enriched nutrition group compared with control nutrition and fasted subjects, although this did not reach statistical significance (Figure 3B).

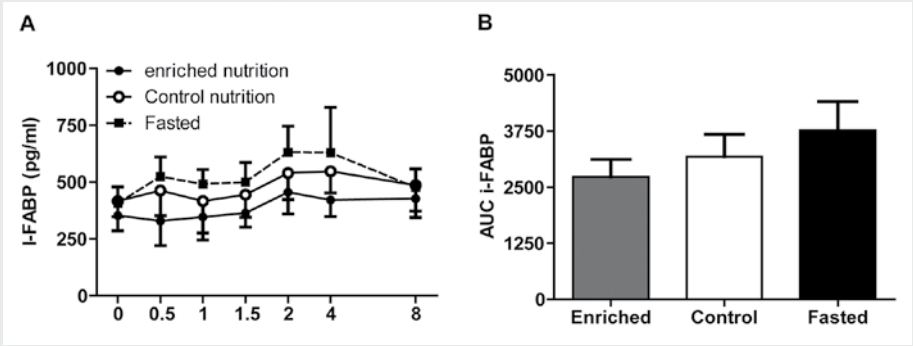

Figure 3 *Administration of LPS results in sub-clinical intestinal damage. Intravenous administration of LPS results in a gradual increase of plasma i-FABP levels in all groups (A). Subjects fed enriched nutrition display a smaller increase in circulating i-FABP levels (A-B), although this does not reach statistical significance.*

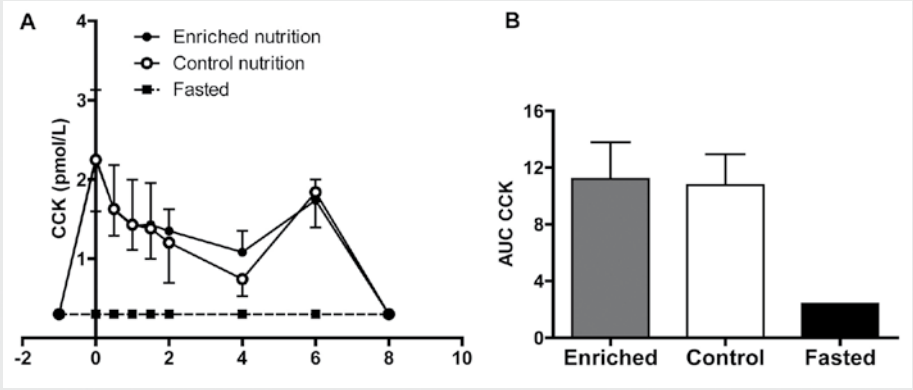

Figure 4 *Postpyloric administration of control or enriched nutrition increases plasma levels of CCK. Continuous enteral administration of both the control and enriched nutrition results in an increase in CCK plasma levels compared with fasted subjects. Both nutritional interventions demonstrate a slight decrease in CCK plasma levels at 4 hours following LPS administration. After cessation of nutrient infusion, plasma CCK levels fall below detection level (A). There is no significant difference in total CCK release between control and enriched nutrition (B). ND, not detectable.*

Increased plasma CCK levels during administration of enteral nutrition

In order to assess the effect of continuous duodenal infusion on CCK release, plasma CCK levels were assessed on indicated time points (Figure 1). CCK levels increased from nondetectable values (< 0.3 pmol/L) before administration of enteral nutrition (T = -1 hrs) to 2.3 ± 0.5 pmol/L at 1 hour after onset of continuous administration of enriched and control nutrition (T= 0; Figure 4A). Four hours after intravenous

LPS injection CCK plasma levels in the control group dropped (0.7 ± 0.2 pmol/L; $p < 0.05$) compared with the levels at $t = 0$. The drop in CCK levels tended to be smaller in the enriched group. There were no significant differences in total plasma CCK release between enriched and control nutrition (Figure 4B). CCK levels dropped to undetectable levels at 8 hours, after cessation of the nutrient infusion. In fasted subjects, plasma CCK levels stayed below the detection level throughout the protocol.

Discussion

The present study is the first to investigate the immediate immunomodulatory effect of continuous enteral administration of nutrition enriched with lipids and protein in man. Our data reveal that enriched nutrition modulates the innate immune response during human endotoxemia, resulting in attenuated plasma levels of TNF-α, IL-6, and IL-1RA, and elevated circulating IL-10, indicating a reduced proinflammatory response and an augmented anti-inflammatory response.

During the last decades, the catabolic state of surgical and critically ill patients increasingly gained interest[24, 25]. The observed negative correlation between catabolism and clinical outcome resulted in more liberal nutritional regimes, such as reduced preoperative fasting and early administration of enteral nutrition[24]. Implementation of these renewed nutritional support regimes reduced morbidity and length of hospital stay[9, 10]. Although the exact mechanisms behind these beneficial effects are not well known, it is assumed that adequate nutritional support prevents immunodeficiency induced by caloric deficits[26]. In addition to caloric support, prolonged ingestion of nutrition enriched with intrinsic anti-inflammatory compounds, such as long-chain n-3 polyunsaturated fatty acids and glutamine results in immune-modulating effects and improves outcome by influencing specific metabolic processes, including eicosanoid production, glutathione synthesis, and generation of heat shock proteins[27, 28]. Our group has revealed a novel pathway in which enteral nutrition modulates the immune response via a fast-acting hard-wired pathway. Enteral lipid-enriched nutrition was shown to limit inflammation and reduce organ damage via a CCK-mediated activation of the cholinergic anti-inflammatory pathway in several rodent models[16-19, 29]. Herein, we present the first evidence of the effectiveness of the nutritional anti-inflammatory mechanism in man.

Virtually every surgical, trauma, and ICU patient suffers from systemic inflammation. The complex interplay between pro- and anti-inflammatory mechanisms during such a systemic inflammatory response is still incompletely understood[30]. The

human endotoxemia model does not replicate these clinical conditions, but has been extensively employed to study the acute systemic inflammatory response *in vivo*[21]. Administration of endotoxin affects various systemic physiological and metabolical processes in a manner similar to the early phase of injury and infection, making it a suitable human model for proof-of-principle studies[21].

Excess release of TNF-α is known to contribute to the development of systemic inflammatory response syndrome, organ damage, and mortality in sepsis[31]. Furthermore, circulating levels of TNF-α and IL-6 are correlated with the severity of sepsis in patients[32]. In line with our animal data[16, 17], the current study demonstrates that enriched nutrition limits inflammation during human experimental endotoxemia by attenuating circulating levels of TNF-α and IL-6. Moreover, the intervention with enriched nutrition resulted in decreased IL-1RA plasma levels. These data are conform previous reports, demonstrating that TNF-α and IL-6 enhance IL-1RA release during endotoxemia, while inhibition of these cytokines lowers circulating IL-1RA[33, 34]. In accordance, attenuation of the inflammatory response using epinephrine or glucocorticoids downregulates IL-1RA and IL-1 plasma levels[35, 36]. In parallel with these reports, our findings that enriched nutrition not only decreases plasma levels of TNF-α and IL-6, but also of IL-1RA, reflect an overall reduced proinflammatory state. Interestingly, postpyloric administration of enriched nutrition amplified the anti-inflammatory response to endotoxin, as evidenced by a pronounced increase in circulating IL-10 compared with fasted subjects. IL-10 is considered to be part of the host-protective mechanism that counterbalances the proinflammatory response during acute infection and inflammation[36]. Furthermore, administration of IL-10 has been shown to reduce endotoxin-induced lethality in mice[37]. The beneficial effect of enriched nutrition on the immune response during human endotoxemia is in line with previous studies using well-known pharmacological agents, including epinephrine and glucocorticoids, which inhibit plasma levels of proinflammatory cytokines and augment circulating IL-10[36, 38]. Together, these data indicate that enriched enteral nutrition is a promising and physiological intervention to control acute inflammation.

Intestinal epithelial cell damage often accompanies sepsis, trauma, and major surgery and is related to the degree of gastrointestinal hypoperfusion[39-41]. Additionally, intestinal compromise has been implicated in the development of inflammatory complications following injury[42]. Here, we show that intravenous administration of LPS resulted in increased i-FABP levels. The rise in plasma i-FABP levels tended to be smaller in subjects treated with enriched nutrition compared with control nutrition

or fasted subjects, although this did not reach statistical significance. These data are supported by rodent studies demonstrating that lipid-enriched nutrition preserves intestinal integrity[17, 19]. The small increase in i-FABP plasma levels is likely attributable to the relative low dose of LPS and limited hypoperfusion due to the prehydration protocol. Future studies are therefore needed to ascertain the gut-protective effect of enriched nutrition in man.

CCK-mediated activation of vagal afferents plays a dominant role in nutrient-induced digestive, metabolic, and immunologic feedback[19, 43, 44]. Intestinal release of CCK is predominantly triggered by the luminal presence of lipid and protein[20, 45], while termination of nutrient exposure results in a rapid drop of CCK levels (45). Taking these considerations into account, we chose to continuously administer nutrition enriched with lipids and proteins to induce a prolonged stimulation of the CCK-mediated anti-inflammatory pathway. Our nutritional intervention resulted in detectable circulating CCK levels during the entire endotoxin-induced inflammatory response. The fact that bolus administration of a lipid-rich milkshake containing 100 g fat prior to endotoxin challenge failed to affect the immune response[46], underlines the importance of continuous nutrient administration. In comparison, continuous infusion of enriched nutrition delivered approximately 60 g fat in total. Although enriched nutrition displayed a more powerful anti-inflammatory effect than control nutrition, significant differences in CCK levels could not be detected in plasma. This might be explained by the fact that circulating CCK levels do not reflect local intestinal concentrations and subsequent activation of afferent vagal fibres in the gut. In this context, it is interesting that plasma concentrations of exogenous CCK have to be at least 10-fold higher compared with postprandial CCK levels to obtain a similar satiety effect[47]. Future studies using specific CCK-1 receptor antagonists, which are currently not available, should specify the role of local CCK levels. In addition, these studies should also focus on the role of other intestinal peptides, including glucagon-like peptide 1 (GLP-1). Recently, our group implicated GLP-1 as co-stimulatory peptide of the nutritional anti-inflammatory pathway in rodents, because administration of GLP-1 receptor antagonists partially reduced the inhibitory effect of lipid-rich nutrition on systemic inflammation[48].

In conclusion, the current proof-of-principle study demonstrates for the first time that: 1) continuous administration of enteral nutrition instantly modulates inflammation in man, and 2) enrichment of the nutritional composition with lipid and protein enforces this anti-inflammatory potential. Our findings show that the anti-inflammatory

effects of enriched nutrition previously observed in rodents also apply to the human situation. Taken together, the current study implicates continuous administration of enriched nutrition as a promising intervention to modulate inflammatory conditions in the clinical setting.

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Chapter 10

The influence of concentration/meditation on autonomic nervous system activity and the innate immune response: a case study

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Submitted

Abstract

Background It might be possible to influence autonomic nervous system activity and the innate immune response through concentration/meditation techniques. A Dutch individual known as 'the iceman' holds several world records with regard to tolerating extreme cold, and claims he can influence his autonomic nervous system and thereby his innate immune response through a special concentration/meditation technique.

Methods We performed a case study of the iceman. The subject's *ex vivo* cytokine response (stimulation of peripheral blood mononuclear cells with lipopolysaccharide [LPS]) was determined before and after an 80-minute full-body ice immersion during which the subject practiced his concentration/meditation technique. Furthermore, the subject's *in vivo* inflammatory response was studied while practicing his concentration/mediation technique during experimental endotoxemia (i.v. administration of 2 ng/kg LPS). The results from the endotoxemia experiment were compared with a historical cohort of 112 subjects who participated in endotoxemia experiments in our institution.

Results The *ex vivo* pro- and anti-inflammatory cytokine response was greatly attenuated by concentration/meditation during ice immersion, accompanied by high levels of cortisol. In the endotoxemia experiment, concentration/meditation resulted in increased circulating concentrations of catecholamines, and plasma cortisol concentrations were higher than in any of the previously studied subjects. The subject's *in vivo* cytokine response and clinical symptoms following LPS administration were remarkably low compared with previously studied subjects.

Conclusions The concentration/meditation technique employed by the iceman volunteer appears to evoke a controlled stress response. This response is characterized by sympathetic nervous system activation and subsequent catecholamine/cortisol release, which appears to attenuate the innate immune response.

Introduction

The innate immune system embodies the first defence mechanism against invading pathogens[1]. While this system is crucial to our survival, an excessive inflammatory response, characterized by high levels of inflammatory mediators and activated effector immune cells, can result in tissue damage and organ injury[2]. Examples of excessive or inappropriate inflammatory responses include sepsis and major trauma, as well as autoimmune disorders such as inflammatory bowel disease and rheumatoid arthritis[3]. In these conditions, limiting the innate immune response could reduce disease burden and improve outcome.

It has become increasingly clear that the autonomic nervous system (ANS) can limit the innate immune response. It has long been known that the sympathetic nervous system can attenuate systemic inflammation directly via activation of β2-adrenoceptors by catecholamines[4-6]. In addition, as part of a stress response, increased levels of catecholamines are often accompanied by elevations in cortisol, a well-known immunodepressant[7-9]. More recently, the parasympathetic nervous system has been shown to modulate innate immunity, as electrical stimulation of the efferent vagus nerve greatly attenuates the inflammatory response in animal models[10-12].

The ANS is generally regarded as a system that cannot be willingly influenced. However, several recent investigations suggest that through certain concentration/meditation techniques, it is possible to modulate autonomic activity. For instance, heart rate variability (HRV; a measure of cardiac ANS activity) was shown to be remarkably different during meditation compared with resting conditions[13]. Other studies have reported increased parasympathetic activity (also measured by HRV) during Zen-meditation in healthy subjects[14] and in subjects with coronary heart disease practicing transcendental meditation[15]. Furthermore, reduced sympathetic nervous system activation (measured by skin conductance level, SCL) was found after mindfulness-based stress-reduction (MBSR) in fibromyalgia patients[16].

In light of the effects of the ANS on the innate immune system, it is conceivable that concentration/meditation techniques can influence inflammatory parameters[17]. There are a few reports that support the existence of such a relation. For example, Guolin Qigong (a combination of meditation, controlled breathing, and physical movement) reduced *ex vivo* inflammatory cytokine production by leukocytes[18]. Likewise, MBSR has been shown to result in decreased proinflammatory cytokine production in *ex vivo* stimulated leukocytes of breast and prostate cancer patients[19].

A Dutch individual known as 'the iceman'[20] holds several world records with regard to withstanding extreme cold, including the fastest half marathon barefoot on ice/snow[21] and standing fully immersed in ice for 1 hour and 50 minutes. He claims to achieve these remarkable feats through a special concentration/meditation technique that he developed himself. He claims that, through this technique, he can influence his autonomic nervous system and also his immune response[22]. Herein, we describe a case study of this man, focusing on the effects of his concentration/meditation technique on autonomic nervous system parameters and the innate immune response, both *ex vivo* during ice immersion and *in vivo* during experimental human endotoxemia (lipopolysaccharide [LPS] administration). The experimental human endotoxemia model, a well-characterized standardized model to study the innate immune response[23], has been operational at our institution for several years[24-28]. This allowed us to compare the response of the iceman to a large group of healthy volunteers who underwent the same endotoxemia protocol.

Materials and methods

Study subjects

The iceman is a healthy male, aged 51 years. The cytokine, hemodynamic, temperature, and illness score results from the endotoxemia experiment in the iceman (Clinical Trial Register number NCT01352871) were compared with a historical cohort of 112 healthy male volunteers that participated in human endotoxemia trials in our institution (NCT00246714, NCT00513110, NCT00783068, NCT00785018, NCT00916448, NCT01349699, NCT01091571). Demographic characteristics of the iceman and the 112 previously studied subjects are listed in Table 1. Only subjects who, besides endotoxin, received placebo-treatment or an intervention that had no effect on the endotoxin-induced inflammatory response were included for comparison. Neither the iceman nor the 112 volunteers used any medication. Other exclusion criteria were: cardiovascular disease, a (family) history of cerebrovascular disease, previous vagal collapse, hypertension (Riva Rocci (RR) systolic > 160 or RR diastolic > 90 mmHg), hypotension (RR systolic < 100 or RR diastolic < 50 mmHg), renal impairment (plasma creatinine > 120 μmol/l), liver enzyme abnormalities, and positive hepatitis or HIV serology.

Table 1 *Demographic characteristics of the iceman and the 112 healthy male volunteers previously studied used for comparison*

Parameter	iceman	112 volunteers
Age (years)	51	22.2 ± 0.2
Length (cm)	182	184 ± 0.6
BMI (kg/m²)	24.8	22.8 ± 0.2

Data are presented as mean ± SEM

Study protocols

We performed three distinct experiments on three separate days to study the effect of concentration/meditation on the autonomic nervous system and innate immune response:

1) Concentration/meditation during ice immersion

Before ice immersion, a cannula was placed in the antecubital vein to permit blood withdrawal. Approximately 30 minutes after the iceman started practicing his concentration/meditation technique, an initial blood sample was obtained for cortisol determination and *ex vivo* stimulation experiments. Subsequently, iceman was fully immersed in ice (with the exception of his head) for 80 minutes (starting at 2.45 PM). Throughout the ice immersion, he practiced his concentration/meditation technique. Immediately after cessation of ice immersion (and the concentration/meditation period), only a very limited amount of blood could be collected due to vasoconstriction (this was only sufficient for cortisol analysis). Therefore, he subsequently took a hot shower and blood was collected 30 minutes later for cortisol determination and *ex vivo* stimulation experiments.

2) Concentration/meditation without ice immersion

At 9.45 AM, a cannula was placed in the antecubital vein to permit blood withdrawal and infusion of 25 mL/hour 2.5% glucose/ 0.45% saline solution. Furthermore, a recorder for heart rate variability (HRV) registration and electroencephalography (EEG) electrodes were connected. EEG was performed to monitor vigilance and for signs of drowsiness or sleep before, during, and after meditation. Heart rate was continuously monitored using a 3-lead ECG. Cuff blood pressure and tympanic temperature was measured every 30 minutes. At 10.45 AM, a baseline blood sample was obtained and subsequently, the iceman started the sham concentration/meditation period during which he watched television. After 1.5 hours, a blood sample was

obtained and the iceman started his concentration/meditation technique. 1.5 hours later, the final blood sample was obtained. Blood was used for *ex vivo* stimulation experiments and cortisol and catecholamine determination.

3) Concentration/meditation during human endotoxemia

The study protocol was approved by the Ethics Committee of the Radboud University Nijmegen Medical Centre and complies with the Declaration of Helsinki, including current revisions and the Good Clinical Practice guidelines. The subject had not been exposed to ice-immersion for more than 6 weeks before the experiment and signed written informed consent. Screening of the subject 14 days before the experiment revealed no abnormalities in medical history or upon physical examination. The subject refrained from food 12 hours before the start of the experiment, and caffeine or alcohol containing substances 24 hours before the start of the experiment. The experiment was performed at the research unit of the intensive care department, with the subject in supine position. After local anaesthesia (lidocaine HCl 20 mg/ml) the radial artery was cannulated using a 20 Gauge arterial catheter and connected to an arterial pressure monitoring set. The arterial line was used for continuous monitoring of blood pressure and blood sampling. A cannula was placed in the antecubital vein to permit infusion of 2.5% glucose/ 0.45% saline solution; the subject received 1.5 L during one hour starting one hour before endotoxin infusion (prehydration), followed by 150 ml/h until 6 hours after endotoxin infusion, and 75 ml/h until the end of the experiment. A recorder for HRV registration, muscle sympathetic nerve activity (MSNA) electrodes and EEG electrodes were connected. EEG was performed to assess for possible encephalopathy after LPS infusion and monitor vigilance and for signs of drowsiness or sleep before, during, and after meditation. Tympanic temperature was measured every 30 minutes. Heart rate and respiratory rate were monitored by a 3-lead ECG. Blood pressure, heart rate, and respiratory rate data were stored every 30 seconds by a custom in-house developed data recording system (ICweb). Subjects were asked to score the severity of the endotoxin-induced flulike symptoms (nausea, headache, shivering, muscle, and back pain) every 30 min up to 6 hrs after the administration of endotoxin. Symptoms were scored on a scale ranging from 0 (symptom not present) to 5 (worst ever experienced) and these scores were added, forming an arbitrary total symptom score. 30 minutes before LPS administration (T=-0.5 hrs), the subject started concentrating/meditating for 3 hours (until T=2.5 hrs). The subject received U.S. Reference *E. coli* endotoxin (lipopolysaccharide; LPS derived from *Escherichia coli* O:113, Clinical Centre Reference Endotoxin, National Institute of Health (NIH), Bethesda, MD). Endotoxin, supplied as a lyophilized powder, was reconstituted in 5 ml saline 0.9% for injection and vortex-mixed for at

least 10 minutes after reconstitution. The endotoxin solution was administered as an intravenous bolus injection at a dose of 2 ng/kg of body weight at 10.50 AM.

The endotoxemia protocols for the 112 healthy volunteers to which the iceman was compared (historical cohort) were identical, with the exception of practicing concentration/meditation. All of these study protocols were approved by the Ethics Committee of the Radboud University Nijmegen Medical Centre and comply with the Declaration of Helsinki, including current revisions and the Good Clinical Practice guidelines. All volunteers signed written informed consent. The circadian variation in cortisol levels did not confound the comparison between the iceman and the historical cohort, because in all endotoxemia studies, LPS was administered between 10-11 AM.

Ex vivo stimulation experiments

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Diegem, Belgium). Cells at a concentration of $5x10^6$/mL in RPMI 1640 medium (Dutch modification; Invitrogen, Paisley, UK), supplemented with 1 mM sodium pyruvate, 2 mM L-Glutamine and 50 μg/mL gentamicin, were incubated in round-bottom 96-wells plates at 37 °C and 5% CO_2. After 24 hrs (for TNF-α and IL-6) or 60 hrs (for IL-10) of incubation with LPS (10 ng/mL), supernatants were collected and stored at −80 °C until further analysis. For macrophage experiments, PBMCs at a concentration of $5x10^6$/ml in the abovementioned RPMI 1640 medium, additionally containing 10% pooled human serum, were kept at 37 °C and 5% CO_2. After 6 days of incubation, cells were stimulated with LPS (10 ng/mL) for 24 hrs, after which supernatants were collected and stored at −80 °C until further analysis. All stimulation experiments were performed in duplicate. LPS (*E. Coli* serotype 055:B5) was purchased from Sigma-Aldrich, and an additional purification step was performed as described previously[29].

Cortisol, catecholamine, and cytokine measurements

Cortisol levels were determined in EDTA plasma using a luminometric immunoassay on a random access analyzer (Architect® *i* System, Abbott, Illinois, USA). The cortisol response was compared with that of a subgroup of the aforementioned cohort of 112 subjects in which cortisol was determined (n=15, NCT00513110)[30]. Blood samples for catecholamine determination were collected into chilled lithium-heparin tubes and were immediately placed on ice. Plasma norepinephrine and epinephrine concentrations were measured using sensitive and specific high-performance liquid chromatography with fluorometric detection as described previously[31]. TNF-α, IL-6, and IL-10 concentrations in EDTA plasma obtained from the endotoxemia experiment

of the iceman and the historical cohort were measured using a simultaneous Luminex assay according to the manufacturer's instructions (Milliplex, Millipore, Billerica, MA, USA). Concentrations of TNF-α, IL-6, and IL-10 in *ex vivo* stimulated cell culture supernatants were determined by commercial ELISA's according to the manufacturer's instructions (TNF-α: R&D systems, Minneapolis, MN, USA; IL-6 & IL-10: Pelikine Compact, Sanquin, Amsterdam, The Netherlands).

