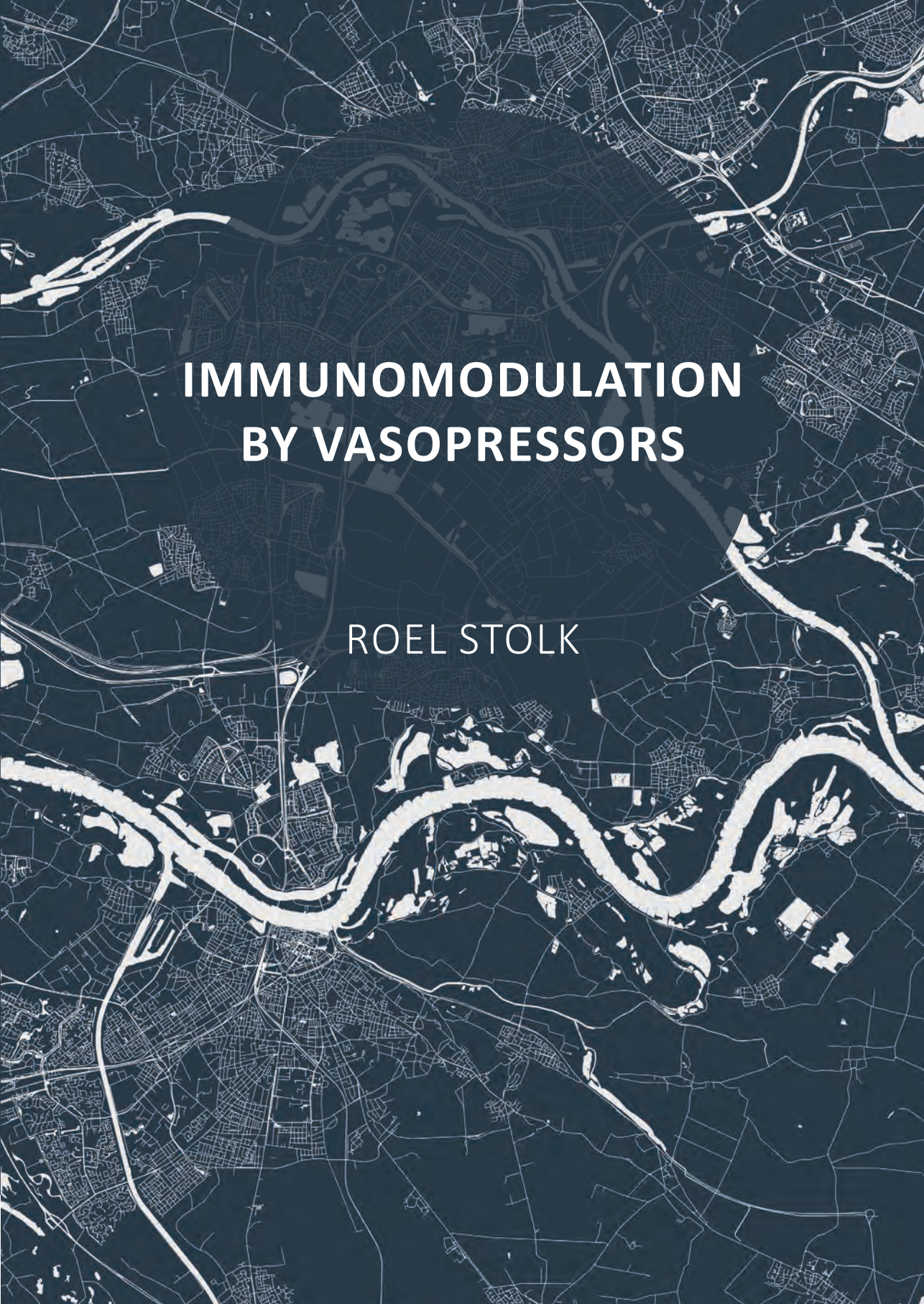

IMMUNOMODULATION BY VASOPRESSORS

ROEL STOLK

IMMUNOMODULATION BY VASOPRESSORS

Roeland Frederik Stolk

COLOFON

Immunomodulation by vasopressors

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The proper function of man is to live, not to exist.
Jack London, The Road

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CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

INTRODUCTION

HOST DEFENSE

The body's protection against invading pathogens, such as bacteria, viruses and fungi, is organized along several 'lines of defense'. The first is represented by our senses and ability to avoid pathogens (one generally does not eat rotten food). The second line consists of physical barriers such as the skin and mucosa. The final frontier for pathogens to overcome