Electroencephalography (EEG) analysis

EEG was determined using a standard 21-lead recording with surface Ag/AgCl cup electrodes as described previously[9, 30]. The full-length recording was analyzed visually by an experienced clinical neurophysiologist (NvA) and scored using a five category classification system for septic encephalopathies. Additionally, EEG background activity was monitored during concentration/meditation and the presence of drowsiness or sleep was noted, as were signs of shivering indicated by the presence of tremorous muscle activity artifacts at both the scalp and chest electrodes. For further quantitative analysis at least once per 30 minutes a one-minute artefact-free raw EEG sample (10-second epoch) of the subject lying awake with his eyes closed was selected.

Heart rate variability (HRV) analysis

Short-term HRV (5-min recordings) was measured in supine position and during quiet circumstances. A 3-lead ECG signal was obtained using a Medilog AR12 recorder (Huntleigh Healthcare, Cardiff, UK) and analyzed as described previously[32]. Normalized units of low and high frequency spectral power (LFnu and HFnu, representing sympathetic and parasympathetic modulation, respectively[33]) were calculated by dividing LF or HF power by the sum of LF and HF power. The HRV data of the iceman was compared with a subgroup of the aforementioned cohort of 112 subjects where HRV was measured (n=40, NCT00513110 and NCT00783068)[32].

Muscle sympathetic nerve activity

Muscle sympathetic nerve activity (MSNA) was measured as described before[34]. However, the subject exhibited quite vigorous movement during the concentration/meditation period, which led to dislocation of the electrodes. Therefore, only a reliable baseline recording was obtained.

Data analysis and calculations

Analyses were performed using Graphpad Prism 5.0 (Graphpad Software, San Diego, CA, USA).

Results

Experiment 1. Concentration/meditation during ice immersion

Thirty minutes after the start of concentration/meditation (just before ice-immersion), cortisol levels were already relatively high (0.69 µmol/L at 2.15 PM, which is above the reference limits for both 8 AM and 5 PM which are 0.55 and 0.38 µmol/L in our centre, respectively) and rose to slightly higher levels immediately after the end of the ice immersion period (0.75 µmol/L at 4.05 PM). 30 Minutes later (4.35 PM), cortisol levels decreased to 0.53 µmol/L. LPS-induced cytokine production of *ex vivo* stimulated peripheral blood mononuclear cells (PBMCs) obtained after ice immersion was greatly attenuated compared with PBMCs obtained before (Figure 1A). Both pro- (TNF-α and IL-6) and anti-inflammatory (IL-10) responses were equally affected. This effect was not only observed after stimulation with LPS, but also when PBMCs were stimulated with other (components of) heat-killed pathogens such as *Candida albicans* and *Staphylococcus aureus* (data not shown). Strikingly, in monocyte-derived macrophage cultures stimulated with LPS 6 days after the ice immersion, a similar pattern was observed (Figure 1B).

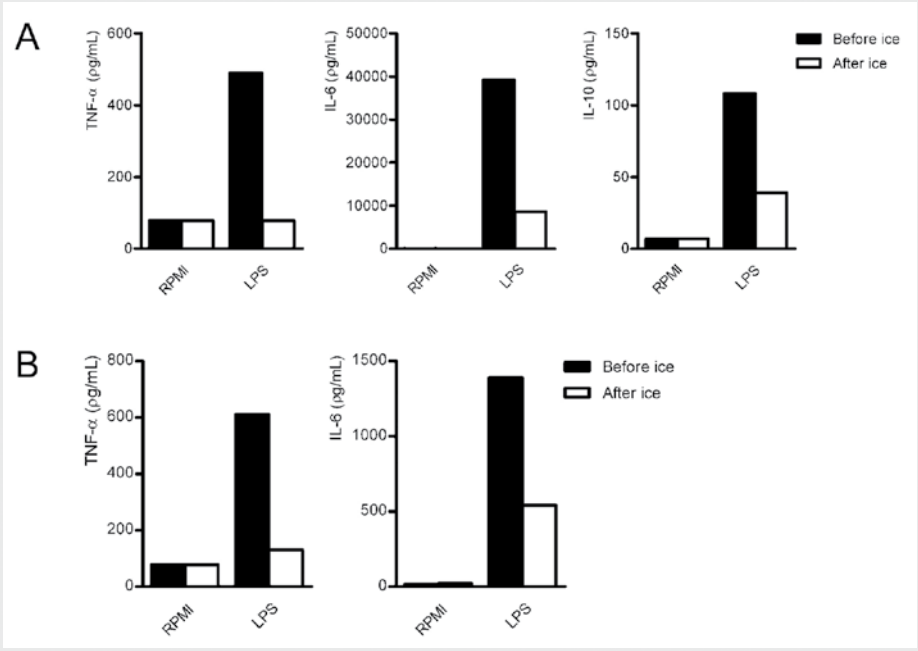

Figure 1 *Production of pro- (TNF-α and IL-6) and anti-inflammatory (IL-10) cytokines in peripheral blood mononuclear cells (A) and monocyte-derived macrophages (B) obtained before and after ice-immersion that were ex vivo stimulated with RPMI (control) or LPS (10 ng/mL) for 24 hours (TNF-α and IL-6) or 60 hours (IL-10). Macrophages were obtained by culturing monocytes for 6 days in the presence of 10% pooled human serum.*

Experiment 2. Concentration/meditation without ice immersion

To investigate whether the remarkable reduction in *ex vivo* cytokine production was an effect of the subject's concentration/meditation technique or a direct effect of the cold exposure, we studied the subject while practicing his technique without exposure to ice. Cortisol and norepinephrine levels were not increased by concentration/meditation (cortisol: sham concentration/meditation: 0.39 μmol/L, concentration/meditation: 0.36 μmol/L; norepinephrine: sham concentration/meditation: 2.12 nmol/L, concentration/meditation: 2.25 nmol/L). In contrast, the plasma epinephrine concentration during sham concentration/meditation was 0.23 nmol/L (normal value: 0.2 nmol/L), and showed a pronounced rise after the concentration/meditation period to 0.36 nmol/L. HRV analysis revealed that, while total spectral power increased considerably during concentration/meditation compared with sham (sham concentration/meditation: 2584 ms^2; concentration/meditation: 5798 ms^2), no differences in cardiac autonomic balance were observed (sham concentration/meditation: LFnu 79%, HFnu 21%; concentration/mediation: LFnu: 77%, HFnu: 23%). The EEG recording showed normal background cortical activity during baseline and sham concentration/meditation (i.e. watching tv). After 6 cycles of concentration/meditation with hyperventilation the EEG background showed slight slowing that turned in to drowsiness after the 8th cycle, and a very short period of stage II sleep before the subject was woken up by ambient noise. No abnormalities were found. Stimulation of PBMCs or macrophages revealed no effects of concentration/meditation on cytokine production *ex vivo* (data not shown).

Experiment 3. Concentration/meditation during human endotoxemia

To determine the effects of concentration/meditation on the inflammatory response *in vivo*, the subject participated in a human endotoxemia experiment while practicing his concentration/meditation technique for three hours starting 30 minutes before LPS administration (from T=-0.5 to T=2.5). MSNA before the start of the concentration/meditation period was: total MSNA: 28.3 ± 2.1 bursts/min; frequency corrected MSNA: 47.5 ± 5.5 bursts/100 beats (mean ± SD), which is comparable to what was previously reported in subjects under resting conditions[35]. Figure 2 depicts the breathing and hemodynamic pattern during the first 30 minutes of concentration/meditation (T=-0.5 - T=0). The technique is characterized by cycles consisting of a few minutes of hyperventilation followed by breath holding for up to 1-2 minutes. 30 Minutes after the start of concentration/meditation (T=0), blood gas analysis revealed a distinct alkalosis in combination with a low pCO_2 (Table 2), consistent with hyperventilation. This pattern was even more pronounced at T=1. After cessation of concentration/meditation, blood gas values gradually returned to normal.

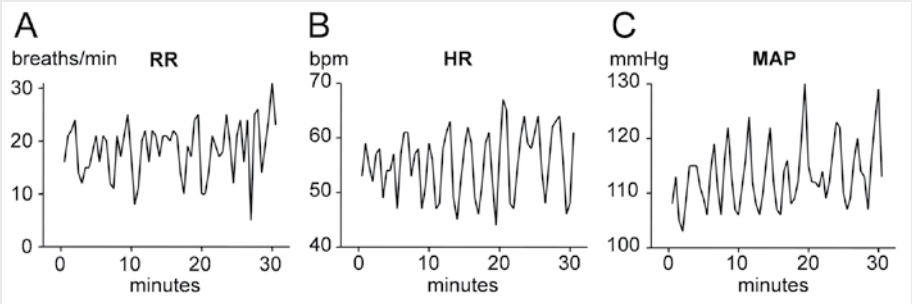

Figure 2 *Respiratory rate (A), heart rate (B) and mean arterial pressure (C) during the first 30 minutes of concentration/meditation (T=-0.5 – T=0).*

Table 2 *Iceman's blood gas parameters during human endotoxemia*

Time (hours post-LPS)	pH	pCO_2 (kPa)
0	7.56	3.1
1	7.57	2.9
2	7.54	3.8
4	7.48	4.0
6	7.50	3.7
8	7.46	4.3

LPS administration in the iceman resulted in remarkably few symptoms, he only reported a mild headache for 10 minutes at T=1.5 (the time-point at which the symptom score normally peaks), yielding a symptom score of 1 compared with a symptom score of 6.6 ± 2.8 (mean ± SD) at T=1.5 in the 112 previously studied subjects. Of note, only one out of the 112 persons studied before exhibited an equally low symptom score. The LPS-induced rise in body temperature was comparable to that of the 112 previously studied subjects (iceman: 1.3 °C; 112 subjects: 1.6 ± 0.6 °C [mean ± SD]). The LPS-induced decrease in mean arterial pressure and increase in heart rate was similar to that of the historical cohort as well. Plasma norepinephrine levels peaked immediately after the start of concentration/meditation and gradually returned to baseline levels (Figure 3A). Epinephrine showed a similar, but delayed pattern: levels started to rise at T=0 and peaked one hour after LPS administration (Figure 3A). Plasma cortisol levels at the time of LPS administration were similar to those of 15 subjects (a subgroup from the historical cohort of 112 subjects) not practicing concentration/meditation (Figure 3B). However, the increase in cortisol after LPS administration was much more pronounced in the iceman compared with

the other subjects. The area under curve (AUC) of the iceman's cortisol time-course (only using time-points also measured in the previously studied subjects: T=0, T=2, T=4 and T=8) was outside the reference range (mean + 1.96*SD) of the other 15 subjects.

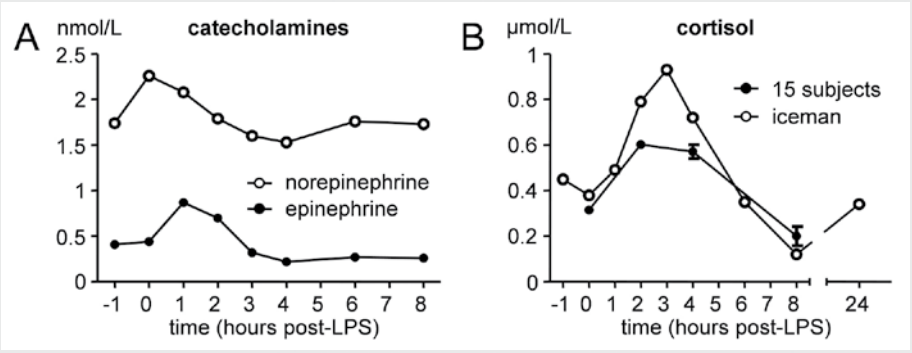

Figure 3 *Plasma levels of catecholamines (A) and cortisol (B) during experimental human endotoxemia. Cortisol data (mean ± SEM) of 15 previously studied subjects are shown for comparison.*

LPS-induced plasma levels of inflammatory cytokines were remarkably low in the iceman (Figure 4). The AUCs of the 112 subjects were ranked and the iceman's AUC cytokine response lied within the 18th percentile for TNF-α, 5th percentile for IL-6 and 13th percentile for IL-10.

Besides an initial increase in total spectral power and SDNN (which is the time-domain correlate of total spectral power), no clear-cut effects of concentration/meditation on HRV indices were observed (SDNN and total spectral power depicted in Figure 5). Furthermore, the common decrease in HRV indices following LPS administration was also present in the iceman and to a similar extent as in 40 previously studied subjects.

The EEG showed normal cortical background activity at baseline, with an occipitoparietotemporal alpha rhythm of 8.8 - 9.3 Hz. During the first 4 hours of the experiment, including baseline, LPS infusion, and meditation period afterwards, there were no signs of encephalopathy, nor a drop in vigilance shown as either drowsiness or sleep. During the subjects' meditation cycle short periods (10-15 s) of focused attention were found in the EEG, reflected by a disappearance of the occipitoparietal alpha rhythm and diffuse irregular beta frequencies associated with cognitive activity. Quantitative EEG showed no relevant changes during the sample periods.

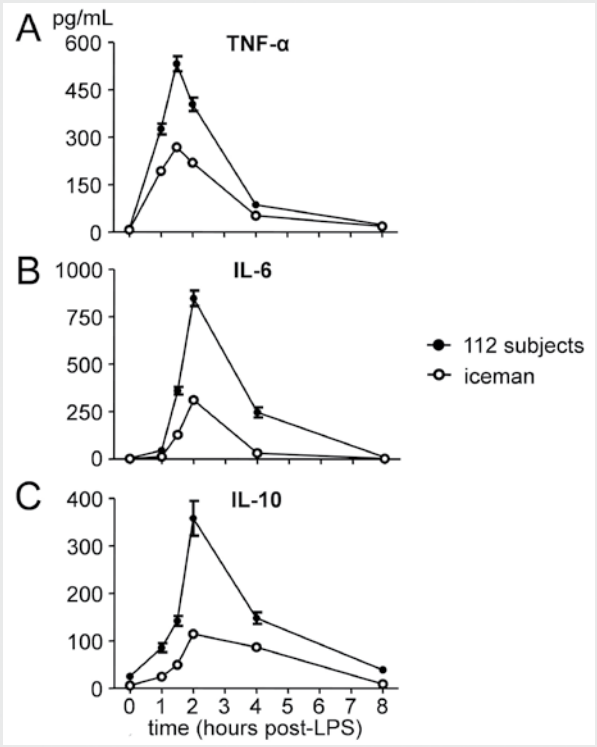

Figure 4
Plasma levels of pro- (A: TNF-α and *B: IL-6) and anti-inflammatory (C: IL-10) cytokines during experimental human endotoxemia. Data (mean ± SEM) of 112 previously studied subjects are shown for comparison.*

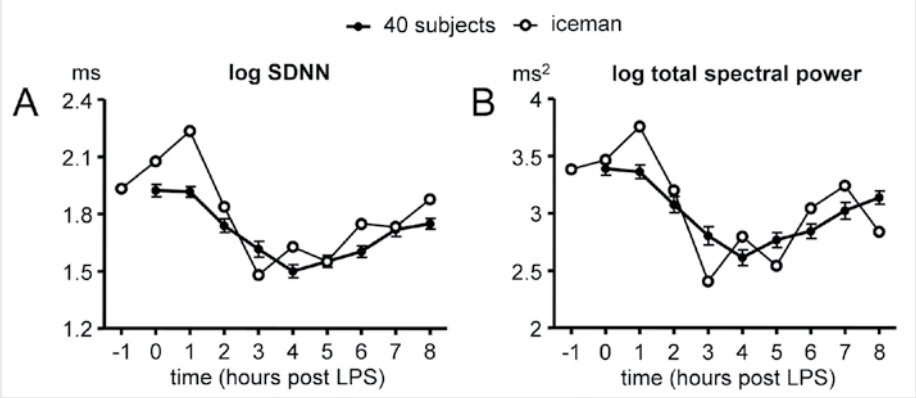

Figure 5 *Log SDNN (A) and total spectral power (B) heart rate variability indices during experimental human endotoxemia. Data (mean ± SEM) of 40 previously studied subjects are shown for comparison.*

Discussion

In the present study, we describe the effects of a special concentration/meditation technique developed by an individual known as 'the iceman' on the autonomic nervous system and innate immune response. This individual holds several world records with regard to extreme tolerance to cold exposure. Concentration/meditation during ice immersion resulted in high cortisol levels and suppressed cytokine production *ex vivo*. Although elevated levels of epinephrine were found during concentration/meditation without ice immersion, no effects on norepinephrine, cortisol levels, or the *ex vivo* cytokine response were observed. Finally, we demonstrate that concentration/meditation during experimental endotoxemia resulted in elevated levels of (nor) epinephrine and an amplified cortisol response, associated with a remarkably mild inflammatory response.

In the presence of an exogenous stimulus (ice immersion and LPS administration), cortisol levels well above the reference values were observed upon concentration/meditation. In the endotoxemia study, this was preceded by an increase in (nor) epinephrine. These data indicate that the iceman's concentration/meditation technique evokes a stress response characterized by activation of the sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA) axis. Both catecholamines and cortisol are well-known immunosupressants; administration of both of these mediators has been shown to attenuate the inflammatory response *in vivo* in the human endotoxemia model[4-8]. Furthermore, in heart failure patients, plasma cortisol levels correlated negatively with *ex vivo* cytokine production[36]. Therefore, this concentration/meditation-induced stress response might explain the observed immunosupression *ex vivo* in the ice immersion experiment and the remarkably mild inflammatory response *in vivo* during endotoxemia.

Increased cortisol levels following LPS administration have been reported by ourselves[30] and others[7, 37]. Cortisol was already elevated in the sample obtained after 30 minutes of concentration/meditation, but before ice immersion. While baseline cortisol levels in the endotoxemia experiment were comparable to those of the young healthy volunteers that were previously studied, the LPS-induced increase was more pronounced than in any of the subjects we have previously studied. The remarkable differences in the *ex vivo* cytokine responses between the leukocytes obtained before and after ice immersion may be explained by the fact that the leukocytes obtained after ice-immersion had been subjected to high cortisol and/or catecholamine levels for a prolonged period (>2 hours) compared with the cells obtained before.

Interestingly, macrophages cultured from monocytes derived after ice immersion that were stimulated with LPS 6 days later still showed a vastly attenuated cytokine response. It might be speculated that monocytes exposed to high concentrations of cortisol and/or catecholamines are deactivated for a prolonged period. Along these lines, it has been shown that short incubations with the corticosteroid dexamethasone have long-lasting effects in monocytes[38].

In the endotoxemia experiment in the iceman, catecholamines levels peaked at the time of endotoxin administration (norepinephrine) and 1 hour after endotoxin (epinephrine), thereafter declining to baseline or even below baseline levels. In contrast, others have demonstrated peak catecholamine levels 1.5 (norepinephrine) and 3 (epinephrine) hours following endotoxin administration[35]. This strengthens the notion that the concentration/meditation technique is responsible for this increase in (nor)epinephrine. The absence of increased levels at 3 hours post-endotoxin might be the result of the relatively mild inflammatory response in the iceman.

The iceman reported exceptionally few symptoms during endotoxemia. However, the symptom score is a highly subjective parameter and the iceman may be used to extreme circumstances, which makes it difficult to compare these results to those of the previously studied healthy volunteers. Despite a very mild inflammatory response, the LPS-induced changes in heart rate, blood pressure, and temperature were comparable to those of the previously studied subjects, which appears conflicting. However, we have recently shown that the cytokine response does not correlate with hemodynamic parameters, temperature, and illness score during endotoxemia[26].

Meditation is generally associated with reduction of stress and catecholamine/cortisol levels[16, 19, 39-41]. These discrepant results compared with our study might be explained by the fact that the other studies, in contrast to ours, have evaluated effects of meditation in the long term (weeks to months). Furthermore, the concentration/meditation technique practiced by the iceman is very different to those employed in other studies. This particular technique is not targeted at relaxation of the body, but rather appears to activate it. This is supported by data from a previous report showing that the iceman's oxygen consumption doubled during ice immersion[42], indicating that practicing this technique results in increased metabolism. The increased energy expenditure (and therefore heat production) might play a role in the subject's extreme tolerance towards cold; despite 80 minutes of full-body ice immersion and significant heat loss through the skin, core body temperature was maintained[42]. Furthermore,

the iceman reported that ice-immersion results in significant weight-loss (personal communication). Signs of increased energy expenditure were also apparent during the last hours of the endotoxemia experiment; after cessation of concentration/meditation the subject was noticeably tired and very hungry. Hyperventilation (followed by breath holding) is an obvious element of the iceman's concentration/meditation technique and might have direct effects on sympathetic nervous system activity and/or stress hormones. One study in healthy subjects found relatively large increases in (nor) epinephrine, but only a marginal increase in cortisol immediately after 20 minutes of hyperventilation[43]. In another study, three minutes of hyperventilation resulting in a pH of 7.65 increased cortisol levels 1.5-fold[44]. Interestingly, 3-minute hyperventilation followed by maximal voluntary breath holding resulted in an even more pronounced and longer-lasting increase in cortisol[44]. The remarkably low cytokine response is unlikely to result from the hyperventilation-induced low pCO_2 and high pH levels, because hypocapnic alkalosis, as opposed to hypercapnic acidosis[45], is not associated with anti-inflammatory effects.

With respect to the subject's particular concentration/meditation technique, our findings might better be compared with studies investigating the effects of acute stress on the inflammatory response. Subjects exposed to a standardized laboratory stressor (Trier social stress test, TSST) displayed elevated levels of cortisol[40]. Furthermore, it was recently reported that acute psychological stress results in increased levels of norepinephrine and cortisol accompanied by decreased TNF-α mRNA [46] and protein[47] expression in *ex vivo* LPS-stimulated leukocytes. In accordance, a very recent and remarkable study demonstrated a profound rise in catecholamines and cortisol during bungee-jumping, which was associated with significantly reduced cytokine production in *ex vivo* LPS-stimulated whole blood[48]. These results are in line with ours. In contrast to acute stressors, prolonged stress, such as in depression or post-traumatic stress syndrome, is associated with increased levels of inflammatory cytokines[49-51]. A possible explanation for this might be the opposing effects of short- and long-term exposure to catecholamines. While acute exposure to or infusion of β-adrenergic agonists attenuates proinflammatory cytokine production *in vitro*[52] and *in vivo*[5, 6], prolonged exposure increases production of these mediators *in vitro*[52]. In accordance, 24-hour norepinephrine infusion results in increased plasma concentrations of proinflammatory mediators in rats[53]. Another explanation for increased cytokine levels upon long-term stress might be development of glucocorticoid and/or catecholamine insensitivity due to receptor downregulation or desensitization. Loss of leukocyte glucocorticoid receptors is well-described in patients with depression[54, 55]. Furthermore, sustained

heightened activation of the sympathetic nervous system, reflected by high levels of circulating catecholamines, results in β-receptor downregulation and uncoupling in congestive heart failure patients[56, 57].

We did find an increase in HRV total spectral power during concentration/meditation, which was not surprising in light of the large fluctuations in heart rate during this period. However, we did not find evidence for increased sympathetic activity using HRV analysis. It has been reported that while certain HRV indices (in particular high frequency power) correlate well with parasympathetic modulation[58], sympathetic correlates of HRV are still disputed[59]. Therefore, HRV analysis might not be an appropriate tool to investigate sympathetic activity. Furthermore, HRV is known to be affected by breathing patterns/frequency[60], therefore, the iceman's extremely irregular breathing may have influenced the measurements.

Our study has several limitations. First an foremost, we describe a set of studies on a single subject making it impossible to determine a cause effects relationship between concentration/meditation and the autonomic nervous system and/or the innate immune response. Nevertheless, case reports with remarkable findings can yield valuable information[61] and are important in hypothesis generation for further research. This study is further limited by the absence of an additional endotoxemia experiment in which the iceman did not practice his concentration/meditation technique. This is rather a limitation of the human endotoxemia model; our group has demonstrated that repeated LPS administrations result in the development of endotoxin tolerance[26, 62], therefore, data from a cross-over experiment would be impossible to interpret. Finally, the iceman's age is considerably higher than that of the 112 healthy volunteers used for comparison. This could have biased our results. However, a human endotoxemia study comparing a group with a median age of 24 to a group with a median age of 66 revealed that ageing was associated with a slightly higher cytokine response to LPS[63]. Therefore, age appears not to explain the relatively low cytokine levels observed in the iceman.

In conclusion, the iceman's autodidact concentration/meditation technique appears to result in a consciously controlled stress response, characterized by sympathetic nervous system activation and subsequent catecholamine/cortisol release. This response appears to attenuate the innate immune response. The iceman claims that he can teach others this technique within a relatively short time-frame (one week). Therefore, further investigations should establish whether the results obtained in the iceman can be reproduced in larger groups of individuals.

Acknowledgements

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Part three

Methodological considerations

Chapter 11

Influence of different breathing patterns on heart rate variability indices and reproducibility during experimental endotoxaemia in human subjects

Matthijs Kox, Jan C. Pompe, Johannes G. van der Hoeven,
Cornelia W. Hoedemaekers, Peter Pickkers

Clinical Science 2011; 121(5):215-22

Abstract

Background HRV (heart rate variability) analysis is a widely employed method to assess cardiac autonomic nervous system activity. Accurate HRV measurement is critical to its value as a diagnostic and prognostic tool. Different breathing patterns may affect HRV, but results obtained under static conditions are conflicting. HRV indices decrease considerably during systemic inflammation evoked by experimental endotoxaemia, enabling the determination of the effects of different breathing patterns on HRV in a dynamic setting. We investigated the impact of different breathing patterns on short-term HRV measurements during experimental endotoxaemia. Furthermore, we assessed whether paced breathing improved HRV reproducibility.

Methods Twelve healthy male volunteers received an intravenous bolus (2 ng/kg of body weight) of endotoxin (LPS [lipopolysaccharide], derived from *Escherichia coli* O:113) on two occasions with an interval of 2 weeks (visits 1 and 2). Five-minute HRV recordings were performed just prior to LPS administration and hourly thereafter until 8 hours post-LPS. Three breathing protocols were employed every hour: 1) spontaneous breathing, 2) metronome-guided breathing at the subject's normal respiratory rate (paced), and 3) metronome-guided breathing at 150% of the subject's normal respiratory rate (mild hyperventilation).

Results LPS administration resulted in a sharp decrease of all of the HRV indices measured, which was similar during both LPS administrations. Neither paced breathing nor mild hyperventilation influenced HRV indices compared with spontaneous breathing. Paced breathing did not improve reproducibility, as it did not exert a significant effect on intra-subject coefficients of variation and intra-class correlation coefficients (calculated between both visits).

Conclusions Over a wide range of HRV magnitudes during experimental endotoxaemia, neither paced breathing nor mild hyperventilation affected HRV indices. Moreover, paced breathing did not result in a significant improvement in reproducibility. Therefore, employing a paced breathing protocol is not required to obtain valid HRV data during endotoxaemia.

Introduction

HRV (heart rate variability) analysis is a widely employed method to assess cardiac autonomic nervous system activity[1]. Owing to its noninvasive nature, HRV, and especially short-term HRV recordings (5 min), are easily obtainable from patients or healthy volunteers. Changes in HRV, in particular a reduction in HRV, are associated with increased mortality after myocardial infarction and heart failure[2-5]. In systemically inflamed critically ill patients, such as in sepsis, HRV is diminished and inversely correlated with disease severity[6-9]. Moreover, reduced HRV is a predictor of MODS (multi-organ dysfunction syndrome) and death in these patients[10, 11]. Therefore, HRV analysis could represent a valuable diagnostic and prognostic tool.

Accurate measurements of HRV are critical for its interpretation and clinical use. For example, it is well-documented that postural changes have a major impact on HRV[12-14]. Therefore, all HRV measurements within a study should be performed in the same position and data cannot be compared with subjects measured in another position. Less attention has been paid to effects of different breathing patterns, although this is considered to have a significant impact on HRV. The parasympathetic component of HRV (respiratory sinus arrhythmia, reflected by HF [high-frequency] power and r-MSSD [root-mean-square differences of successive NN (normal-to-normal) intervals]) is thought to be predominantly mediated by respiration-induced blood pressure changes, which are sensed by carotid baroreceptors leading to (de)activation of cardiac vagal fibres[15, 16]. An increased respiratory rate has been linked to a reduction in LF (low-frequency) and HF power[17]; however, increased HF and reduced LF power with increased breathing rate have also been reported[18]. Metronome-guided (paced) breathing appears to result in increased HF and reduced LF power compared with spontaneous breathing in some[19, 20], but not all studies[21]. In addition, paced breathing may[19, 22] or may not[23, 24] increase HRV analysis reproducibility. These conflicting results indicate that the effects of different breathing patterns on HRV are not well established yet.

To date, all studies investigating effects of different breathing patterns on HRV have been performed under relatively static conditions, i.e. when HRV does not vary to a large extent. Experimental endotoxaemia (LPS [lipopolysaccharide] administration in healthy volunteers) is a well-characterized standardized model of systemic inflammation widely used to study the innate immune response in man[25]. HRV indices greatly fluctuate during experimental endotoxaemia[26-29], which makes it a very suitable model to investigate the effects of different breathing patterns on

HRV in a dynamic setting. Therefore, the aim of our present study was to investigate whether paced breathing or mild hyperventilation affect short-term HRV indices during experimental endotoxaemia. Furthermore, because subjects in our study were administered endotoxin twice with an interval of 2 weeks, we assessed whether paced breathing improved HRV measurement reproducibility during experimental endotoxaemia.

Materials and methods

Subjects

Twelve healthy young male non-smoking volunteers were enrolled in a cross-over experimental endotoxaemia study (Clinical Trial Register number NCT00783068) in which they received LPS twice (LPS visits 1 & 2) with a mean interval of 14 days (range, 11-18 days). The study protocol was approved by the local ethics committee of the Radboud University Nijmegen Medical Centre and is in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Written informed consent was obtained from all study participants. The findings of the physical examinations, electrocardiography, and routine laboratory studies on all the volunteers before the start of the experiment showed normal results. Volunteers were not taking any prescription medications, and they were negative for hepatitis B surface antigen and HIV infection.

Experimental endotoxaemia model

Subjects refrained from food 12 hours before the start of the experiment, and from caffeine or alcohol containing substances 24 hours before the start of the experiment. The experiments were performed at the research unit of the intensive care department, with subjects in supine position. After local anaesthesia (lidocaine HCl 20 mg/ml), the radial artery was cannulated using a 20-Gauge arterial catheter (Angiocath, Becton Dickinson, Sandy UT, USA) and connected to an arterial pressure monitoring set (Edwards Lifesciences LLC, Irvine CA, USA), connected to a Phillips IntelliVue MP70 monitor (Philips Medical Systems, Eindhoven, The Netherlands). The arterial line was used for continuous monitoring of blood pressure and blood sampling. A cannula was placed in the antecubital vein to permit infusion of 2.5% glucose/0.45% saline solution; subjects received a bolus of 1.5 litres during 1 hour before LPS infusion (prehydration), followed by 150 ml/h until 6 hours after LPS infusion and 75 ml/h until the end of the experiment. Heart rate was continuously monitored using a three-lead ECG. Body temperature was measured every 30 minutes using an infrared tympanic thermometer (FirstTemp Genius, Sherwood Medical,

Crawley/Sussex, UK). Leukocyte counts were determined using flow cytometry (Sysmex XE-2100; Goffin Meyvis, Etten-Leur, the Netherlands). U.S. Reference *Escherichia coli* endotoxin (*E. coli* O:113, Clinical Center Reference Endotoxin, NIH [National Institute of Health], Bethesda, MD) was used. Lot Ec-5 endotoxin, supplied as a lyophilized powder, was reconstituted in 5 ml saline 0.9% for injection and vortex-mixed for at least 10 minutes after reconstitution. The endotoxin solution was administered as an intravenous bolus injection at a dose of 2 ng/kg of body weight.

HRV measurements

HRV was measured hourly by 5-min recordings starting just before LPS administration (T=0 or baseline) up to 8 hours after LPS administration. HRV was measured in supine position and in a quiet environment. HRV measurements were performed at the same time of day during both visits (T=0 at 11 am). Each subject's normal spontaneous breathing rate was determined in rest before LPS administration. We employed three breathing protocols, repeated every hour: 1) 5 minutes of spontaneous breathing, 2) five minutes of metronome-guided breathing at the subject's spontaneous respiratory rate (paced) and 3) five minutes of metronome-guided breathing at 150% of the subject's spontaneous respiratory rate (mild hyperventilation). A three-lead ECG signal was obtained using a Medilog AR12 recorder (Huntleigh Healthcare, Cardiff, UK). R-peak position was determined at a sample rate of 4096 Hz. HRV was analyzed using dedicated software (Medilog Darwin HRV, Huntleigh Healthcare, Cardiff, UK). In each 5-min recording, QRS complexes were detected, and only NN beat intervals were tabulated, yielding an interval tachogram (from which the mean heart rate was calculated). Recordings with artefacts such as extrasystolic or supraventricular beats, or other arrhythmias comprising more than 5% of the total epoch were discarded. After linear detrending, power spectral density was determined by fast Fourier transformation of interval tachograms using the Welch method and a FFT (fast Fourier transform) width of 1024. We chose to solely analyze 'raw' HRV indices, not calculated indices such as LF/HF, LFnu (LF power in normalized units) and HFnu (HF power in normalized units), because effects on calculated values are a direct consequence of their impact on raw values and therefore do not provide additional information. We analyzed time domain indices SDNN (SD of NN intervals) and r-MSSD, and frequency domain indices LF power, HF power and total power. VLF (very-LF) power (0.0033-0.04 Hz) was not analyzed because this parameter cannot be reliably obtained from 5-min recordings[30]. The HRV indices analyzed, their definitions, and their physiological correlates are listed in Table 1.

Table 1 *HRV indices definitions and physiological correlates*

domain	*HRV index (unit)*	*Definition*	*Physiological correlate*
Time domain	SDNN (ms)	Standard deviation of all NN intervals	Sympathetic and parasympathetic activity
	r-MSSD (ms)	Square root of the mean squared difference of successive NN intervals	Parasympathetic activity
Frequency domain	LF (ms^2)	Low frequency power (0.04-0.15 Hz)	Sympathetic and parasympathetic activity
	HF (ms^2)	High frequency power (0.15-0.4 Hz)	Parasympathetic activity
	total (ms^2)	Total spectral power (0-0.15 Hz)	Sympathetic and parasympathetic activity

ms, milliseconds.

Calculations and statistical analysis

None of the measured HRV indices was normally distributed (calculated using the Shapiro-Wilk test) and therefore they were log-transformed. Comparison of the HRV indices between both LPS visits were made by repeated measures two-way ANOVA. For Bland-Altman analysis, paced breathing was designated as the gold standard[31]. The Grubbs test (extreme studentized deviate method) was performed to test for significant outliers. Percentage intraCVs (intra-subject coefficients of variation) were calculated by the following formula: 100 X (standard deviation [visit 1, visit 2] / mean [visit1, visit 2]). We performed a power calculation to determine the power achieved in case of actual intraCV differences of 1 and 2%. Using a SD of 4% [32], a sample size of 108 observations (12 subjects measured at nine time points), and a two-tailed α of 0.05, we would achieve a 73,1% power to detect an intraCV difference of 1%, and 99.9% to detect an intraCV difference of 2%. Percentage intraCVs between spontaneous and paced breathing were tested by paired, two-sided Student's t-tests. A p-value <0.05 was considered significant. Statistical analysis was performed using Graphpad Prism 5 (Graphpad software, San Diego, CA, USA) and MedCalc 11.3.1.0 (MedCalc software, Mariakerke, Belgium).

Results

Effects of endotoxin administration on hemodynamic, clinical, haematological, and HRV parameters

LPS administration resulted in a typical changes in haemodynamic, clinical, and haematological parameters (data of visit 1 depicted in Figure 1), as well as increased plasma levels of pro- and anti-inflammatory cytokines (data not shown).

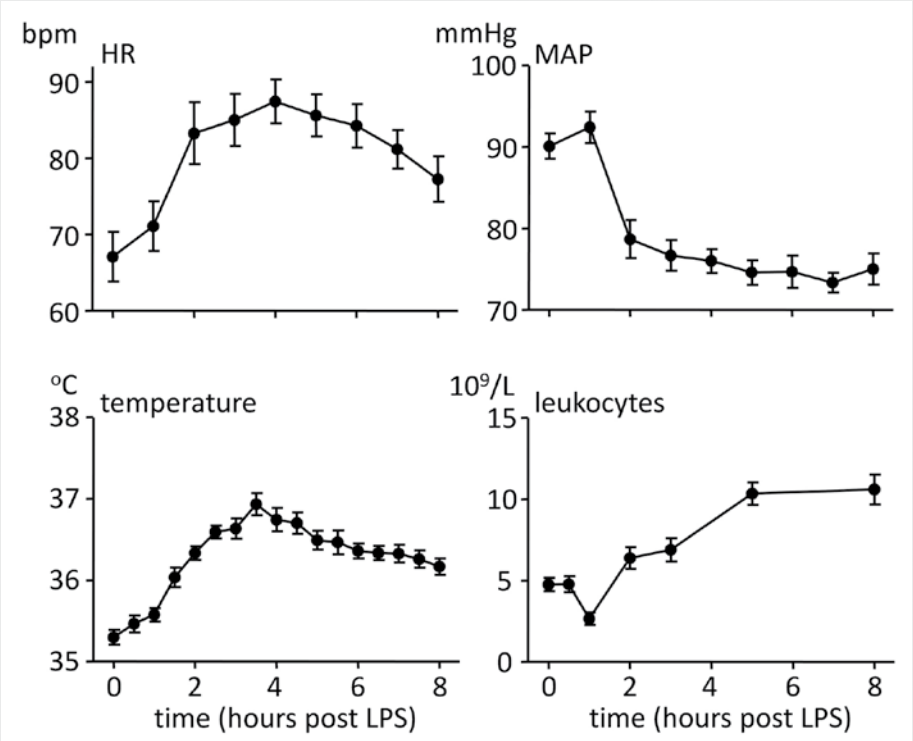

Figure 1 *Haemodynamic, clinical, and haematological parameters during experimental endotoxaemia. Values are mean ± SEM of 12 subjects during LPS visit 1. HR, heart rate; MAP, mean arterial pressure.*

Endotoxaemia also resulted in a typical sharp decrease of all HRV indices (data of spontaneously breathing subjects during visit 1 are depicted in Figure 2). There were no differences in the hemodynamic, clinical, haematological, and HRV responses to LPS between both visits (data not shown).

Influence of paced breathing on HRV during endotoxaemia

We constructed Bland-Altman plots of the difference between spontaneous breathing vs. metronome-guided paced breathing at a subject's normal respiratory rate (mean rate 11, range 8-15 breaths/min) against their average values from 0 to 8 hours post-LPS administration (Figure 3A). All indices displayed a symmetrical distribution around the zero line, indicating the absence of a systematic error. The bias was not dependent on HRV magnitude which greatly decreased following LPS administration.

Influence of mild hyperventilation on HRV during endotoxaemia

As depicted in Figure 3B, there was no difference in HRV indices between paced breathing at a subject's normal respiratory rate compared with paced breathing at 150% of this rate (mean rate 17, range 12-22 breaths/min) during experimental endotoxaemia. Again, the bias was not dependent on HRV magnitude. In the subject with the lowest normal respiratory rate (8 breaths/min), a large increase in HF power was observed during mild hyperventilation (12 breaths/min; bias: 20.6%, significant outlier compared with the 11 other subjects whose mean bias was -3.2% with a range of -11.6 to 3.6%).

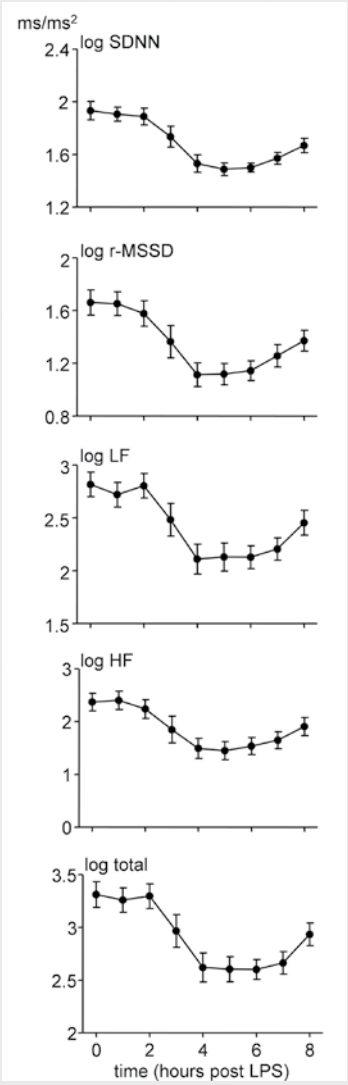

Figure 2 *HRV indices during experimental endotoxaemia. Values are mean ± SEM of 12 subjects during LPS visit 1. ms indicates milliseconds. Log SDNN and r-MSSD are in ms; log LF, HF, and total are in ms².*

Effect of paced breathing on HRV measurement reproducibility

IntraCVs (calculated between visit 1 and 2) did not differ between paced and spontaneous breathing (Table 2). Similarly, ICCs (intra-class correlation coefficients) of paced and spontaneous breathing were not significantly different.

Discussion

In the present study, we investigated the effects of different breathing patterns on short-term HRV and its reproducibility in a dynamic setting during experimental endotoxaemia in humans. Compared with spontaneous breathing, neither paced breathing nor mild hyperventilation affected HRV indices significantly. Furthermore, paced breathing did not improve HRV reproducibility.

To the best of our knowledge, this is the first study assessing the effects of different breathing patterns on HRV during experimental endotoxaemia. The effects of endotoxin administra-

tion on HRV are well-established. Similar to other studies[26-29], we observed a distinct decrease in all measured HRV indices. Consequently, the experimental endotoxaemia model uniquely allowed us to investigate the effects of different breathing patterns on HRV and its reproducibility in a wide range of HRV magnitudes, as opposed to previous studies that examined it during relatively static conditions[17-19, 21-24, 32, 33].

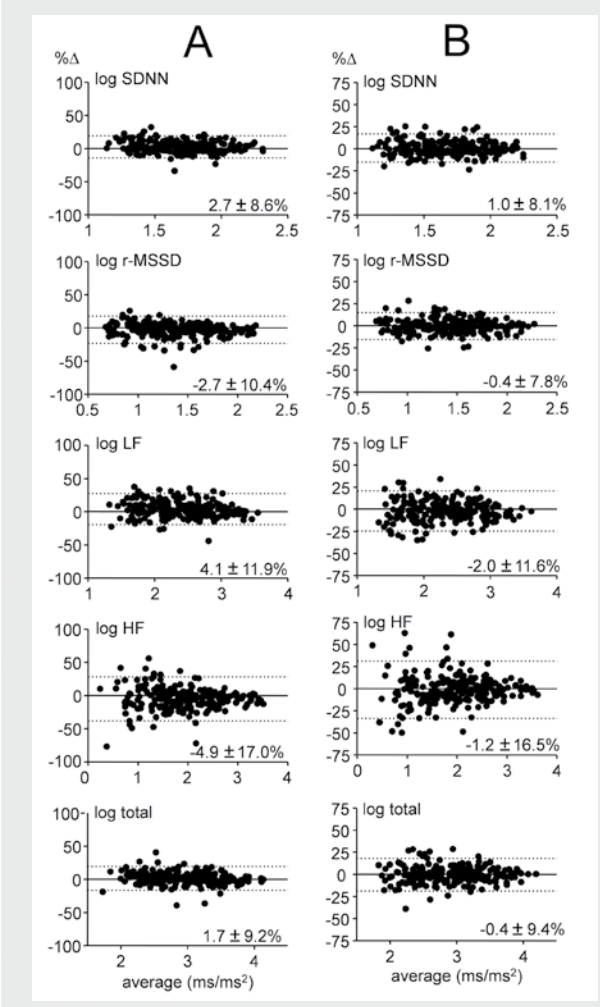

Figure 3 *Bland-Altman plots of A) the % bias (100*[spontaneous-paced]/average on the y-axis) of HRV indices between spontaneous breathing and paced breathing against their average (x-axis) during experimental endotoxaemia. B) the % bias (100*[hyperventilation-paced]/average on the y-axis) of HRV indices between paced breathing and paced breathing at 150% of this rate against their average (x-axis) during experimental endotoxaemia.*
Dotted lines indicate upper and lower 95% limits of agreement. Data from 12 subjects who were administered LPS on two separate occasions (visit 1 and visit 2) with an interval of two weeks are depicted. HRV indices (SDNN and r-MSSD in ms; LF, HF, and total power in ms²) were measured during spontaneous and paced breathing, and mild hyperventilation every hour starting at T=0 until T=8, yielding a total of 216 data points. Mean ± SD *bias is shown in each panel.*

Table 2 *% intraCVs and intra-class correlation coefficients (ICC) of HRV indices between spontaneous and paced breathing during experimental endotoxaemia*

HRV index	*reproducibility measure*	*spontaneous (95% CI)*	*paced (95% CI)*
log SDNN	% intraCV	5.78 (4.83 – 6.73)	6.69 (5.73 – 7.65)
	ICC	0.78 (0.68 – 0.84)	0.78 (0.69 – 0.85)
log r-MSSD	% intraCV	9.11 (7.53 – 10.70)	8.32 (6.90 – 9.74)
	ICC	0.82 (0.74 – 0.87)	0.86 (0.79 – 0.91)
log LF	% intraCV	9.45 (8.01 – 10.89)	8.45 (7.16 – 9.74)
	ICC	0.69 (0.58 – 0.78)	0.79 (0.70 – 0.85)
log HF	% intraCV	13.86 (11.08 – 16.65)	11.57 (9.12 – 14.01)
	ICC	0.83 (0.76 – 0.88)	0.88 (0.83 – 0.92)
log total	% intraCV	7.00 (5.85 – 8.15)	6.80 (5.78 – 7.81)
	ICC	0.74 (0.63 – 0.81)	0.81 (0.72 – 0.87)

Values of 12 subjects who were administered LPS on two separate occasions (visits 1 and 2) with an interval of 2 weeks are listed. HRV indices were measured during spontaneous and paced breathing every hour starting at T=0 until T=8, yielding a total of 108 paired observations from which ICCs and % intraCVs were calculated.

Our first objective was to investigate the effects of different breathing patterns on HRV. We employed a paced breathing protocol in which subjects were paced at their normal respiratory rate, measured in rest before LPS administration. In all other investigations on the subject, a fixed respiratory rate of 12 or 15 breaths/min was employed[19, 21, 32-34]. Paced breathing has been shown to lead to an increased HF and a decreased LF power[19, 32, 33], whereas others found no differences[21, 34]. The absence of a significant bias during spontaneous breathing in our present study could result from the fact that subjects breathed extremely regularly during the spontaneous breathing measurements, implying that breathing patterns were virtually identical during spontaneous and paced breathing measurements. However, we did not find any differences when the subjects were paced at 150% of their normal respiratory rate either. Interestingly, the only subject in whom a relatively large bias (increased HF power) was observed upon mild hyperventilation had a low spontaneous respiratory rate of 8 breaths/min. This observation can be explained by the fact that this subject's respiratory rate corresponds to a frequency of 0.13 Hz, which is just outside of the HF power spectrum (0.15-0.4 Hz), while all other subjects' spontaneous breathing frequencies were within the HF band. Mild hyperventilation moved this subject's breathing frequency well within the HF band (0.2 Hz). These findings are corroborated by a recent investigation in which subjects, as part of a HRV biofeedback protocol, were paced at low respiratory frequencies outside the HF band (0.1 Hz) during endotoxaemia[35]. In these subjects, large differences in HRV parameters were found compared with subjects paced at 0.25 Hz. Effects of paced respiration (within the HF band) on HRV might also be observed in subjects

breathing spontaneously at frequencies above 0.4 Hz, which has been reported during experimental endotoxaemia[26]. However, we can only speculate on this, because we did not measure the spontaneous respiratory rate throughout the study protocol.

The second objective of this study was to determine the effects of paced breathing on HRV measurement reproducibility during repeated experimental endotoxaemia. There is a large body of literature on the stability of HRV over time and the effect of paced breathing on it. Although the definition of a categorical rating of relative reproducibility based on ICC is still controversial, values found during spontaneous breathing in the present study can be considered moderate-to-good (0.69-0.83)[22, 36, 37]. These results are in line with those found by others, using intervals between HRV measurements of 1 day[22, 23], 5 days[38], and 3 weeks[24]. Paced breathing did not result in significant reproducibility improvements. Our findings are in line with several other investigations, where either no differences or small nonsignificant ICC & intraCV improvements were found with measurement intervals of 1 day[22, 23], 5 days[38], 3 weeks[24], and two months[33]. In one small study, significantly reduced intraCVs were found for LF and HF power for paced breathing (12 breaths/min) compared with spontaneous breathing[19]. However, in that study, indices were not log-transformed, which is surprising in light of the commonly described skewed distribution of HRV indices[22, 33, 38]. By definition, skewed data results in incorrect and falsely high SD, which consequently overestimates the variation of the measurements.

In the present study, we provide strong evidence for the lack of an effect of paced breathing or mild hyperventilation on HRV in healthy subjects exposed to experimental endotoxaemia. It is not clear, however, whether our findings can be extrapolated to different patient groups, because several conditions might alter the relationship between respiration and HRV. For instance, hypertension and diabetes are associated with a decreased baroreflex sensitivity, which may significantly affect the influence of respiration on the parasympathetic component of HRV[39, 40].

In conclusion, over a wide range of HRV magnitudes during experimental endotoxaemia, paced breathing and mild hyperventilation did not affect HRV indices, provided that breathing frequencies lay within the HF band. Furthermore, paced breathing did not improve HRV measurement reproducibility. Therefore, employing a paced breathing protocol is not required to obtain valid HRV data during experimental endotoxaemia. In subjects whose respiratory frequency lies outside the HF band, paced breathing at frequencies within the HF band might be considered.

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Chapter 12a

Differential *ex vivo* and *in vivo* endotoxin tolerance kinetics following human endotoxemia

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Abstract

Background Endotoxin (lipopolysaccharide) tolerance is characterized by a transient refractory state to a subsequent lipopolysaccharide challenge. Following human endotoxemia, *ex vivo* tolerance of circulating leukocytes to LPS resolves within 24 hours. However, the duration of *in vivo* tolerance, assumed to be primarily mediated by tissue-resident macrophages, is unknown.

Methods We performed a clinical experimental study in 16 healthy male volunteers at an Intensive care research unit. To compare *ex vivo* and *in vivo* tolerance kinetics, whole blood from healthy volunteers was stimulated with lipopolysaccharide before, 4 hours after and 1, 2, 3, and 4 weeks following *in vivo* endotoxin (2 ng/kg; lipopolysaccharide derived from *E coli* O:113) administration. Furthermore, we compared the inflammatory response during two subsequent endotoxemia experiments in healthy volunteers with an interval of two weeks. The cytokines TNF-α, IL-6, IL-10, IL-1RA, and TGF-β were measured.

Results Four hours after *in vivo* lipopolysaccharide administration, production of TNF-α, IL-6, and IL-10, but not IL-1RA in *ex vivo* lipopolysaccharide-stimulated whole blood was diminished. *Ex vivo* lipopolysaccharide tolerance completely resolved within one week. In contrast, *in vivo* lipopolysaccharide tolerance was still apparent after two weeks. Compared with the first lipopolysaccharide administration, plasma peak levels of TNF-α, IL-6, IL-10, IL-1RA, and TGF-β were attenuated by 46%, 36%, 45%, 10%, and 14%, respectively (all $p<0.05$).

Conclusions While *ex vivo* lipopolysaccharide tolerance quickly resolves, *in vivo* lipopolysaccharide tolerance persists for at least two weeks. These findings strengthen the notion that the *in vivo* response to lipopolysaccharide is mediated by tissue-resident macrophages and that *ex vivo* stimulation does not accurately reflect the *in vivo* innate immune response. Intervention studies utilizing the human endotoxemia model should be performed using parallel groups rather than a cross-over design.

Introduction

Lipopolysaccharide (LPS) or endotoxin tolerance is characterized by a blunted inflammatory response to subsequent LPS challenges[1]. The most compelling evidence for this phenomenon has been established in mice, where pretreatment with a sublethal dose of LPS prevented mortality induced by a normally lethal LPS challenge[2]. Clinically, the immune paralysis frequently observed in e.g. sepsis/trauma patients may be related to endotoxin tolerance and this hyporesponsive state observed after the initial hyperinflammatory phase may render them vulnerable for secondary infections[3].

Endotoxin tolerance is commonly objectified by the measurement of inflammatory cytokines following consecutive LPS incubations/administrations, and tolerance studies can be divided into three categories. First, *in vitro* models in which inflammatory cells pre-exposed to LPS display a diminished cytokine response to a subsequent LPS challenge (*in vitro* tolerance)[4]. Second, *in vivo* exposure to LPS followed within hours by *ex vivo* LPS restimulation of circulating leucocytes harvested from the host show a diminished cytokine response (*ex vivo* tolerance)[5-8]. Finally, subsequent *in vivo* LPS challenges result in a diminished *in vivo* cytokine response (*in vivo* tolerance)[2, 9]. Importantly, *ex vivo* tolerance may not correlate with the *in vivo* situation because the *in vivo* response to endotoxin is thought to be primarily mediated by tissue-resident cells and not by circulating leukocytes, as illustrated by elevated cytokine levels in LPS-challenged neutropenic mice[10].

Our group recently showed that LPS administrations for five consecutive days almost completely abolished pro- and anti-inflammatory cytokine release[11]. While the hyporesponsiveness of *ex vivo* stimulated leukocytes from septic and trauma patients persists for several days to weeks, it is thought that *in vivo* tolerance after a single LPS challenge is shortlived and as such, pharmacological intervention trials during experimental human endotoxemia are frequently designed in a cross-over manner, using an interval of 1-2 weeks[12-15]. Also, hyporesponsiveness of *ex vivo* stimulated leukocytes is generally accepted to reflect *in vivo* tolerance (and hence a subject's immune status), thereby ignoring the role of tissue resident cells such as macrophages, which play an important role in host defense. In this study, we compared the kinetics of *ex vivo* and *in vivo* LPS tolerance in humans. We used inflammatory cytokine levels as the primary measure of tolerance, but also investigated clinical parameters such as symptom score, hemodynamics, temperature response, and leukocyte counts.

Materials and methods

Subjects

Ex vivo endotoxin tolerance was studied in 4 healthy male nonsmoking volunteers who participated in a human endotoxemia trial in which volunteers were administered LPS once (Clinical Trial Register number NCT00513110). *In vivo* endotoxin tolerance was examined in 12 healthy male nonsmoking volunteers who participated in a crossover human endotoxemia study (Clinical Trial Register number NCT00783068) in which they received LPS twice (visit 1 & 2) with a mean interval of 14 days (range 11-18 days). The study protocols were approved by the local ethics committee of the Radboud University Nijmegen Medical Centre. Written informed consent was obtained from all study participants. The findings of the physical examinations, electrocardiography, and routine laboratory studies on all the volunteers before the start of the experiment showed normal results. Volunteers were not taking any prescription medications, and they were negative for hepatitis B surface antigen and human immunodeficiency virus infection.

Human endotoxemia model

Human endotoxemia protocols for the *ex vivo* and *in vivo* endotoxin tolerance studies were identical. Subjects refrained from food 12 hours before the start of the experiment, and caffeine or alcohol containing substances 24 hours before the start of the experiment. The experiments were performed at the research unit of the intensive care department, with subjects in supine position. After local anesthesia (lidocaine HCl 20 mg/ml) the radial artery was cannulated using a 20 Gauge arterial catheter (Angiocath, Becton Dickinson, Sandy UT, USA) and connected to an arterial pressure monitoring set (Edwards Lifesciences LLC, Irvine CA, USA), connected to a Phillips IntelliVue MP70 monitor (Philips Medical Systems, Eindhoven, The Netherlands). The arterial line was used for continuous monitoring of blood pressure and blood sampling. A cannula was placed in the antecubital vein to permit infusion of 2.5% glucose/ 0.45% saline solution; subjects received 1.5 L during one hour starting one hour before LPS infusion (prehydration), followed by 150 ml/h until 6 hours after LPS infusion, and 75 ml/h until the end of the experiment. Body temperature was measured every 30 minutes using an infrared tympanic thermometer (FirstTemp Genius, Sherwood Medical, Crawley/Sussex, UK). Heart rate was continuously monitored using a 3-lead electrocardiogram. Subjects were asked to score the severity of the endotoxin-induced flulike symptoms (nausea, headache, shivering, muscle, and back pain) every 30 min up to 6 hrs after the administration of endotoxin. Symptoms were scored on a scale ranging from 0 (symptom not present)

to 5 (worst ever experienced) and these scores were added, forming an arbitrary total symptom score. U.S. Reference *E. coli* endotoxin (*Escherichia coli* O:113, Clinical Center Reference Endotoxin, National Institute of Health [NIH], Bethesda, MD) was used. Ec-5 endotoxin, supplied as a lyophilized powder, was reconstituted in 5 mL saline 0.9% for injection and vortex-mixed for at least 10 minutes after reconstitution. The endotoxin solution was administered as an intravenous bolus injection at a dose of 2 ng/kg of body weight.

Whole blood stimulation

To investigate *ex vivo* endotoxin tolerance, blood of 4 subjects that received LPS *in vivo* was rechallenged with LPS *ex vivo* immediately after withdrawal. Arterial (day of LPS administration) or venous (1, 2, 3, and 4 weeks after human endotoxemia) blood was drawn into 5 mL lithium-heparin containing vacutainers (Vacutainer System, BD Biosciences). Whole blood was diluted 1:5 in RPMI and stimulated for 24 hours with 1 ng/mL *E. coli* LPS (serotype O55:B5, Sigma-Aldrich, St Louis, MO, USA) or medium (control). LPS was further purified as described previously[16]. After stimulation, whole blood cultures were centrifuged (14000 rpm, 5 min) after which supernatants were stored at -80 °C until assayed.

Cytokine measurements

During human endotoxemia experiments, EDTA anticoagulated blood was collected from the arterial line and immediately centrifuged at 2000g for 10 minutes at 4 °C to obtain plasma. Concentrations of TNF-α, IL-6, IL-10, and IL-1RA in plasma and whole blood stimulation supernatants were measured using a simultaneous Luminex Assay according to the manufacturer's instructions (Bio-plex cytokine assay, BioRad, Hercules, CA, USA). Plasma levels of TGF-β were determined by ELISA according to the manufacturer's instructions (TGF-β1 Duoset, R&D systems, Minneapolis, MN, USA).

Calculations and statistical analysis

The Shapiro-Wilk test was used to test for normality. Whole blood stimulation cytokines were normalized for monocyte count as these are the main producers of cytokines in whole blood stimulations[17]. Furthermore, cytokine production in unstimulated whole blood samples was subtracted from cytokine production in LPS-stimulated whole blood to correct for circulating cytokines and basal stimulation. *Ex vivo* tolerance data was analyzed using repeated measures one-way analysis of variance with Dunnett's Multiple Comparisons post-hoc test. To test for the presence of *in vivo* tolerance, the response to the first and second in vivo LPS administration (cytokines, hemodynamic

data, temperature, leukocyte count, symptom score) were compared using repeated measures two-way analysis of variance (interaction term). The % decreases in peak plasma cytokine levels between visit 1 and 2 for the different cytokines measured were compared using repeated measures one-way analysis of variance with Newman-Keuls post-hoc test. Correlation between the decrease in peak cytokine levels during both LPS challenges and the interval between both challenges was tested using Pearson correlation coefficients. Data are expressed as mean ± SEM. A *p*-value of less than 0.05 was considered statistically significant.

Results

The ex vivo cytokine response following LPS administration is restored within one week

Ex vivo stimulation with LPS of whole blood obtained from four LPS-challenged subjects demonstrated a significant reduction of proinflammatory cytokines TNF-α and IL-6 4 hours after *in vivo* LPS administration, compared with stimulated blood drawn before LPS administration. Subsequent *ex vivo* whole blood stimulations after 1, 2, 3, and 4 weeks showed that the *ex vivo* proinflammatory cytokine response was restored within one week (Figure 1, left panel). While the *ex vivo* IL-10 production was also attenuated 4 hours after *in vivo* LPS administration, one week later it was significantly higher compared with before the *in vivo* LPS challenge (Figure 1, right panel). *Ex vivo* production of the anti-inflammatory cytokine IL-1RA was not impaired at any time point following LPS administration.

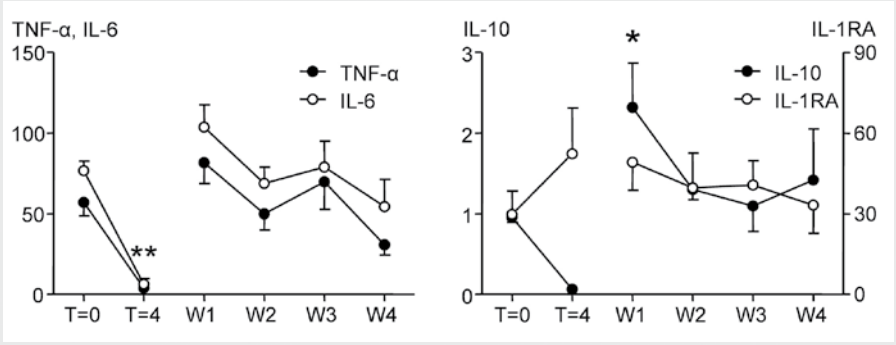
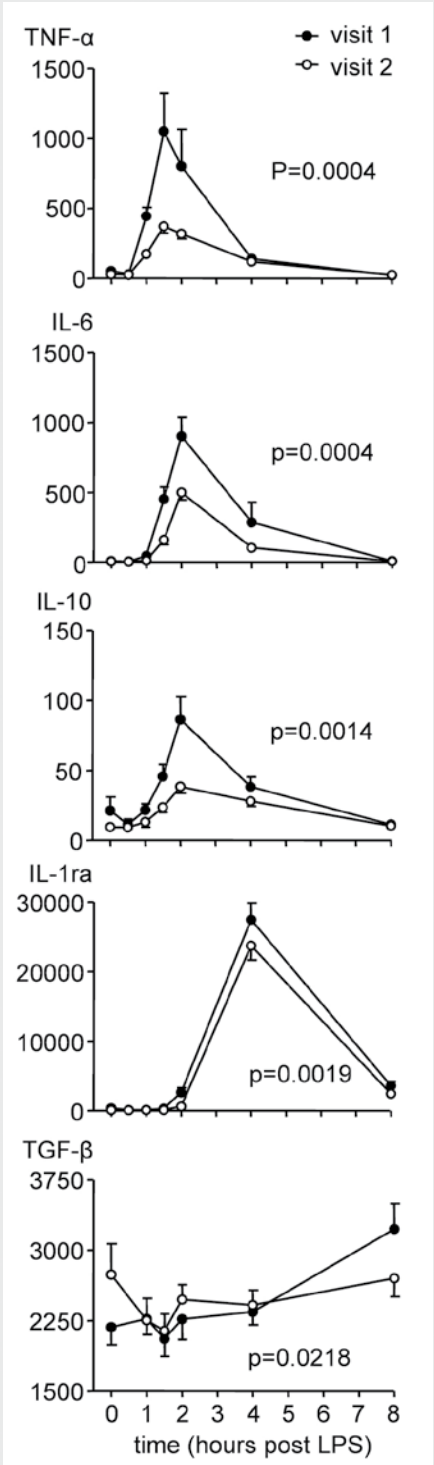


*Figure 1 Proinflammatory (TNF-α and IL-6, left panel) and anti-inflammatory (IL-10 and IL-1RA, right panel) cytokine production in ex vivo LPS-stimulated whole blood of subjects on the day of LPS administration (T=0 and T=4 after LPS administration) and 4 weeks afterwards (W1-W4). Data are expressed as pg/1000 monocytes and as mean ± SEM of four subjects. * indicates p<0.05 vs. T=0 (repeated measures one-way analysis of variance with Dunnet's Multiple Comparisons post-hoc test).*

Figure 2 *Plasma concentrations (in pg/mL) of cytokines (TNF-α, IL-6, IL-10, IL-RA, and TGF-β) during human endotoxemia of subjects who were administered LPS on two separate occasions (visits 1 and 2) with an interval of approximately 2 weeks. Data are expressed as mean ± SEM of 12 subjects. p-values were calculated using repeated measures two-way analysis of variance (interaction term).*



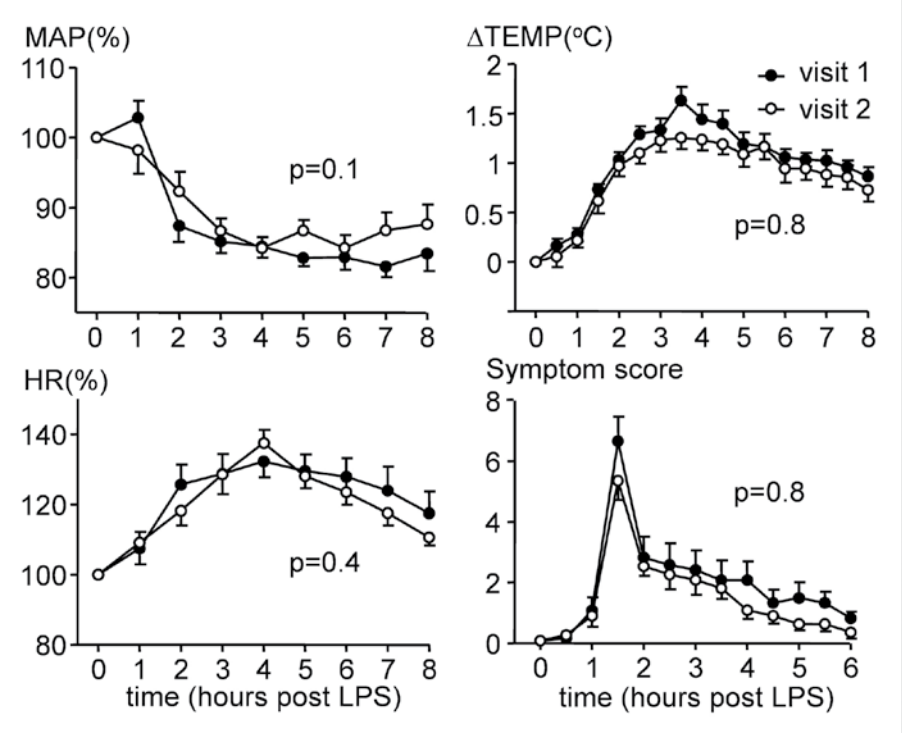


Figure 3 *Changes in mean arterial pressure (MAP, upper left panel), temperature (TEMP, upper right panel), heart rate (HR, lower left panel) and symptom score (lower right panel) during human endotoxemia of subjects who were administered LPS on two separate occasions (visits 1 and 2) with an interval of approximately 2 weeks. Data are expressed as mean ± SEM of 12 subjects. p-values were calculated using repeated measures two-way analysis of variance (interaction term).*

Table 1 *Leukocyte counts (X 10^9) during human endotoxemia of subjects who were administered LPS on two separate occasions (visits 1 and 2) with an interval of approximately 2 weeks.*

Time (hours post LPS)	visit 1			visit 2		
T=0	4.76	±	0.41	4.32	±	0.36
T=1	2.66	±	0.37	3.19	±	0.23
T=2	6.38	±	0.66	7.81	±	0.75
T=5	10.34	±	0.67	9.72	±	0.58
T=8	10.59	±	0.90	9.33	±	0.73

Values are mean ± SEM of 12 subjects.

The in vivo cytokine response following LPS administration is still impaired after 2 weeks

The first *in vivo* LPS administration (visit 1) resulted in a typical transient increase in plasma concentrations of the proinflammatory cytokines TNF-α and IL-6 and the anti-inflammatory cytokines IL-10 and IL-1RA (Figure 2). TGF-β plasma levels only showed an increase 8 hours post-LPS (Figure 2). Compared with the first LPS administration, the second LPS administration led to an attenuation of peak plasma levels of TNF-α, IL-6, and IL-10 by 46±10%, 36±9%, and 45±8%, respectively. Although statistically significant, peak levels of IL-1RA and TGF-β were only attenuated by 10±7% and 14±6%, respectively. The attenuation in peak concentrations of IL-1RA and TGF-β was significantly less pronounced than that of the other cytokines measured ($p<0.05$). There was no correlation between the attenuation of peak levels of the various cytokines and the interval between the two LPS experiments (TNF-α: $r=0.16$; IL-6: $r=0.06$; IL-10: $r=0.14$, IL-1RA: $r=-0.12$, TGF-β: $r=0.09$).

In contrast to the cytokine data, LPS-induced changes in mean arterial pressure, heart rate, temperature, and symptom score were similar during both endotoxemia experiments (Figure 3). Likewise, leukocyte counts did not differ between both visits (Table 1).

Discussion

In the present study, we demonstrate that following a single *in vivo* LPS challenge, the *ex vivo* cytokine response to LPS is restored within one week, whereas attenuation of the *in vivo* cytokine response persists for at least two weeks. The attenuated cytokine response did not result in less pronounced clinical symptoms, hemodynamic changes, rise in temperature, and leukocyte counts.

Reports on *in vivo* LPS tolerance in humans are scarce. In a previous study, 0.06 ng/kg LPS (as opposed to 2 ng/kg in the present study) was administered twice with an interval of two weeks. Although very low circulating cytokine levels were found, a small, but significant attenuation of TNF-α, IL-6, and IL-1RA plasma levels was observed upon the second LPS administration[18]. Earlier work from our group has shown that after LPS administration for 5 consecutive days in healthy volunteers, the cytokine response is virtually nullified at day 5[11, 19], and similar results were observed in LPS-treated cancer patients[20].

Rapid reversal of *ex vivo* endotoxin tolerance after a single *in* vivo LPS challenge has been demonstrated before by ourselves and others[5, 6, 8]. *Ex vivo* hyporesponsiveness was not restricted to LPS, but also observed using other Toll-like receptor stimuli,

indicating cross-tolerance[5, 6]. In line with these data, we confirm that the *ex vivo* TNF-α, IL-6 and IL-10 response is severely impaired 4 hours after LPS administration, but is restored after one week and remains unaltered in the following 3 weeks. *Ex vivo* hyporesponsiveness to LPS was not mediated by high levels of circulating TGF-β, a well-described suppressor of LPS-induced proinflammatory cytokine production[21], because plasma TGF-β levels were not increased until 8 hours post-LPS. The limited duration of *ex vivo* endotoxin tolerance after a single LPS administration might be due to the relatively short lifespan of monocytes in the circulation. Especially during inflammation they leave the circulation and extravasate into tissues[22, 23]. Subsequently, the blood pool is replenished by 'new' monocytes originating from the bone marrow or from a splenic reservoir[22-24]. These new monocytes may not have been exposed to LPS and therefore appear not to have developed tolerance. In contrast to our data, the tolerant state of *ex vivo* stimulated leukocytes of septic or injured patients can persist for several weeks[3, 25]. The discrepancy in duration of *ex vivo* tolerance between patients and human endotoxemia volunteers may be due to the fact that human disease states complicated by or induced by endotoxin are often episodic (multi-hit theory) or continuous. Repeated activation of the immune system is likely to result in the development of a more profound tolerance, both *in vivo* and *ex vivo*, compared with a single hit.

The difference between the *ex vivo* and *in vivo* LPS tolerance duration signifies the notion that the blood compartment does not contribute significantly to the *in vivo* cytokine response to LPS, and the fact that tissue-resident macrophages are its main source. This hypothesis is strengthened by several findings. First, our group has demonstrated that there exists no correlation between the *ex vivo* and *in vivo* inflammatory response to LPS, indicating that these responses are mediated by two different compartments[26]. Second, increased cytokine levels have been found in neutropenic mice and patients[10, 27, 28]. Third, macrophages are unable to produce IL-1β in response to solely LPS[29], whereas monocytes produce large amounts of IL-1β upon LPS stimulation[30]. In the human endotoxemia model, IL-1β is barely detectable[31], implicating a limited role for monocytes in the cytokine production to an *in vivo* LPS challenge. The turnover of tissue-resident macrophages is slow, which may explain the relatively lengthy refractory state after a single hit[32]. Furthermore, as our data indicate, the hyporesponsive state *in vivo* is not reflected by *ex vivo* cytokine production of stimulated leukocytes. Therefore, conclusions regarding the immune status of patients or subjects based on *ex vivo* stimulation experiments should be met with caution.

Interestingly, tolerance was not observed in the production of IL-1RA *ex vivo*, and only very moderately *in vivo*. In *ex vivo* stimulated whole blood of patients with meningococcal infection, TNF-α, IL-1β, and IL-6 production is severely depressed at admission and gradually restores upon patient recovery, whereas this pattern is reversed for IL-RA production[33]. Furthermore, *ex vivo* LPS-stimulated leukocytes of septic shock patients produce significantly less amounts of IL-1β compared with leukocytes obtained from control subjects, whereas IL-1RA production is unaltered[34]. It was demonstrated that while the LPS-induced increase in transcription of IL-1β and IL-1RA is similarly attenuated in leukocytes of septic patients, IL-1RA mRNA is more stable than IL-1β mRNA. As a result, the slowly decaying 'pool' of IL-RA mRNA is still effectively translated upon LPS stimulation[34].

In contrast to the *in vivo* LPS tolerance measured by attenuated cytokine release, endotoxemia-induced changes in hemodynamics, temperature, leukocyte counts, and symptom score were not blunted during the second endotoxemia experiment. During LPS administrations for 5 consecutive days, cytokine levels were virtually nullified at day 5, whereas significant LPS-induced changes in temperature, heart rate, mean arterial pressure, and white blood cell counts persisted, albeit it to a lesser extent[11]. Therefore, it appears likely that mediators or processes other than the cytokines measured are responsible for generation of these effects and that these do not become tolerant to LPS. The disparity between clinical symptoms and cytokine levels are of particular interest from a clinical point of view. In a sepsis trial, patients are usually included when systemic inflammatory response syndrome criteria are present. If the effects of, e.g., an anti-cytokine intervention are studied, our data illustrate that the target analyte cytokine of interest may not be elevated in the presence of systemic inflammatory response syndrome criteria. Therefore, evidence that the analyte target is present should be one of the inclusion criteria prior to enrolment.

This study may also have important consequences for the design of pharmacological intervention trials using the human endotoxemia model. Our data show that a cross-over design with an interval of 1-2 weeks[12-15] is importantly hindered by development of *in vivo* LPS tolerance. At this moment it is unclear which wash-out period between LPS administrations is sufficient. As our data show, tolerance will be present within the 2 weeks following the first LPS administration, whereas a diminished response to LPS due to antibody formation may occur at a later stage[35]. Therefore, we suggest to use parallel groups to investigate interventions aimed at modulation of the inflammatory response during human endotoxemia.

Our study has several limitations. First, our study design has not permitted us to investigate the duration of *in vivo* LPS tolerance. In order to determine this, multiple parallel groups of healthy volunteers that are administered LPS on two separate occasions with different wash-out periods would be needed. Also, we cannot draw definitive conclusions regarding the tolerant state of tissue-resident macrophages or mediators/mechanisms of tolerance, such as the recently implicated role of IL-1 receptor-associated kinase-M (IRAK-M) and suppression of tumorigenicity 2 (ST2)[8, 36], because it requires invasive procedures to harvest these cells.

In conclusion, our results imply that the *in vivo* response to LPS is mediated by tissue-resident macrophages that remain tolerant for a much longer period than circulating leukocytes. Therefore, *ex vivo* stimulation of circulating leukocytes, a frequently used method to investigate a subject's or patient's immune status, might not be indicative of the *in vivo* innate immune response to a pathogenic stimulus. The disparity between cytokine levels and clinical symptoms may have implications for the design of sepsis trials in which patients are often enrolled based on clinical symptoms. Finally, due to *in vivo* tolerance, intervention studies utilizing the human endotoxemia model should employ an unpaired parallel group design.

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Chapter 12b

Letter to the editor

Nitric oxide inhalation and glucocorticoids as combined treatment in human experimental endotoxemia: it takes not always two to tango

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Abstract original article

Nitric oxide inhalation and glucocorticoids as combined treatment in human experimental endotoxemia

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Critical Care Medicine 2008; 36(11):3043-7

Background Inhaled nitric oxide and glucocorticoids as a combination therapy may attenuate endotoxin-induced inflammatory responses in humans as indicated by levels of cytokines and clinical signs. Because other authors have shown that combined inhaled nitric oxide and steroids improved the histologic damage both in pulmonary and systemic organs in a porcine endotoxin model, we examined if an anti-inflammatory interaction could be demonstrated in humans.

Methods We performed a double-blind, crossover, placebo-controlled randomized study in fifteen healthy white volunteers (4 women, 11 men) at the intensive care unit of a university hospital. Endotoxin (2 ng/kg) was administered intravenously. Thirty minutes thereafter the volunteers were given glucocorticoids (2 mg/kg) intravenously and inhaled nitric oxide 30 ppm or placebo (nitrogen) administered through a nasal cannula. Blood samples and clinical signs were collected before and up to 5.5 hrs after the endotoxin infusion.

Results Following endotoxin body temperature and heart rate increased significantly compared with baseline. There were no differences observed between the treatments. Endotoxin challenge also markedly elevated the plasma levels of tumor necrosis factor-alpha, interleukin (IL)-6, IL-10, and IL1-ra concentrations during the study period. No difference between placebo/glucocorticoids and inhaled nitric oxide/glucocorticoids treatment was seen in the cytokine response.

Conclusions In a human experimental inflammatory model using endotoxin, inhaled nitric oxide and glucocorticoids in low doses given after the endotoxin challenge did not modify the inflammatory cascade as monitored in this study.

To the editor

We read with interest the article by Hållström et al[1] about the effects on the innate immune response of inhaled nitric oxide (iNO) during experimental endotoxemia. All 15 volunteers received hydrocortisone 30 mins after the endotoxin challenge. The study design was crossover, randomized, double-blind, and placebo-controlled regarding iNO. At least 3 to 4 wks elapsed between the experiments. This is the first study in healthy volunteers in which two potential immune modulating therapies are combined in the human endotoxin model and in our view, several issues concerning this complex study design and its subsequent influence on the results should be addressed. We recently performed a comparable trial in which we induced experimental human endotoxemia twice in 12 healthy male volunteers, in the presence or absence of a pharmacological intervention or placebo. Similarly, this study was also performed in a crossover design, 10 to 18 days apart (mean, 14 days). After deblinding of the study, however, we discovered an important tolerance effect of the administration of endotoxin, with a lower inflammatory response during the second visit regardless of the pharmacological intervention (active or placebo). We would like to know whether such effects were also encountered during the study by Hållström et al. Previous crossover studies with the human endotoxin model generally chose a washout period of 2 wks on average[2] or a washout period of 6 wks on average[3] to avoid the potential effects of lipopolysaccharide (LPS)-antibody formation. Why did they choose a washout period of 3 to 4 wks?

Inhaled nitric oxide may modulate the acute neutrophilic inflammation of the lung parenchyma and dysfunction of the alveolar-capillary membrane that characterizes acute respiratory distress syndrome at several levels. Given the fact that inhaled nitric oxide is inactivated within seconds (by hemoglobin in blood, by haptoglobin-hemoglobin complexes in plasma, and by a reaction with heme ferrous iron and ferric iron that forms nitrosyl-hemoglobin), the question is if the very low concentrations of iNO can attenuate the (parenterally administrated) LPS-mediated cytokine production outside of the lung. There is no human data that 30 ppm iNO in fact can attenuate the LPS-mediated cytokine production in the lung, which could be examined in humans with use of LPS inhalation[4]. In addition, it is unclear to us why hydrocortisone was administered to all of the volunteers. Why examine the possible effect of iNO during a suppressed innate immune response if the effect of iNO alone is questionable? It now remains possible that iNO in the absence of steroid treatment may influence the LPS-induced response. Furthermore, in this protocol hydrocortisone was injected 30 mins after endotoxin infusion as a bolus (2 mg/kg), while other studies that investigate

the immune modulating effects of glucocorticoids in the human endotoxin model use pretreatment with continuous infusion of hydrocortisone during a period of at least 6 hrs before endotoxin infusion[5]. It is unclear if and to what extent hydrocortisone administration 30 mins after the induction of endotoxemia will influence the innate immune response, given that no control group for the steroid treatment was tested.

In our view, time control experiments in which LPS is administrated twice to volunteers without another pharmacological intervention are needed to demonstrate the presence of absence of time-related effects on the innate immune response of LPS. In addition, we feel it is preferable to study one pharmacological intervention at the time or to use four study groups (both iNO and cortisone randomized) if combined effects need to be established.

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Chapter 13

Summary

In this thesis, we describe preclinical, translational and clinical studies investigating the interplay between the autonomic nervous system (ANS) and the innate immune response. We focused on the effects of the parasympathetic nervous system, the so-called cholinergic anti-inflammatory pathway, because this is a relatively newly discovered pathway and its mechanisms and effects have been sparsely studied, especially in humans.

Chapter 1 is a short introduction on the ANS and innate immunity, but mainly focuses on the links between these two systems. In the last decades, the ANS has been identified as a regulator of the innate immune system. At first, it was shown that the afferent vagus nerve can detect inflammation in organs and relay this information to the brain, resulting in fever, cortisol release, and increased efferent vagus nerve activity. In the 1990s, it was shown that the efferent sympathetic nervous system can limit the innate immune response via catecholamines acting on β-receptors. This was confirmed in humans *in vivo*: administration of epinephrine resulted in decreased production of proinflammatory cytokines and increased production of anti-inflammatory mediators during human endotoxemia[1]. In 2000, Tracey and colleagues identified the cholinergic anti-inflammatory pathway, in which efferent vagus nerve activity limits the immune response via binding of acetylcholine to the α7nAChR receptor on macrophages[2, 3].

Part one: Autonomic nervous system – innate immune interactions in human health and disease

In the first part of this thesis, we investigated the interplay between the ANS and the innate immune response, both in patients and healthy volunteers.

In **chapter 2a**, we postulated the hypothesis that increased vagus nerve activity results in the observed immune paralysis in traumatic brain injury patients. These patients often suffer from a hyporeactive innate immune system, making them more susceptible for infections[4, 5]. Furthermore, in traumatic brain injury patients, increased vagus nerve activity, measured by heart rate variability (HRV), is reported[6, 7]. We linked these two observations, suggesting that the observed immune paralysis results from persistent increased vagus nerve activity and subsequent excessive dampening of the innate immune response through the cholinergic anti-inflammatory pathway. We proposed that the increased intracranial pressure, often observed in brain injury patients, is the major contributor to the increased activity of the vagus nerve. Pressure-induced compression of the brainstem might lead to vigorous firing of the dorsal motor nucleus in the medulla oblongata, the centre where the vagus nerve originates. This is

supported by findings of increased vagus nerve activity in subarachnoid haemorrhage patients, which often exhibit high intracranial pressures[8], and animal experiments, where vagal activity increased relative to the increase in intracranial pressure induced by inflating a subdural balloon[9].

In the previous chapter, we proposed a relation between increased intracranial pressure, vagus nerve activity, and the innate immune response in subarachnoid haemorrhage patients. With regard to this relation, in **chapter 2b**, we commented on an article describing the relationship between sympathetic nervous system activity, measured by norepinephrine spillover, and circulating inflammatory cytokines in ICU patients with subarachnoid haemorrhage[10]. The authors found increased norepinephrine spillover and increased levels of plasma cytokines, but no quantitative correlation between these parameters. We put forward that measuring circulating cytokines might not properly reflect a patient's innate immune status as these cytokines could merely result from the initial insult and not provide information on the immune system's capacity to respond to a stimulus. We stated that measuring the inflammatory response of *ex vivo* stimulated leukocytes might be a better option. Furthermore, we proposed to the authors that, with regard to the cholinergic anti-inflammatory pathway, not only sympathetic, but also parasympathetic parameters should be assessed when investigating inflammatory parameters in patients with subarachnoid haemorrhage.

Nicotine is a non-specific agonist of the α7nAChR. Therefore, the cholinergic anti-inflammatory pathway might be activated by nicotine administration in patients. In **chapter 2c**, we discussed an article reporting increased mortality in critically ill patients receiving nicotine-replacement therapy[11]. We speculated that nicotine might have suppressed the innate immune response in these patients to the point of immune paralysis, putting them at a higher risk of acquiring concomitant infections leading to worse outcome. This is supported by the fact that in the nicotine-treated group, 8% of the patients died of infections compared with 2% in patients not treated with nicotine[11]. Unfortunately, infection incidence was not reported.

In **chapter 3**, we investigated the hypothesis described in chapter 2a. To this end, we studied the relationship between ANS activity and the innate immune response in intensive care patients with neurological damage in the first 4 days after admission. ANS activity was measured by HRV and the immune response was assessed by cytokine production in *ex vivo* stimulated leukocytes as well as levels of plasma cytokines. We demonstrated a significant inverse correlation of HFnu (a HRV parameter reflecting vagus nerve activity) and *ex vivo* stimulated TNF-α production. These results suggest

that vagus nerve activity attenuates cytokine production by circulating leukocytes. To further investigate effects of intracranial pressure on vagus nerve activity and the innate immune response in brain injury, we compared patients with conditions associated with high (intracranial haemorrhage) and low (subarachnoid haemorrhage with an extraventricular drain) intracranial pressure. *Ex vivo* stimulated cytokine production was significantly lower in patients with intracranial haemorrhage compared with subarachnoid haemorrhage. Furthermore, within the intracranial haemorrhage group, there was a strong significant inverse correlation between HFnu and plasma levels of TNF-α. The fact that patients with intracranial haemorrhage exhibited the most pronounced immune paralysis suggests intracranial pressure-induced activation of the vagus nerve and subsequent dampening of the innate immune response.

In **chapter 4**, the relationship between cardiac ANS activity, measured by HRV, and the innate immune response in a large group of healthy volunteers during human endotoxemia was examined. It is conceivable that a subject's basal vagus nerve or sympathetic activity would correlate with the inflammatory response. Therefore, we first set out to determine whether baseline HRV parameters, determined before LPS administration, predicted a subject's subsequent plasma cytokine response to LPS in this controlled model of the innate immune response. We found no correlations between any of the HRV parameters and plasma cytokines. Upon LPS administration, HRV parameters decreased considerably. We investigated whether the extent of LPS-induced HRV changes correlated with the magnitude of the plasma cytokine response. Again, no relation was found. Further evidence for the lack of a relation between the plasma cytokine response and HRV alterations was obtained from experiments where healthy volunteers received LPS twice with an interval of two weeks. Due to endotoxin tolerance, the plasma cytokine response to LPS was roughly halved upon the second LPS administration, however, HRV changes were identical. These findings suggest that cardiac ANS activity may not be representative for ANS outflow to other organs involved in the inflammatory response and indicates that changes in HRV are not a reliable surrogate measure for the extent of the inflammatory response during human endotoxemia.

Part two: Limiting the innate immune response through the cholinergic anti-inflammatory pathway; *in vitro*, animal, and human studies

In the second part of this thesis, we focused on the effects of stimulation of the cholinergic anti-inflammatory pathway. We investigated this in animals, using models of ventilator-induced lung injury (VILI), and in humans, both *in vitro* using primary human leukocytes and *in vivo* during experimental human endotoxemia.

In **chapter 5**, we investigated the effects of stimulation and ablation of the cholinergic anti-inflammatory pathway on VILI. Mechanical ventilation is a life-saving therapy, that at the same time can induce lung injury. Activation of the innate immune system by mechanical ventilation, so-called biotrauma, has been shown to play a detrimental role in the pathogenesis of VILI[12, 13]. Patients with underlying inflammatory processes, which is common in the ICU, have an increased risk of developing VILI[14]. We investigated the effects of stimulation of the cholinergic anti-inflammatory pathway by electrical vagus nerve stimulation and ablation of this pathway by vagotomy in a rat two-hit VILI model: LPS administration followed by mechanical ventilation. We performed vagus nerve stimulation or vagotomy after LPS administration but before the start of mechanical ventilation. We chose this time-point to simulate a patient with systemic inflammation admitted to the ICU for initiation of mechanical ventilation. We demonstrated that mechanical ventilation with high (15 mL/kg), but not low (8 mL/kg), tidal volumes potentiates the LPS-induced inflammatory response in the lungs. Vagotomy resulted in elevated levels of pulmonary cytokines and reduced oxygenation in spontaneously breathing animals, but did not further enhance the inflammatory response in LPS-treated mechanically ventilated rats. Vagus nerve stimulation did not affect the inflammatory response during spontaneous breathing and mechanical ventilation. These results suggest that delayed stimulation of the cholinergic anti-inflammatory pathway has no beneficial effects on pulmonary inflammation and questions the clinical applicability of stimulation of this pathway in systemically inflamed patients admitted to the ICU where mechanical ventilation is initiated.

In **chapter 6**, we explored the anti-inflammatory effects and underlying mechanisms of α7nAChR stimulation on primary human leukocytes. We demonstrated that both the non-specific α7nAChR agonist nicotine and the specific agonist GTS-21 dose-dependently inhibited proinflammatory cytokine release by human leukocytes stimulated with various Toll-like receptor agonists. GTS-21 was more potent and efficacious than nicotine. Furthermore, we revealed that the anti-inflammatory effects of GTS-21 are mediated at the transcriptional level as it prevented upregulation of cytokine mRNA levels. Finally, qPCR array data and experiments using the JAK2 inhibitor AG490 indicated that the effects of GTS-21 involve activation of the JAK2-STAT3 pathway. These data demonstrate that GTS-21 has potent anti-inflammatory effects on human innate immune cells and that these effects are transcriptionally mediated.

The anti-inflammatory potential of GTS-21 was further examined in **chapter 7**, where we investigated its effects in a murine VILI model. Furthermore, the effects of acetylcholinesterase inhibition with neostigmine, which improved outcome in murine sepsis[15], and nAChR blockade with mecamylamine, which worsened murine pancreatitis[16], were explored. Mice were ventilated with a clinically relevant tidal volume of 8 mL/kg for 4 hours, which resulted in a pulmonary and systemic inflammatory response. Pre-treatment with GTS-21 attenuated pulmonary and systemic TNF-α at the transcriptional level, and lowered the arterial-alveolar gradient, indicating improved gas exchange. Furthermore, GTS-21 inhibited TNF-α mRNA expression and production in isolated alveolar macrophages. Neither neostigmine, nor mecamylamine affected inflammatory or respiratory parameters. This study demonstrates that selective α7nAChR stimulation can limit VILI elicited by mechanical ventilation using clinically relevant ventilator settings. The absence of effects of neostigmine and mecamylamine may be related to the fact that the relatively mild inflammatory response induced by mechanical ventilation is not severe enough to endogenously activate the cholinergic anti-inflammatory pathway.

After the encouraging effects of GTS-21 *in vitro* and in mice, it remained to be determined whether this compound could limit the innate immune response in humans *in vivo* as well. To this end, in **chapter 8**, we performed a double blind placebo-controlled pilot study to investigate the effects of GTS-21 on the innate immune response in healthy volunteers during experimental endotoxemia. Subjects received GTS-21 or placebo orally during 3 days before endotoxin administration, and on the day of the human endotoxemia experiment. We found no differences in plasma cytokine levels between the GTS-21 and the placebo groups. This may be caused by the fact that plasma concentrations of GTS-21 were relatively low and highly variable between subjects. However, within the GTS-21-treated group, higher GTS-21 plasma concentrations correlated with lower levels of TNF-α, IL-6, and IL-1RA, but not IL-10. The correlation between higher plasma levels of GTS-21 and lower concentrations of inflammatory cytokines suggest that, at higher concentrations, GTS-21 can limit the innate immune response in humans *in vivo*.

Apart from GTS-21, we also studied another means of activating the cholinergic anti-inflammatory pathway in humans. In **chapter 9**, we investigated a novel gut-brain-immune axis in healthy volunteers during experimental endotoxemia. Recent animal studies have revealed that the cholinergic anti-inflammatory pathway can be activated by short-term enteral administration of lipid-enriched nutrition devoid of intrinsic

anti-inflammatory properties[17-19]. The luminal presence of lipid-enriched nutrition results in cholecystokinin (CCK) release, which activates CCK-receptors located on afferent vagal fibers[20]. Activation of these CCK receptors triggers a vagovagal reflex, that reduces the innate immune response via the cholinergic anti-inflammatory pathway. In our study, subjects received either lipid- and protein-enriched or isocaloric control nutrition via nasojejunal tube. A third group of subjects was fasted throughout the study. Administration of lipid- and protein-enriched nutrition significantly attenuated plasma levels of the proinflammatory cytokines TNF-α, IL-6 and IL-1RA compared with control nutrition and fasting. Furthermore, enriched nutrition significantly augmented release of the anti-inflammatory cytokine increased IL-10 compared with fasted subjects. These results indicate that enteral administration of protein- and lipid-enriched nutrition can activate the cholinergic anti-inflammatory pathway in humans, resulting in attenuation of the innate immune response.

In the last chapter of part two, **chapter 10**, we investigated whether the ANS, and through it, the innate immune response, can be influenced through concentration/meditation. We describe a case study of a Dutch individual known as 'the iceman'. This individual holds several world records with regard to withstanding extreme cold. He claims to achieve these remarkable feats through a special autodidact concentration/meditation technique through which, he claims, he influences his ANS and also his immune response. We demonstrated that 80 minutes of ice-immersion (during which the iceman practiced his concentration/meditation technique) resulted in high levels of cortisol and a large reduction of cytokine production in *ex vivo* stimulated leukocytes. We also performed a human endotoxemia experiment in this individual while he practiced his concentration/meditation technique. We found elevated levels of catecholamines after initiating concentration/meditation, and the increase in plasma cortisol level was higher than in any of the previously studied healthy volunteers during endotoxemia. Moreover, the LPS-induced plasma cytokine response and symptoms were remarkably mild in comparison to a large group of previously studied subjects. In conclusion, these results suggest that, through concentration/meditation, this individual can elicit a consciously controlled stress response, characterized by sympathetic nervous system activation and subsequent catecholamine/cortisol release which dampens the innate immune response. Moreover, this study implies that the ANS might be consciously influenced through special concentration/meditation techniques.

Part three: Methodological considerations

The use of various measurement techniques and experimental models inevitably leads to various interesting findings concerning these techniques and models. In the third part of this thesis, we describe methodological considerations regarding HRV and the human endotoxemia model.

In **chapter 11**, we investigated the influence of different breathing patterns on HRV and its reproducibility in healthy subjects during human endotoxemia. Different breathing patterns might influence HRV, but results from previous studies are conflicting and have all been obtained under static conditions, when HRV does not vary to a great extent. As we have shown in chapter 4, HRV parameters greatly fluctuate as a result of the systemic inflammatory response to endotoxin. This makes the human endotoxemia model ideal to investigate the effects of different breathing patterns on HRV in a dynamic setting. We demonstrated that, over a wide range of HRV magnitudes, neither paced breathing nor mild hyperventilation influenced any of the HRV parameters compared with spontaneous breathing. Furthermore, paced breathing did not improve HRV measurement reproducibility. We concluded that a paced breathing protocol is not required to obtain valid HRV data during endotoxemia.

Our research group has previously shown that repeated endotoxin administration results in endotoxin tolerance, characterized by a blunted inflammatory response to LPS challenges, both *in vivo* and *ex vivo*[21, 22]. However, while pharmacological intervention trials during experimental human endotoxemia are frequently performed in a cross-over manner using an interval of 1-2 weeks[23-26], it is unknown for how long the refractory state that may lead to carry-over effects persists. In **chapter 12a**, we describe the kinetics of endotoxin tolerance in human volunteers, both *ex vivo* and *in vivo*. We demonstrate that following endotoxin administration *in vivo,* the *ex vivo* response of circulating leukocytes to LPS is restored within a week. However, two weeks after endotoxin administration, the *in vivo* cytokine response to a second endotoxin challenge is still severely suppressed. These findings imply that the *in vivo* response to LPS is mediated by tissue-resident macrophages and that *ex vivo* stimulation does not accurately reflect the *in vivo* innate immune response. Furthermore, this study implicates that intervention studies utilizing the human endotoxemia model should be performed using parallel groups rather than a cross-over design.

In light of the findings reported in chapter 12a, in **chapter 12b** we commented on an experimental human endotoxemia study employing a cross-over design with a washout period of 3-4 weeks[27]. We speculate that this washout period might have been too short and tolerance may still be present, hampering clear interpretation of the data. The authors replied that, upon reviewing their data, a tolerance effect was indeed found, but this did not affect the conclusions of this particular study. Furthermore, they stated that in another study, using a washout period of 6 weeks, no tolerance effects were observed[28].

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Chapter 14

General discussion and future perspectives

General discussion

A well-functioning innate immune system protects us from invading pathogens. Upon recognition of microorganisms, the production of proinflammatory cytokines, such as TNF-α and IL-6, by cells of the innate immune system is one of the initial steps in the orchestration of an inflammatory response aimed to clear the body of invaders. However, as Shakespeare already knew: 'too much of a good thing can be bad for you'[1]. Overproduction of proinflammatory cytokines can lead to a hyperinflammatory state that can have devastating effects on organs. The innate immune system therefore is a double-edged sword: it is essential to our survival, but excessive or persistent activation of this system may be harmful. It has become clear that the autonomic nervous system (ANS) can limit the innate immune response, which might represent new treatment options to counteract excessive inflammation.

In this final chapter, we discuss the findings described in this thesis, also in light of recent studies in the field that were performed during the conduct of our own research. Furthermore, we draw conclusions and present future perspectives.

Autonomic nervous system – innate immune interactions in human health and disease

In the first part of this thesis, we demonstrated that ANS activity, measured by HRV analysis, does not correlate with the innate immune response or vice-versa in healthy subjects during human endotoxemia. However, we did find evidence in support of the cholinergic anti-inflammatory pathway in patients with brain injury.

It is still unclear whether vagal input to the heart (which is reflected by HRV) reflects vagal input to other organs involved in the innate immune response. This has, to the best of our knowledge, never been studied. However, in case of the sympathetic nervous system, several studies are in support of a differentiated output. In animals, systemic inflammation was found to be associated with increased sympathetic nerve activity to the spleen and adrenals, but decreased outflow in other sympathetic branches[2, 3]. A recent study examined different measures of sympathetic nervous system activity during human endotoxemia: muscle sympathetic nerve activity (MSNA) and plasma catecholamines[4]. While MSNA was depressed, circulating catecholamine levels were elevated. HRV analysis, as demonstrated by ourselves and others[5], shows sympathetic predominance after LPS administration, which is in accordance with elevated catecholamine levels. These results suggest that the autonomic nervous system represents a highly differentiated system and outflow is organ-specific. Moreover, it

was shown that, at least in animals, the cholinergic anti-inflammatory pathway can be activated by electrical charges below the threshold required to influence heart rate, making it undetectable by HRV[6]. Taken together, these findings demonstrate that several limitations inherent to HRV limit its use in investigation of the cholinergic anti-inflammatory pathway in humans. Nevertheless, in critically ill patients, relations between HRV and the immune response were found by us and others[7]. It might be speculated that the differentiation of autonomic outflow observed in healthy subjects is (partly) lost in critical illness and/or brain injury. Such a phenomenon is known to occur during vasovagal syncope, when effects of increased vagal outflow are observed on all organs innervated. If the loss of differentiation is indeed dependent on illness severity, the absence of a relation between HRV and the immune response in healthy subjects during human endotoxemia may not be surprising, because this model of inflammation is relatively mild.

Based on our observational study, we cannot draw definitive conclusions regarding our hypothesis that persistent activation of the cholinergic anti-inflammatory pathway accounts for the immune paralysis observed in patients with brain injury, as we did not find increased vagus nerve HRV parameters in these patients. However, in agreement with our hypothesis, the largest suppression of *ex vivo* stimulated cytokines was found in the group of patients with intracranial haemorrhage, a condition associated with increased intracranial pressure. Of interest, in a very recent investigation, nonsurviving subarachnoid haemorrhage patients exhibited significantly higher levels of parasympathetic activity (measured by HRV) compared with survivors[8]. Unfortunately, the cause of death was not reported. It is conceivable that the infection prevalence was higher in the nonsurvivors due to the observed increased vagus nerve activity and that this contributed to the increased mortality. However, the worse outcome might also be a direct result of increased intracranial pressure, of which the high parasympathetic activity in these patients is indicative.

In conclusion, several lines of evidence suggest that the cholinergic anti-inflammatory pathway may play a detrimental role in different conditions in critically ill patients. However, the highly heterogeneous patient population and the only currently available method to assess vagus nerve activity, HRV, does not allow us to draw definitive conclusions regarding the involvement of this pathway.

Limiting the innate immune response through the cholinergic anti-inflammatory pathway

In the second part of this thesis, we demonstrated that pharmacological stimulation of the cholinergic anti-inflammatory pathway by the selective α7nAChR agonist GTS-21 before the start of mechanical ventilation limits VILI in mice. However, delayed stimulation of the pathway by electrical vagus nerve stimulation did not attenuate VILI in a second-hit model in rats. Furthermore, we showed that GTS-21 has potent anti-inflammatory effects in human leukocytes, but its potential to limit the innate immune response *in vivo* in humans appears to be hampered by its pharmacokinetic properties upon oral administration. We further demonstrated a novel gut-brain-immune axis in humans, in which enteral lipid- and protein-enriched nutrition limits the immune response, supposedly via CCK-mediated activation of the cholinergic anti-inflammatory pathway. Finally, we described a case study of a remarkable individual, the iceman, who appears to be able to consciously influence his autonomic nervous system and thereby his innate immune response.

While we confirmed previous findings demonstrating that vagotomy amplifies pulmonary inflammation[9], we did not corroborate the anti-inflammatory effects of vagus nerve stimulation[9] in a second hit model of VILI. This might result from differences in the first hit employed. We used LPS administration, whereas in the other study hemorrhagic shock preceded mechanical ventilation. Furthermore, we performed vagus nerve stimulation at a clinically relevant time point: after the first hit. In the other study, stimulation was performed twice: before haemorrhagic shock as well as before the start of mechanical ventilation. The lack of an effect in our study could therefore result from the timing of stimulation, as the vast majority of studies on vagus nerve stimulation in models of inflammation that have shown beneficial results have performed stimulation before or simultaneously with the insult[6, 9-17]. We know of only two other investigations that assessed delayed stimulation of the cholinergic anti-inflammatory pathway[18, 19]. In one study, the effects of administration of the acetylcholinesterase inhibitor physostigmine was evaluated in a cecal ligation and puncture (CLP) model, a widely used model to mimic polymicrobial sepsis[18]. While administration of physostigmine simultaneous with CLP induction attenuated inflammation and septic shock, administration 6 hours after CLP induction conferred no protective effects[18]. In contrast, the other study did report beneficial effects of administration of nicotine 24 hours after induction of CLP[19]. These conflicting results indicate that further studies are required to establish whether delayed stimulation of the cholinergic anti-inflammatory pathway has beneficial effects. This is an important

question, because it defines the clinical applicability of this pathway in ICU patients, in which pretreatment is often not possible.

It was recently demonstrated that pre-treatment with CNI-1493, a centrally acting drug that increases vagus nerve activity, reduced VILI induced by injurious mechanical ventilation (20 mL/kg, no PEEP) in mice[9]. We expanded on these findings by demonstrating that pre-treatment with GTS-21 attenuates VILI induced by mechanical ventilation with clinically relevant 'protective' ventilator settings (8 mL/kg, 1.5 cmH_2O PEEP). Our findings suggest that real lung-protective mechanical ventilation does not exist, at least not using tidal volumes that maintain normocapnia. Specific pharmacological stimulation of the α7nAChR might have therapeutic potential to limit the mechanical ventilation-induced inflammatory response.

The effects of GTS-21 on human leukocytes presented in this thesis have recently been replicated by others that demonstrated identical results[20]. Interestingly, we could not block GTS-21 effects with antagonists of the α7nAChR. This may implicate the involvement of other receptors in the anti-inflammatory effects of GTS-21. While the α7nAChR is widely regarded as the receptor responsible for the anti-inflammatory effects of the vagus nerve, several recent studies have implicated roles for other nAChRs. For instance, nicotine's effects on phagocytosis in murine peritoneal macrophages were blocked by an antagonist of the α4/β2 nAChR, but not by α7nAChR antagonists[21]. Furthermore, nicotine was recently found to inhibit LPS-induced increases in serum TNF-α levels in both wild-type and α7nAChR knockout mice[22]. An alternative explanation involves differences between the α7nAChR in excitable cells such as neurons, and non-excitable cells such as macrophages and monocytes. For example, while T-cells express an essentially identical transcript for the α7nAChR subunit as neuronal cells, they do not form functional ligand-gated ion channels[23]. In this study, α7nAChR specific antagonists MLA and α-bungarotoxin also failed to inhibit nicotine-induced effects[23]. This might be due to other pharmacological properties of the α7nAChR in these cells. Along these lines, it was shown that primary human leukocytes[24, 25] as well as several monocytic cell lines[25] do not express the normal α7 subunit, but an α7 duplicate nicotinic acetylcholine receptor-related protein (dupα7) which lacks the α-bungarotoxin binding site[24, 26]. The activation of the JAK2-STAT3 pathway downstream of α7nAChR activation has been demonstrated before in murine peritoneal macrophages[12]. Our study confirms these findings in primary human leukocytes. In contrast, it was recently reported that GTS-[21] and nicotine decreased IL-6-induced JAK2-STAT3 activation in endothelial cells. This can

be explained by the fact that the effects of activation of the JAK2-STAT3 pathway are highly dependent on the stimulus and the cell type used; in endothelial[27] cells and hepatocytes[28] it exerts proinflammatory effects, whereas it attenuates the inflammatory response in macrophages[28, 29]. The disparate effects in different cell types as well and the fact that the JAK2-STAT3 pathway is involved in the response to various other stimuli/processes[30] prevents it from being a specific marker for α7nAChR activation.

Oral administration of GTS-21 did not attenuate the innate immune response during human endotoxemia. However, the significant inverse correlations between the plasma concentrations of GTS-21 and plasma cytokines within the GTS-21 treated group strongly suggest that the absence of a group effect was due to low GTS-21 plasma concentrations. We used the maximum dose that has been tested safe in humans[31], but this still resulted in relatively low plasma concentrations. The highest plasma concentration we found was approximately 0.5 μM, which is below the concentration range that was shown by us to be effective in attenuating the cytokine response in leukocytes in vitro (1-100 μM). Nevertheless, the inverse correlations between GTS-21 concentrations and plasma cytokine levels suggest that this drug may be able to modulate the innate immune response in humans *in vivo*. The only other report on pharmacological stimulation of the cholinergic anti-inflammatory pathway in humans describes the effects of transdermal nicotine administration during human endotoxemia[32]. Nicotine exerted no effects on proinflammatory cytokines, but it potentiated plasma levels of the anti-inflammatory cytokine IL-10. Similar to our study, the absence of an effect on proinflammation was likely due to the low plasma levels of nicotine achieved. Furthermore, the authors speculated that the increase in IL-10 might result from increased cortisol levels found in the nicotine-treated group rather than a direct effect of α7nAChR stimulation, because glucocorticosteroids have been shown to enhance IL-10 production during human endotoxemia[33, 34].

We demonstrated remarkable anti-inflammatory effects of enteral administration of lipid- and protein-enriched nutrition during human endotoxemia. We hereby present, for the first time, human *in vivo* evidence for a recently described gut-brain-immune axis. Nutritional stimulation of the cholinergic anti-inflammatory pathway had, up till now, only been investigated in animal models[35-37]. In these studies, it was shown that vagotomy, as well as nAChR and CCK receptor antagonists, inactivate this pathway. In addition, a recent study demonstrated that mesenteric afferent vagus nerve firing significantly increased in response to lipid-enriched nutrition[36]. These findings have led to the characterization of a gut-brain-immune axis in which lipid-rich nutrition

results in intestinal release of CCK, which activates vagal afferents, resulting in a vago-vagal reflex that limits the immune response via activation of nAChRs on immune cells. Animal studies revealed that low-lipid nutrition also dampens the innate immune response compared with fasted animals, albeit to a lesser extent than enriched nutrition[36], which we confirm in our human study. However, we must remain cautious in our conclusions, as the role of the vagus nerve was not assessed in our study. It is interesting to discuss our findings in light of the superior effects of early enteral versus parenteral nutrition on outcome in critically ill patients[38-41], of which the mechanisms are largely unknown. The gut-brain-immune axis could play a role in this observed difference, as parenteral nutrition does not lead to CCK release in the intestine and activation of this pathway, while enteral nutrition does. In this respect, attenuation of excessive inflammation by activation of the cholinergic anti-inflammatory pathway might contribute to the better outcome associated with early enteral nutrition. The fact that early (< 24 hour) administration of enteral nutrition is associated with better outcome than late[38, 42, 43] is in support of the notion that limiting the innate immune response in the initial phase of ICU admission might be beneficial, while it might have detrimental effects in the later phase.

The ANS is regarded as a system that we cannot consciously influence. However, several recent investigations suggest that through certain concentration/meditation techniques, it is possible to modulate autonomic activity[44-47]. In light of the immunomodulatory effect of the ANS, it is conceivable that by influencing the ANS, the innate immune system can also be modulated. We set out to investigate this in the iceman, and demonstrated remarkable results of a concentration/meditation technique that appears to activate the stress response and thereby the sympathetic nervous system, resulting in suppression of the innate immune response. However, it is important to emphasize that a set of experiments on a single subject does not enable us to demonstrate a cause-effect relationship between concentration/meditation, the autonomic nervous system, and the innate immune response. It was not possible to repeat the experimental endotoxemia study in the same subject without concentration/meditation because of the development of endotoxin tolerance (this thesis and [48]). Nevertheless, case studies with remarkable findings can yield valuable information and are important in hypothesis generation for further research.

In conclusion, stimulation of the cholinergic anti-inflammatory pathway can exert beneficial effects in VILI, but the timing of stimulation of this pathway is crucial, which might limit its clinical applicability. Our studies further indicate that stimulation

of the cholinergic anti-inflammatory pathway is a feasible option to limit the innate immune response in humans, either via nutritional stimulation or via α7nAChR agonists. However, the only currently available specific agonist in humans, GTS-21, has unfavourable pharmacokinetic properties. Concentration/meditation might represent a novel method to consciously activate the sympathetic nervous system and thereby attenuate the innate immune response, but further studies are necessary to confirm this single observation.

Methodological considerations

In the final part of this thesis, we demonstrated that different breathing patterns do not affect HRV parameters or reproducibility over a wide range of HRV magnitudes during human endotoxemia. Lastly, we demonstrated discrepant kinetics of endotoxin tolerance *in vivo* and *ex vivo*.

Paced breathing did not affect HRV parameters. This confirms the validity of the HRV data obtained in the spontaneously breathing healthy subjects presented in this thesis. Hyperventilation did not affect HRV indices as well. This was surprising, because the parasympathetic component of HRV (respiratory sinus arrhythmia) is thought to be predominantly mediated by respiration-induced blood pressure changes which are sensed by carotid baroreceptors, leading to (de)activation of cardiac vagal fibres[49, 50]. Interestingly, in one subject, a relatively large bias (an increase in HF power, a measure of vagus nerve activity) was observed upon mild hyperventilation. This subject had a very low spontaneous respiratory rate (8 breaths/min), corresponding to a frequency of 0.13 Hz, just outside of the HF power spectrum (0.15-0.4 Hz). Hyperventilation moved this subject's breathing frequency within the HF band, leading to the observed increase in HF power. In conclusion, HRV measurements in spontaneously breathing subjects yield valid and reproducible HRV data provided that the subjects respiratory rate lies within the HF frequency band (9-24 breaths/min).

The observed differential *ex vivo* and *in vivo* endotoxin tolerance kinetics following human endotoxemia teaches us various things. The fact that *ex vivo* endotoxin tolerance quickly subsides, while the *in vivo* response to endotoxin is still severely impaired two weeks later indicates that the blood compartment does not contribute significantly to the *in vivo* cytokine response to LPS, and that tissue-resident macrophages are its main source. This is supported by previous data from our group demonstrating that there exists no correlation between the *ex vivo* and *in vivo* inflammatory response to LPS[51]. This is an important observation, because in many studies, a subject's or patient's

LPS-stimulated leukocyte response *ex vivo* is used as a measure of the immune status *in vivo*. Our data indicate that, while a patient's or subject's *ex vivo* response might be normal, the *in vivo* response to a pathogenic stimulus, vital in host defence, may still be severely impaired. It is also important to mention that endotoxemia-induced changes in hemodynamics, temperature, leukocyte counts and symptom score were similar during both endotoxemia experiments. These responses were apparently not subject to tolerance. This disparity between clinical symptoms and cytokine levels are of particular interest from a clinical point of view. In a clinical sepsis trial, patients are usually included when SIRS criteria are present. If the effects of, e.g. an anti-cytokine intervention are studied, our data illustrate that the target cytokine of interest may not be elevated in the presence of SIRS criteria. Therefore, in our view, evidence that the target analyte is present should be one of the inclusion criteria prior to enrolment. Furthermore, our data have important implications for the design of pharmacological intervention trials using the human endotoxemia model. We show that a washout of 2 weeks is not enough and the author's response in chapter 12a indicates that 4 weeks is not enough as well. They state that 6 weeks is an appropriate window, but by then, antibodies to LPS may have been formed. We therefore recommend to refrain from cross-over designs but use parallel groups instead in trials using the experimental endotoxemia model.

Future perspectives

Investigation of the cholinergic anti-inflammatory pathway is seriously hindered by the lack of a proper readout for vagus nerve activity or activation of the α7nAChR. The only available method to determine vagus nerve activity in humans *in vivo* is HRV, and we and others[52] debate its value. In addition and to the best of our knowledge, there are no measures of specific activation of the α7nAChR on immune cells, such as mRNA or protein expression patterns. This makes it difficult to assess the beneficial or pathological role of this pathway in health and disease. Therefore, novel techniques to measure vagus nerve activity or α7nAChR activation are highly warranted.

Given the difficulties outlined above, the role of intracranial pressure on vagus nerve activity and the immune response should perhaps also be investigated using animal models of increased intracranial pressure[53-56]. In these models, electrophysiological recordings[57] of efferent vagus nerve discharges can shed light on the effects of increased intracranial pressure on vagus nerve activity. These experiments can also be combined with an inflammatory challenge (LPS or live bacterial sepsis) to investigate the effects of intracranial pressure-induced vagus nerve activity on the innate immune response. Moreover, an electrophysiological approach can elucidate organ-specific outflow and allow for comparison with vagal HRV parameters, which can be obtained in animals as well[58, 59].

The only two studies to date on the effects of stimulation of the cholinergic anti-inflammatory pathway in single hit VILI models (our study and [9]) are promising. Therefore, it could represent a novel therapeutic option to limit mechanical ventilation-induced pulmonary inflammation in previously healthy lungs, for instance in mechanical ventilation during elective surgery. Limiting the first mechanical ventilation-induced hit might reduce the risk of complications in the later phase. For example, inflammation of the lungs can spread systemically and lead to multiple organ dysfunction syndrome (MODS)[60].

We did not demonstrate that pharmacological stimulation of the cholinergic anti-inflammatory pathway with GTS-21 attenuated the innate immune response during human endotoxemia. Nevertheless, with regard to the low plasma levels of GTS-21 (relative to the concentrations needed to attenuate the inflammatory response *in vitro*), and the observed correlations between higher GTS-21 plasma concentrations and lower cytokine levels in the *in vivo* study, we feel that selective pharmacological stimulation of the cholinergic anti-inflammatory pathway might have therapeutic

potential. In case of GTS-21, higher dosages or other routes (parenteral) of administration should be evaluated. Furthermore, the therapeutic potential of other specific α7nAChR agonists such as AR-R17779, CAP55, PNU-282987 in humans should be explored if or when they are safe and suitable for human use.

Furthermore, alternative means of stimulation of the cholinergic anti-inflammatory pathway should be investigated in humans. We have shown that lipid- and protein-enriched nutrition greatly limits the innate immune response during human endotoxemia, supposedly via CCK-mediated activation of the cholinergic anti-inflammatory pathway. This mechanism should be further expanded on using specific CCK receptor antagonists, which are currently not available for human use. On the effector side, studies with mecamylamine, a nAChR antagonist used for the treatment of hypertension and in smoking cessation, could establish the role of nAChRs. Besides nutritional or pharmacological activation of this pathway, other means, such as transcutaneous vagus nerve stimulation should be evaluated. This technique is feasible in humans[61] and has recently been shown to be equally effective in limiting the innate immune response as electrical vagus nerve stimulation in mice[6].

The lack of an effect in our second-hit VILI model questions the clinical applicability of stimulation of the cholinergic anti-inflammatory pathway in critically ill patients with an already present systemic inflammatory process, such as in sepsis. Given the scarcity of studies on the effects of delayed stimulation of the cholinergic anti-inflammatory pathway, further preclinical investigations should be performed before a translation to the clinic is in sight. However, in patients where pretreatment is feasible, such as the inflammatory response caused by major elective surgery, therapies based on stimulation of the cholinergic pathway might be introduced earlier in the clinical setting. Stimulation of this pathway may also be of benefit in autoimmune diseases where timing of treatment is not as vital. The clinical translation of therapies based on stimulation of the cholinergic anti-inflammatory pathway may therefore turn out to be analogous to that of the many therapies aimed to limit the innate immune response in sepsis that also showed great promise in animal models. While these treatments have ultimately failed in the treatment of sepsis, they are very effective in the treatment of autoimmune diseases such as rheumatoid arthritis. Such a role for therapies based on stimulation of the cholinergic anti-inflammatory pathway is supported by the promising results recently obtained in animal models of rheumatoid arthritis[62, 63]. The first study investigating the effects of electrical vagus nerve stimulation in rheumatoid arthritis patients is currently being performed in Amsterdam.

The effects of the iceman's concentration/meditation technique on the sympathetic nervous system and innate immune response are remarkable. However, as we already pointed out, we describe a study in just one individual. The iceman claims that he can teach others his technique within a short time frame. We are currently investigating the possibilities to perform a study to reproduce these findings in a larger group of individuals.

Finally, while the long-lasting *in vivo* tolerance that we demonstrated in this thesis prohibits the use of a superior cross-over design in pharmacological intervention trials using the human endotoxemia model, it provides a novel tool to study effects of therapeutic modalities aimed to stimulate the immune response and reverse immune paralysis in humans *in vivo*, currently a hot topic in the sepsis literature[64]. The persisting hyporesponsive state provides an ample window for administration of drugs. This study has thereby opened the way for translational research into immunostimulatory therapies that are currently conducted in our department. A more nuanced evaluation of the sepsis patient may eventually result in tailor-made therapies to modulate the innate immune response: inhibition in patients suffering from hyperinflammation and stimulation in immune paralyzed patients. This could prove to be the way to go for the treatment of sepsis patients and presents an exciting challenge for future research.

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Chapter 15

Nederlandse samenvatting, algehele conclusies en toekomstperspectieven

Nederlandse samenvatting

Een goed functionerend aangeboren immuunsysteem beschermt het lichaam tegen ziekteverwekkende micro-organismen zoals bacteriën en virussen. Na herkenning van micro-organismen door receptoren, met name de Toll-like receptoren, aanwezig op cellen van het aangeboren immuunsysteem produceren deze cellen ontstekingseiwitten, ook wel cytokinen genoemd. Deze cytokinen, zoals tumour necrosis factor-α (TNF-α) en interleukine-6 (IL-6), zijn belangrijk voor het genereren van een effectieve immuunrespons, ook wel ontstekings/inflammatoire respons genoemd, met als doel de micro-organismen op te ruimen. Echter, zoals Shakespeare al zei: 'teveel van iets goeds kan slecht voor je zijn'. Teveel of persisterende productie van cytokinen, zoals bijvoorbeeld bij sepsis, en onnodige activatie van het immuunsysteem zoals bij autoimmuunziekten, kan leiden tot een hyperinflammatoire status, waardoor organen schade kunnen ondervinden. Het aangeboren immuunsysteem heeft daarom twee gezichten: enerzijds is het essentieel voor onze afweer en overleving, terwijl anderzijds over- of langdurige activatie van dit systeem schade veroorzaakt bij de gastheer.

Uit recent onderzoek is gebleken dat het autonome zenuwstelsel het aangeboren immuunsysteem kan remmen. De bestaande gedachte dat een ontstekingsreactie niet door het brein beïnvloed kon worden blijkt onjuist. Dit mechanisme wordt tot op heden niet benut om ziektebeelden geassocieerd met overmatige inflammatie te behandelen, maar biedt daar wel mogelijkheden toe. Het autonome zenuwstelsel bestaat uit twee delen: het sympathische zenuwstelsel en het parasympathische zenuwstelsel, het laatste vrijwel geheel bestaand uit de nervus vagus (de 10^{e} hersenzenuw). Het autonome zenuwstelsel beïnvloedt nagenoeg alle organen in het lichaam.

Dit proefschrift bevat preklinische, translationele en klinische studies gericht op de interactie tussen het autonome zenuwstelsel en de aangeboren immuunrespons. We hebben ons voornamelijk gericht op de effecten van de nervus vagus op het immuunsysteem, de zogenaamde 'cholinerge anti-inflammatoire pathway'. Deze pathway is recent ontdekt en de onderliggende mechanismen en effecten van de beïnvloeding van deze pathway zijn, vooral bij de mens, nog nauwelijks onderzocht.

Hoofdstuk 1 geeft een korte introductie op het autonome zenuwstelsel, het aangeboren immuunsysteem en de interactie tussen beide systemen. Eerder is aangetoond dat de afferente tak van de nervus vagus, van organen naar het brein, een ontsteking in perifere organen kan detecteren en zo het brein kan informeren, hetgeen resulteert in koorts, productie van cortisol en verhoogde activiteit van de

efferente nervus vagus, van het brein naar de organen. In de jaren negentig van de vorige eeuw is ontdekt dat efferente activiteit van het sympathische zenuwstelsel de immuunrespons remt door effecten van catecholamines, de neurotransmitters van het sympathisch zenuwstelsel, op β-receptoren. Deze resultaten zijn bevestigd bij mensen *in vivo,* in het gehele lichaam, dus niet in geïsoleerde cellen of weefsels in het laboratorium. Middels intraveneuze toediening van lipopolysaccharide, LPS, een bestanddeel van de celwand van een Gram negatieve bacterie is het mogelijk de aangeboren immuunrespons bij de mens op te wekken en te onderzoeken. Dit wordt experimentele endotoxinemie genoemd. Toediening van de catecholamine adrenaline leidt tot verminderde productie van cytokinen tijdens experimentele endotoxinemie in gezonde vrijwilligers. In 2000 is door Tracey en collega's de cholinerge anti-inflammatoire pathway bij dieren beschreven. Stimulatie van de efferente nervus vagus remt de aangeboren immuunrespons, via binding van acetylcholine, de vagale neurotransmitter, aan de α7 nicotinerge acetycholine receptor (α7nAChR) op cellen van het aangeboren immuunsysteem, zoals macrofagen. Remming van de efferente nervus vagus heeft het tegenovergestelde effect. Hoe belangrijk de rol van deze pathway bij de mens is, is niet bekend.

Deel 1: Interacties tussen het autonome zenuwstelsel en het aangeboren immuunsysteem

Het eerste deel van dit proefschrift beschrijft onderzoek naar de interactie tussen het autonome zenuwstelsel en het aangeboren immuunsysteem. We onderzochten dit zowel bij gezonde vrijwilligers als bij patiënten met hersenletsel.

In **hoofdstuk 2a** postuleren we de hypothese dat verhoogde activiteit van de nervus vagus een rol speelt in het optreden van immuunparalyse bij patiënten met traumatisch hersenletsel. Deze patiënten hebben vaak een verminderd functionerend immuunsysteem, ook wel immuunparalyse genoemd, met als gevolg verhoogde vatbaarheid voor infecties. Bij deze patiënten met hersenletsel is tevens verhoogde activiteit van de nervus vagus aangetoond, gemeten door middel van hartritmevariabiliteit (HRV). We koppelen deze twee observaties aan elkaar: we stellen dat de immuunparalyse bij deze patiënten veroorzaakt kan worden door (langdurig) verhoogde activiteit van de nervus vagus, resulterend in een remming van de immuunrespons via de cholinerge anti-inflammatoire pathway. We veronderstellen verder dat verhoogde intracraniële druk (hersendruk), vaak voorkomend bij patiënten met hersenletsel, een belangrijke rol speelt in de verhoogde activiteit van de nervus vagus. Deze druk kan leiden tot compressie van de hersenstam met als gevolg verhoogde

activiteit van de dorsale motor nucleus in de medulla oblongata, het centrum waar de nervus vagus ontspringt. Dit wordt ondersteund door bevindingen van verhoogde activiteit van de nervus vagus in patiënten met subarachnoïdale bloedingen, een aandoening die vaak gepaard gaat met hoge intracraniële drukken. Verder is middels dierexperimenteel onderzoek waarbij de intracraniële druk kunstmatig wordt verhoogd door het opblazen van een subdurale ballon aangetoond dat de stijging in activiteit van de nervus vagus correleert met de stijging van de intracraniële druk.

In het voorgaande hoofdstuk veronderstellen we een relatie tussen verhoogde intracraniële druk, activiteit van de nervus vagus en de aangeboren immuunrespons bij patiënten met een subarachnoïdale bloeding. Met het oog op deze relatie becommentariëren we in **hoofdstuk 2b** een artikel waarin de relatie tussen de activiteit van het sympathisch zenuwstelsel, gemeten door noradrenaline spillover, en circulerende cytokinen bij intensive care patiënten met een subarachnoïdale bloeding wordt beschreven. De auteurs tonen verhoogde noradrenaline spillover en verhoogde concentraties van cytokinen in het bloed aan bij deze patiënten, maar vinden geen kwantitatieve correlatie tussen deze twee. We stellen dat het meten van cytokinen in het bloed wellicht geen goede maat is van de status van het aangeboren immuunsysteem van patiënten, omdat de productie van deze cytokinen waarschijnlijk slechts het gevolg is van het initiële insult en niet weergeeft in hoeverre het immuunsysteem nog in staat is om te reageren op een volgende infectie door een bacterie of een virus. Meten van de immuunrespons van *ex vivo,* dat wil zeggen buiten het lichaam in het laboratorium, gestimuleerde leukocyten geeft in dit geval een beter beeld van de status van het aangeboren immuunsysteem. Daarnaast stellen we voor dat het interessant is om, met het oog op de cholinerge anti-inflammatoire pathway, naast parameters van het sympathische zenuwstelsel, ook die van het parasympathische zenuwstelsel te bepalen bij onderzoek naar het immuunsysteem bij patiënten met een subarachnoïdale bloeding.

Nicotine is een aspecifieke agonist van de α7nAChR. Daarom kan de cholinerge anti-inflammatoire pathway mogelijk geactiveerd worden door behandeling van patiënten met nicotine. In **hoofdstuk 2c** becommentariëren we een artikel waarin verhoogde mortaliteit wordt gerapporteerd bij intensive care patiënten die behandeld zijn met nicotine vervangingstherapie. We speculeren dat nicotine de aangeboren immuunrespons onderdrukt bij deze patiënten, waardoor ze via de cholinerge anti-inflammatoire pathway immuunparalytisch worden. De daaruit volgende verhoogde vatbaarheid voor infecties kan bijgedragen hebben aan de verhoogde kans op overlijden

van deze patiënten. Dit wordt ondersteund door de bevinding dat 8% van de groep patiënten die behandeld werden met nicotine overleed aan infecties vergeleken met 2% in de groep patiënten die niet behandeld werden met nicotine. Helaas wordt de infectie-incidentie niet vermeld in het artikel.

In **hoofdstuk 3** wordt onze hypothese uit hoofdstuk 2a onderzocht. In deze studie hebben we de relatie onderzocht tussen de activiteit van het autonome zenuwstelsel en de aangeboren immuunrespons bij intensive care patiënten met hersenschade tijdens de eerste vier dagen na opname. De activiteit van het autonome zenuwstelsel werd gemeten door middel van HRV en de immuunrespons door middel van zowel cytokineproductie van *ex vivo* gestimuleerde leukocyten als concentraties van cytokinen in het bloed. We vonden een significante correlatie tussen hogere niveaus van HFnu, een HRV parameter die correspondeert met activiteit van de nervus vagus, en lagere *ex vivo* gestimuleerde TNF-α productie. Om de invloed van intracraniële druk op de activiteit van de nervus vagus en de aangeboren immuunrespons verder te onderzoeken hebben we patiëntgroepen vergeleken met verschillende aandoeningen, geassocieerd enerzijds met hoge intracraniële druk (intracraniële bloeding) en anderzijds met normale intracraniële druk (subarachnoïdale bloeding met een extraventriculaire drain). De *ex vivo* gestimuleerde cytokineproductie was significant lager bij patiënten met een intracraniële bloeding vergeleken met patiënten met een subarachnoïdale bloeding. Daarnaast was er een sterke correlatie tussen hogere niveaus van HFnu en lagere concentraties van TNF-α in het bloed in de groep patiënten met een intracraniële bloeding.
Deze resultaten suggereren een verband tussen hoge intracraniële druk, verhoogde activiteit van de nervus vagus en remming van de immuunrespons en dus een rol voor de cholinerge anti-inflammatoire pathway bij de mens.

Hoofdstuk 4 beschrijft de relatie tussen activiteit van het autonome zenuwstelsel, gemeten door middel van HRV, en de aangeboren immuunrespons bij een grote groep gezonde vrijwilligers die deelnamen aan experimentele endotoxinemie studies. Om te bepalen of de basale activiteit van de nervus vagus of het sympathisch zenuwstelsel de mate van de aangeboren immuunrespons voorspelt, onderzochten we of baseline HRV parameters, bepaald vóór LPS toediening, correleerden met concentraties van cytokinen in het bloed na LPS toediening. We vonden geen enkele correlatie tussen HRV parameters en cytokinen in het bloed. Na LPS toediening daalden HRV parameters aanzienlijk. Daarom onderzochten we vervolgens of de activatie van het aangeboren immuunsysteem het autonome zenuwstelsel beïnvloedt. Wederom toonden we geen

relatie aan. Het ontbreken van een relatie tussen concentraties van plasma cytokinen en HRV veranderingen bleek verder uit experimenten waarbij gezonde vrijwilligers tweemaal LPS toegediend kregen met een interval van twee weken. Als gevolg van endotoxine-tolerantie waren de cytokineconcentraties in het bloed ongeveer half zo hoog na de tweede LPS toediening vergeleken met de eerste LPS toediening. Echter, de HRV veranderingen waren identiek na beide LPS toedieningen, hetgeen aantoont dat er geen directe relatie is tussen de concentratie cytokinen in het bloed en de HRV veranderingen.
Onze bevindingen wijzen er enerzijds op dat de vagale activiteit, gemeten op het hart door middel van HRV, niet de mate van de ontstekingsrespons op LPS voorspelt. Anderzijds tonen onze resultaten aan dat de mate van de ontstekingsreactie niet gerelateerd is aan de veranderingen in HRV.

Deel twee: remmen van de aangeboren immuunrespons door middel van de cholinerge anti-inflammatoire pathway; *in vitro*, bij proefdieren en bij de mens.
Het tweede deel van dit proefschrift is gericht op de effecten van stimulatie van de cholinerge anti-inflammatoire pathway. We onderzochten dit bij proefdieren, met behulp van muis- en ratmodellen van beademingsgeïnduceerde longschade, en bij de mens, zowel *in vitro* bij leukocyten van de mens en *in vivo* tijdens experimentele endotoxinemie.

In **hoofdstuk 5** beschrijven we de effecten van stimulatie en het uitschakelen van de cholinerge anti-inflammatoire pathway op beademingsgeïnduceerde longschade. Mechanische beademing is een levensreddende ingreep, maar kan ook longschade veroorzaken. Deze schade wordt mede veroorzaakt door activatie van het aangeboren immuunsysteem door de mechanische beademing met positieve druk, zogenaamd biotrauma. Een onderliggend ontstekingsproces, dat vaak aanwezig is bij intensive care patiënten, geeft een verhoogd risico op beademingsgeïnduceerde longschade. In onze studie stimuleerden we de cholinerge anti-inflammatoire pathway door elektrische stimulatie van de nervus vagus met een elektrode in de hals. Het uitschakelen van deze pathway werd bereikt door de nervus vagus door te snijden (vagotomie). We onderzochten de effecten van deze behandelingen in de volgorde die in de kliniek ook vaak voorkomt: eerst een inflammatoire respons, bijvoorbeeld door een infectie, gevolgd door beademing. We stimuleerden de nervus vagus of voerden de vagotomie uit na LPS toediening, maar vóór het starten van de beademing. Voor dit tijdspunt werd gekozen om een patiënt met systemische (in het gehele lichaam) ontsteking te simuleren die op de intensive care is gebracht voor beademing. Beademing met een

hoog (15 ml/kg), maar niet met een laag (8 ml/kg) teugvolume versterkte de LPS-geïnduceerde ontstekingsreactie in de longen. Vagotomie resulteerde in verhoogde concentraties van cytokinen in de long en verminderde zuurstofspanning in het bloed bij spontaan ademende dieren, maar leidde niet tot verdere verergering van de LPS-geïnduceerde ontstekingsreactie in beademde ratten. Stimulatie van de nervus vagus had geen invloed op de ontstekingsreactie in zowel spontaan ademende als beademde dieren.
Deze resultaten suggereren dat, in aanwezigheid van een onderliggend ontstekingsproces, stimulatie van de cholinerge anti-inflammatoire pathway geen gunstige effecten meer heeft op beademingsgeïnduceerde longschade. Dit roept twijfels op over het klinisch nut van stimulatie van deze pathway bij patiënten met een onderliggend systemisch ontstekingsproces die op de intensive care worden gebracht voor beademing.

Hoofdstuk 6 beschrijft de ontstekingsremmende effecten en onderliggende mechanismen van α7nAChR stimulatie in leukocyten van de mens. Zowel de niet-specifieke α7nAChR agonist nicotine als de specifieke agonist GTS-21 remde dosisafhankelijk de cytokineproductie in leukocyten gestimuleerd met verschillende Toll-like receptor agonisten. GTS-21 was effectiever dan nicotine. De ontstekingsremmende effecten van GTS-21 werden gereguleerd op het transcriptionele niveau, dat wil zeggen dat GTS-21 de afschrijving van cytokinegenen (mRNA expressie) remde. Tenslotte, uit qPCR array data en experimenten met de JAK2 remmer AG490 bleek dat de effecten van GTS-21 waarschijnlijk via de JAK2-STAT3 pathway verlopen.
Onze data tonen aan dat GTS-21 sterke ontstekingsremmende effecten op primaire menselijke cellen van het aangeboren immuunsysteem en dat deze effecten op het transcriptionele niveau gereguleerd zijn.

In **hoofdstuk 7** beschrijven we de ontstekingsremmende effecten van GTS-21 in een muismodel van beademingsgeïnduceerde longschade. Daarnaast onderzochten we de effecten van zowel neostigmine, een medicijn dat acetylcholine concentraties verhoogt en in eerder onderzoek effectief is gebleken in de behandeling van sepsis bij muizen, als de effecten van het blokkeren van nAChRs door middel van mecamylamine, een medicijn dat in eerder onderzoek pancreatitis in muizen bleek te verergeren. In onze studie werden muizen beademd met een klinisch relevant teugvolume van 8 ml/kg gedurende 4 uur, wat resulteerde in een ontstekingsreactie, zowel in de longen als in het bloed. Behandeling met GTS-21 voor de start van beademing verminderde de

concentraties van TNF-α in de longen en in het bloed en verlaagde de arteriële-alveolaire gradiënt, hetgeen een verbeterde longfunctie aanduidt. Verder remde GTS-21 TNF-α mRNA expressie en productie in geïsoleerde alveolaire macrofagen. Neostigmine en mecamylamine lieten geen effecten zien op ontstekings- of respiratoire parameters.
Onze studie toont aan dat selectieve farmacologische stimulatie van de α7nAChR longschade, geïnduceerd door beademing met klinisch relevante beademingsinstellingen, kan remmen. Een verklaring voor het feit dat neostigmine en mecamylamine geen effecten laten zien, kan zijn dat de relatief milde ontstekingsreactie in ons model niet ernstig genoeg is voor endogene activatie van de cholinerge anti-inflammatoire pathway.

Na de bemoedigende resultaten met GTS-21 in onze vorige studies *in vitro* en bij muizen, hebben we onderzocht of deze stof ook in staat is de aangeboren immuunrespons bij de mens *in vivo* te remmen. Daartoe hebben we in **hoofdstuk 8** een dubbelblinde placebo-gecontroleerde pilot-studie uitgevoerd bij gezonde vrijwilligers tijdens experimentele endotoxinemie. In onze studie slikten proefpersonen GTS-21 of een placebo gedurende 3 dagen vóór, en op de dag van endotoxine-toediening. We vonden geen verschillen in cytokineconcentraties in het bloed tussen de groep proefpersonen behandeld met GTS-21 en de groep behandeld met placebo. Dit kan te wijten zijn aan de relatief lage, en de, tussen de proefpersonen, zeer variabele concentraties van GTS-21 in het bloed. Echter, binnen de groep proefpersonen die behandeld waren met GTS-21 correleerden hogere GTS-21 plasmaconcentraties met lagere plasmaconcentraties van de cytokinen TNF-α, IL-6 en IL-1RA.
De correlatie tussen hogere plasmaspiegels van GTS-21 en lagere concentraties van cytokinen suggereren dat GTS-21, in hogere concentraties, de aangeboren immuunrespons bij de mens *in vivo* kan remmen.

Naast GTS-21 hebben we ook andere methoden onderzocht om de cholinerge anti-inflammatoire pathway in de mens te activeren. **Hoofdstuk 9** beschrijft onderzoek naar een nieuwe darm-hersen-immuun pathway bij gezonde vrijwilligers tijdens experimentele endotoxinemie. Uit eerdere dierstudies is gebleken dat de cholinerge anti-inflammatoire pathway kan worden geactiveerd door enterale toediening van vetverrijkte voeding. Vetverrijkte voeding resulteert in cholecystokinine (CCK) release in de darm, wat lokaal in de darm CCK-receptoren op afferente vagale vezels activeert. Activatie van deze receptoren leidt tot een vagovagale reflex die de aangeboren immuunrespons remt via de cholinerge anti-inflammatoire pathway. In ons onderzoek kregen proefpersonen vet- en eiwitverrijkte voeding of isocalorische controle

voeding toegediend via een nasojejunale sonde. Een derde groep proefpersonen vastte gedurende de studie. Toediening van vet- en eiwitverrijkte voeding resulteerde in lagere concentraties van de cytokinen TNF-α, IL-6 en IL-1RA in het bloed vergeleken met controle voeding en vasten. Bovendien was de concentratie van de ontstekingsremmende stof IL-10 verhoogd in de groep behandeld met eiwit- en vetverrijkte voeding vergeleken bij de gevaste proefpersonen.
Deze resultaten geven aan dat enterale toediening van vet- en eiwitverrijkte voeding de cholinerge anti-inflammatoire pathway kan activeren in de mens en zo de aangeboren immuunrespons kan remmen.

In het laatste hoofdstuk van deel twee, **hoofdstuk 10**, hebben we onderzocht of het autonome zenuwstelsel, en daardoor de aangeboren immuunrespons, kan worden beïnvloed door concentratie/meditatie. We beschrijven een case-study van een individu bekend als 'the iceman'. Deze man heeft diverse uitzonderlijke prestaties op zijn naam staan met betrekking tot extreme koudetolerantie. Hij beweert dat hij deze opmerkelijke prestaties bereikt door middel van een speciale concentratie/meditatie techniek, die hij zichzelf heeft aangeleerd. Hij claimt verder dat hij middels deze techniek zijn autonome zenuwstelsel en daardoor zijn immuunrespons kan beïnvloeden. In onze studie resulteerde tachtig minuten lang zitten in een bak met ijs, waarin 'the iceman' zijn concentratie/meditatie techniek beoefende, in hoge concentraties van plasma cortisol en remming van de cytokineproductie in *ex vivo* gestimuleerde leukocyten. Ook hebben we een endotoxinemie-experiment uitgevoerd bij deze persoon, terwijl hij zijn concentratie/ meditatie techniek beoefende. Na het starten van concentratie/ meditatie vonden we verhoogde concentraties van catecholamines in het bloed en de toename van cortisol in het bloed van 'the iceman' was hoger dan dat van alle eerder onderzochte gezonde vrijwilligers tijdens endotoxinemie. Bovendien waren de symptomen na LPS-toediening bij 'the iceman' opvallend mild in vergelijking met een grote groep proefpersonen die we eerder hebben onderzocht gedurende endotoxinemie en lagen de gemeten piekconcentraties van de cytokinen in het bloed op 1/3 tot 1/2 van de gemiddelden van de groep proefpersonen die we eerder gemeten hebben.
Deze resultaten suggereren dat the iceman, door concentratie/meditatie, een gecontroleerde stressreactie kan opwekken, die gekenmerkt wordt door activering van het sympathische zenuwstelsel en de daarop volgende catecholamine- en cortisolproductie die vervolgens de aangeboren immuunrespons remt. Bovendien impliceert deze studie dat het autonome zenuwstelsel bewust kan worden beïnvloed door middel van concentratie/meditatie technieken.

Deel drie: studies met betrekking tot de gebruikte methoden in dit proefschrift
Het gebruik van verschillende meettechnieken en experimentele modellen leidt onvermijdelijk tot allerlei interessante bevindingen over deze technieken en modellen. In het derde deel van dit proefschrift beschrijven we studies met betrekking tot HRV-analyse en het experimentele endotoxinemiemodel.
In **hoofdstuk 11** hebben we de invloed van verschillende ademhalingspatronen op HRV parameters onderzocht, evenals de reproduceerbaarheid van HRV parameters, bij gezonde proefpersonen tijdens experimentele endotoxinemie. Verschillende ademhalingspatronen zouden van invloed kunnen zijn op HRV. Resultaten uit eerdere studies spreken elkaar echter tegen. Deze studies zijn allemaal uitgevoerd onder statische omstandigheden, waarin HRV niet in grote mate varieert. In hoofdstuk 4 hebben we laten zien dat HRV parameters sterk variëren als gevolg van de ontstekingsrespons geïnduceerd door toediening van LPS. Dit maakte het voor ons mogelijk om de effecten van verschillende ademhalingspatronen op HRV te onderzoeken in een dynamische setting. Gestuurde ademhaling, dat wil zeggen ademen op het ritme van een metronoom, of milde hyperventilatie hadden geen invloed op HRV vergeleken met spontane ademhaling. Bovendien verbeterde gestuurde ademhaling de reproduceerbaarheid van HRV metingen niet.
Onze data laten zien dat gestuurde ademhaling niet noodzakelijk om valide HRV data te verkrijgen.

Onze onderzoeksgroep heeft al eerder aangetoond dat herhaalde toediening van LPS leidt tot endotoxine-tolerantie, gekenmerkt door een verminderde respons op LPS, zowel *in vivo* als *ex vivo*. Farmacologische interventiestudies, die gebruik maken van het experimentele endotoxinemie model, worden vaak uitgevoerd in een cross-over wijze met een interval van één à twee weken. Het is echter niet bekend hoe lang de staat van endotoxine-tolerantie, die kan leiden tot carry-over effecten, aanhoudt. In **hoofdstuk 12a** wordt de kinetiek van endotoxine-tolerantie beschreven, zowel *ex vivo* en *in vivo*. Na toediening van LPS *in vivo* herstelde binnen een week de initieel gedempte *ex vivo* gestimuleerde cytokineproductie van leukocyten uit het bloed van de vrijwilligers die LPS toegediend hadden gekregen. Twee weken na toediening van LPS was de *in vivo* productie van cytokinen na een tweede LPS toediening echter nog altijd sterk onderdrukt. Opvallend genoeg waren de veranderingen in hemodynamiek, lichaamstemperatuur, leukocytenaantal en symptomen vergelijkbaar na beide LPS-toedieningen; hier werd, in tegenstelling tot de cytokineproductie, geen tolerantie gezien.

Deze bevindingen geven aan dat circulerende immuuncellen niet de belangrijkste bron zijn van cytokineproductie na LPS toediening, maar dat deze cytokinen waarschijnlijk hoofdzakelijk worden geproduceerd door weefselmacrofagen. Verder wijzen onze resultaten erop dat *ex vivo* stimulatie van leukocyten de status van de aangeboren immuunrespons *in vivo* niet goed weergeeft. Tenslotte impliceren de resultaten van dit onderzoek dat interventiestudies die gebruik maken van het experimentele endotoxinemiemodel moeten worden uitgevoerd met parallelle groepen in plaats van een cross-over design, omdat de endotoxine-tolerantie die ontstaat de interpretatie van de effecten van de farmacologische interventie bemoeilijkt.

Gezien de bevindingen van hoofdstuk 12a hebben we in **hoofdstuk 12b** een artikel becommentarieerd waarin een experimentele endotoxinemiestudie beschreven wordt die gebruik maakt van een cross-over design met een periode van drie à vier weken tussen de twee LPS toedieningen. We opperen dat deze wash-out periode te kort is, waardoor endotoxine-tolerantie nog steeds aanwezig kan zijn. Dit belemmert de interpretatie van de studiegegevens. De auteurs antwoordden dat ze, na een herziening van hun gegevens, inderdaad een tolerantie-effect vonden. Dit had echter volgens hen geen invloed op de conclusies van dit specifieke onderzoek.

Algehele conclusies en toekomstperspectieven

In het eerste deel van dit proefschrift hebben we aangetoond dat activiteit van de nervus vagus of het sympathisch zenuwstelsel, gemeten met HRV, niet correleert met de aangeboren immuunrespons of vice-versa in gezonde vrijwilligers. We hebben echter wel correlaties gevonden tussen HRV parameters die de activiteit van de nervus vagus weergeven en parameters van het aangeboren immuunsysteem bij patiënten met hersenletsel. Dit suggereert dat de cholinerge anti-inflammatoire pathway bij de zieke mens wel een rol speelt. Echter, op basis van deze observationele studie kunnen we geen definitieve conclusies trekken met betrekking tot onze hypothese dat de immuunparalyse bij deze patiënten veroorzaakt wordt door activatie van de cholinerge anti-inflammatoire pathway, mede omdat we geen verhoging hebben gevonden van de HRV parameters die de activiteit van de nervus vagus weergeven bij deze patiënten. In overeenstemming met onze hypothese vonden we wel de grootste onderdrukking van *ex vivo* cytokineproductie in de groep patiënten met een intracraniële bloeding, een aandoening geassocieerd met een verhoogde intracraniële druk. Helaas wordt onderzoek naar de cholinerge anti-inflammatoire pathway in de mens nog in grote mate beperkt door het ontbreken van een goede uitleesmaat voor activiteit van de nervus vagus of activatie van de α7nAChR. De enige beschikbare methode is HRV, een methode waarvan de betrouwbaarheid betwist wordt. HRV weerspiegelt namelijk slechts de effecten van de nervus vagus op het hart en het is onduidelijk of deze activiteit ook de effecten weergeeft van de nervus vagus op andere organen betrokken bij de aangeboren immuunrespons. Dit is, voor zover ons bekend, nog nooit onderzocht. Er zijn echter wel een aantal studies die laten zien dat de outflow van het sympathische zenuwstelsel verschillend is per orgaan, hetgeen suggereert dat het autonome zenuwstelsel een sterk gedifferentieerd systeem is. Bovendien is bij proefdieren aangetoond dat de cholinerge anti-inflammatoire pathway geactiveerd kan worden door elektrische stimulatie met een intensiteit die onder het niveau ligt dat nodig is voor beïnvloeding van de hartslag, waardoor het mogelijk niet te detecteren is met HRV. Deze bevindingen tonen aan dat HRV een aantal inherente beperkingen heeft, die onderzoek naar de cholinerge anti-inflammatoire pathway bij de mens belemmeren. Niettemin vinden wij en anderen correlaties tussen HRV en de immuunrespons bij ernstig zieke patiënten. Een mogelijke verklaring voor dit fenomeen is dat differentiatie van de outflow van het autonome zenuwstelsel wel aanwezig is bij gezonde proefpersonen, maar dat deze, deels, verloren gaat bij ernstige ziekte en/of hersenletsel. Als het verlies van differentiatie inderdaad afhankelijk is van de ernst van de ziekte, is het ontbreken van correlaties tussen HRV en de immuunrespons bij gezonde proefpersonen tijdens experimentele endotoxinemie niet

opmerkelijk, aangezien dit model relatief mild is.
Gezien de problemen inherent aan HRV zijn nieuwe technieken om de activiteit van de nervus vagus of α7nAChR activatie te bepalen zeer gewenst voor toekomstig onderzoek naar de cholinerge anti-inflammatoire pathway. Verder moet de rol van intracraniële druk op de activiteit van de nervus vagus en de immuunrespons wellicht ook worden onderzocht met behulp van diermodellen waarin de intracraniële druk kunstmatig verhoogd wordt. In deze modellen kunnen elektrofysiologische metingen van de efferente nervus vagus, dat wil zeggen directe zenuwmetingen met behulp van elektrodes, de effecten van verhoogde intracraniële druk op de activiteit van de nervus vagus definitief vaststellen. Deze experimenten kunnen worden gecombineerd met een ontstekingsstimulus (LPS of bacteriële sepsis) om de effecten van de nervus vagus op de aangeboren immuunrespons te onderzoeken. Bovendien kan door elektrofysiologische zenuwmetingen orgaanspecifieke outflow bepaald worden en kan een vergelijking worden gemaakt met HRV parameters die de activiteit van de nervus vagus weergeven.

In het tweede deel van dit proefschrift hebben we aangetoond dat farmacologische stimulatie van de cholinerge anti-inflammatoire pathway door de selectieve α7nAChR agonist GTS-21, toegediend vóór de start van beademing, beademingsgeïnduceerde longschade bij muizen beperkt. Elektrische stimulatie van de nervus vagus, uitgevoerd na LPS toediening maar vóór de start van beademing, had geen gunstige effecten in ratten. Dit zou het gevolg kunnen zijn van de timing; zoals gezegd stimuleerden wij de zenuw tussen twee ontstekingsprikkels (LPS en beademing) in, terwijl in de overgrote meerderheid van studies waarin positieve effecten gevonden werden, de zenuw gestimuleerd werd vóór, of gelijktijdig met het insult. We hebben verder laten zien dat GTS-21 sterke ontstekingsremmende effecten op leukocyten van de mens *in vitro* heeft. Helaas konden we geen ontstekingsremmende effecten van GTS-21 bij mensen *in vivo* aantonen. Dit is hoogstwaarschijnlijk te wijten aan de lage plasmaconcentraties van GTS-21 die bereikt werden na orale toediening van de maximaal toegestane dosering. Deze concentraties zijn namelijk lager dan de concentraties die ontstekingsremmende effecten *in vitro* lieten zien. We vonden wel omgekeerde correlaties tussen hogere bloedspiegels van GTS-21 en lagere concentraties van cytokinen in het bloed. Helaas konden we geen hogere doseringen van GTS-21 gebruiken, omdat de door ons gebruikte dosering de hoogste is die tot nu toe bij mensen veilig is gebleken. De gevonden correlaties suggereren wel dat farmacologische stimulatie van de cholinergic anti-inflammatoire pathway met specifieke agonisten de aangeboren immuunrespons in de mens kan remmen. Ook beschrijven we voor de eerste keer een nieuwe darm-

hersen-immuun pathway bij de mens, waarin enterale toediening van eiwit- en vetverrijkte voeding de aangeboren immuunrespons remt via CCK-gemedieerde activatie van de cholinerge anti-inflammatoire pathway. Deze pathway was tot op heden alleen bij dieren onderzocht. Buiten het feit dat dit mechanisme een nieuwe therapie kan opleveren om overmatige ontsteking te remmen, zijn onze bevindingen interessant in het licht van de superieure effecten van vroeg enteraal versus parenteraal voeden op de prognose van intensive care patiënten. De mechanismen hierachter zijn namelijk grotendeels onbekend. Mogelijk speelt de darm-hersen-immuun pathway hierin een rol, omdat, in tegenstelling tot enterale voeding, parenterale voeding niet leidt tot het vrijkomen van CCK in de darm en activatie van deze pathway, omdat er simpelweg geen voeding in de darm komt. Het is mogelijk dat de activatie van deze pathway door enterale voeding overmatige ontsteking kan remmen en daardoor kan bijdragen aan een betere prognose van deze patiënten. In het laatste hoofdstuk van deel twee presenteren we resultaten die suggereren dat 'the iceman', via zijn concentratie/meditatie techniek, bewust invloed kan uitoefenen op zijn autonome zenuwstelsel en daardoor op zijn aangeboren immuunrespons. Het is echter belangrijk om te benadrukken dat een serie experimenten bij één proefpersoon geen oorzaak-gevolg relatie tussen de concentratie/meditatie techniek, het autonome zenuwstelsel, en de aangeboren immuunrespons aan kan tonen, maar de bevindingen zijn wel opmerkelijk genoeg om vervolgonderzoek bij groepen proefpersonen na te streven.
Met het oog op de toekomst blijkt uit het tweede deel van dit proefschrift dat stimulatie van de cholinerge anti-inflammatoire pathway een mogelijke nieuwe therapie is om overmatige ontsteking te verminderen bij de mens. In het geval van GTS-21 zouden hogere doseringen, als deze veilig zijn, of andere routes, bijvoorbeeld intraveneus, moeten worden geëvalueerd. Bovendien moet het therapeutisch potentieel van andere specifieke α7nAChR agonisten bij de mens worden onderzocht wanneer ze veilig en geschikt zijn gebleken voor gebruik in de mens. Ook zouden alternatieve manieren van stimulatie van de cholinerge anti-inflammatoire pathway, zoals de door ons onderzochte darm-hersen-immuun pathway, verder moeten worden uitgediept. Naast activatie van de cholinerge anti-inflammatoire pathway door voeding of farmaca kunnen ook andere methoden, zoals transcutane stimulatie van de nervus vagus stimulatie, worden geëvalueerd. Deze techniek is haalbaar bij de mens en non-invasief. Onlangs is bij proefdieren aangetoond dat het even effectief kan zijn in het remmen van de aangeboren immuunrespons als elektrische stimulatie van de nervus vagus. Beïnvloeding van het autonome zenuwstelsel en de immuunrespons door concentratie/meditatie behoeft onderzoek bij grotere groepen om de door ons gevonden effecten definitief vast te kunnen stellen. 'The iceman' zegt dat hij

anderen zijn techniek binnen een kort tijdsbestek kan aanleren. We onderzoeken momenteel de mogelijkheden voor een studie met een groep proefpersonen, getraind door 'the iceman' om onze bevindingen te reproduceren. Een belangrijke uitdaging voor toekomstig onderzoek is om vast te stellen of stimulatie van de cholinerge anti-inflammatoire pathway in geval van een reeds aanwezig ontstekingsproces gunstige effecten heeft. Dit is een belangrijke vraag, omdat het in grote mate het klinisch nut van stimulatie van deze pathway bij intensive care patiënten bepaalt. De meerderheid van deze patiënten arriveert namelijk op de intensive care met een reeds aanwezig ontstekingsproces, waardoor voorbehandeling vaak niet mogelijk is. Echter, bij patiënten waarbij voorbehandeling wel haalbaar is, zoals bij een grote electieve operatie die een ontstekingsreactie veroorzaakt en waarbij de patiënt beademd moet gaan worden, hetgeen kan leiden tot verdere longschade, kunnen therapieën gebaseerd op stimulatie van de cholinerge anti-inflammatoire pathway wellicht eerder hun weg vinden naar de kliniek. Stimulatie van deze pathway kan ook van nut zijn bij auto-immuunziekten, waar de timing van de behandeling minder stringent is. Het pad van preklinisch onderzoek naar de kliniek voor therapieën gebaseerd op stimulatie van de cholinerge anti-inflammatoire pathway kan derhalve wel eens analoog blijken te zijn aan dat van de vele ontstekingsremmende therapieën die initieel ontwikkeld zijn voor de behandeling van sepsis, maar thans vooral toegepast worden bij patiënten met auto-immuunziekten. Deze veelbelovende therapieën bij proefdiermodellen van sepsis, bleken uiteindelijk niet effectief te zijn bij de behandeling van septische patiënten. Niettemin worden deze therapieën nu in grote mate gebruikt voor de behandeling van auto-immuunziekten zoals reumatoïde artritis, waarin ze wel erg effectief zijn. Een dergelijke toekomst voor therapieën gebaseerd op stimulatie van de cholinerge anti-inflammatoire pathway wordt ondersteund door veelbelovende resultaten onlangs verkregen in verscheidene proefdierstudies naar reumatoïde artritis. In Amsterdam loopt momenteel het eerste onderzoek waarin onderzocht wordt of elektrische stimulatie van de nervus vagus de ziekteactiviteit bij reumapatiënten vermindert.

In het laatste deel van dit proefschrift hebben we aangetoond dat verschillende ademhalingspatronen niet van invloed zijn op HRV parameters en reproduceerbaarheid tijdens experimentele endotoxinemie. We laten verder zien dat de ademhalingsfrequentie van proefpersonen/patiënten binnen de HF-frequentieband (9-24 ademhalingen/minuut) moet liggen voor een goede beoordeling van de HRV parameters die correleren met nervus vagus activiteit. Onze studie bevestigt de validiteit van de HRV data van gezonde vrijwilligers eerder beschreven dit proefschrift. In het laatste hoofdstuk van dit proefschrift tonen we aan dat de kinetiek van *in vivo* en *ex vivo* endotoxine-tolerantie verschillend is. Het feit dat *ex vivo* endotoxine-tolerantie snel afnam, terwijl de *in vivo*

respons op LPS na twee weken nog steeds ernstig verlaagd was, geeft aan dat het bloedcompartiment geen significante bijdrage heeft aan de *in vivo* cytokineproductie op LPS-toediening. Waarschijnlijk zijn de weefselmacrofagen de belangrijkste bron van de cytokinen in het bloed na LPS-toediening. Dit is een belangrijke constatering, omdat in veel studies de cytokineproductie van *ex vivo* gestimuleerde leukocyten wordt gebruikt als een maatstaf voor de immuunstatus van een patiënt. Onze resultaten tonen aan dat, terwijl de *ex vivo* gestimuleerde cytokineproductie normaal is, de *in vivo* respons op een ontstekingsstimulus, van vitaal belang voor de afweer tegen bacteriën of virussen, nog steeds ernstig verminderd kan zijn. Een andere interessante bevinding van onze studie is dat LPS-geïnduceerde veranderingen in hemodynamiek, lichaamstemperatuur, leukocytenaantal en symptomen vergelijkbaar zijn na beide LPS-toedieningen. Het verschil tussen deze symptomen en plasma cytokineconcentraties is van belang vanuit een klinisch oogpunt. In een klinische sepsisstudie worden patiënten namelijk meestal geïncludeerd op basis van systemic inflammatory response syndrome (SIRS) criteria, waarna ze behandeld worden met bijvoorbeeld een antilichaam tegen een cytokine. Onze data laten echter zien dat, hoewel een patiënt wellicht klinisch een ernstige ontstekingsreactie heeft (meer dan twee SIRS criteria), het cytokine waartegen het antilichaam gericht is, wellicht helemaal niet of niet meer verhoogd is. In onze ogen moet daarom eerst bewijs worden geleverd dat de cytokine, waartegen het antilichaam gericht is, verhoogd aanwezig is, voordat de patiënt geïncludeerd wordt. Onze data hebben ook belangrijke implicaties voor het design van farmacologische interventiestudies die gebruik maken van het experimentele endotoxinemiemodel. Deze data laten namelijk zien dat een wash-out periode van twee weken niet genoeg is. In plaats van een cross-over design raden wij daarom aan om parallelle groepen te gebruiken in studies die gebruik maken van het experimentele endotoxinemiemodel.

Tenslotte, met het oog op de toekomst, maakt ons onderzoek duidelijk dat langdurige *in vivo* tolerantie het gebruik van het superieure cross-over design beperkt, maar dat het wel nieuwe mogelijkheden biedt om het effect van medicijnen te onderzoeken die gericht zijn op het stimuleren van de aangeboren immuunrespons en daarmee het behandelen van immuunparalyse bij de mens *in vivo.* Dit is momenteel een hot topic in de sepsisliteratuur. Ons onderzoek heeft daarmee de weg vrijgemaakt voor translationeel onderzoek naar immunostimulerende therapieën, wat momenteel ook wordt uitgevoerd op onze afdeling. De toekomst voor de behandeling van sepsis ligt wellicht in op maat gemaakte therapieën met betrekking tot modulatie van de aangeboren immuunrespons: remming bij patiënten die lijden aan hyperinflammatie en stimulatie bij patiënten met tekenen van immuunparalyse. Voor toekomstig onderzoek naar de behandeling van sepsis ligt hier een grote uitdaging

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The foundations for this PhD were unquestionably laid at St. Mary's Hospital in London. Under the supervision of **Professor Hughes** I first learned about LPS and its inflammatory and vasodilatory effects. Dear Alun, thank you for this invaluable experience!

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List of publications

The effects of brain injury on heart rate variability and the innate immune response in critically ill patients.
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Voor media-aandacht omtrent het onderzoek bij 'the iceman' zie:
http://www.umcn.nl/Research/Departments/intensive%20care/Pages/MediaExposure.aspx

Curriculum Vitae

Matthijs Kox werd geboren op 8 juli 1982 te Casteren. Na het behalen van het VWO diploma aan het Pius X college te Bladel in 2000 begon hij datzelfde jaar aan de studie Elektrotechniek aan de TU Eindhoven. Begin 2001 stapte hij over naar de Fontys Hogescholen in Eindhoven waar hij de propedeuse Elektrotechniek behaalde. In september 2001 startte hij met de studie Biomedische Wetenschappen aan de Katholieke Universiteit Nijmegen (tegenwoordig Radboud Universiteit Nijmegen). Hij koos in de masterfase van de opleiding voor het hoofdvak Pathobiologie en de bijvakken Geneesmiddelenonderzoek en Toxicologie. In het kader van het bijvak Geneesmiddelenonderzoek liep hij 5 maanden stage op de afdeling Clinical Pharmacology van het St. Mary's Hospital (Imperial College) te London. Onder begeleiding van prof. dr. Alun Hughes in Londen en prof. dr. Peter Pickkers vanuit Nijmegen werd onderzoek gedaan naar de effecten van remmers van Src family tyrosine kinases op lipopolysaccharide-geïnduceerde vasculaire hyporeactiviteit. De afsluitende hoofdvakstage werd uitgevoerd op de afdeling Pathologie van het UMC St Radboud waar hij onder begeleiding van dr. Micha Wilhelmus onderzoek deed naar effecten van ApoE op amyloid-beta-geïnduceerde celdood van astrocyten in het kader van de ziekte van Alzheimer. Na het behalen van het masterdiploma werd in november 2006 gestart met een promotietraject bij de afdeling Intensive Care van het UMC St Radboud onder begeleiding van prof. dr. Peter Pickkers, dr. Astrid Hoedemaekers en prof. dr. Hans van der Hoeven. Het project werd mede gefinancierd door Traumaregio Oost (nu Acute Zorgregio Oost) en in de eerste fase ondersteund door prof. dr. Arie van Vugt. De onderzoeksresultaten staan in dit proefschrift beschreven en werden bovendien gepresenteerd op verscheidene (inter) nationale congressen, wat resulteerde in het behalen van de Young Investigator Award op het congres van the International Society of Shock Societies in Keulen in 2008 en de Poster Award op het International Symposium on Intensive Care and Emergency Medicine in Brussel in 2009. Matthijs bezocht tijdens zijn promotieperiode het lab van prof. dr. David Grundy in Sheffield en het lab van prof. dr. Kevin Tracey in New York. Tevens organiseerde hij een minisymposium voor prof. dr. Roger Papke van de Universiteit van Florida. Hij combineerde zijn promotieonderzoek met de taak van projectleider longschadeonderzoek van de afdelingen Anesthesiologie en Intensive Care. Sinds 1 mei 2011 is Matthijs als wetenschappelijk medewerker verbonden aan de afdelingen Anesthesiologie en Intensive Care van het UMC St. Radboud. In deze functie verzorgt hij onderwijs, begeleidt verschillende promovendi en coördineert het dierexperimenteel onderzoek van beide afdelingen.

Matthijs woont samen met Petri van Gastel in Nijmegen. Op 26 mei 2012 gaan ze trouwen!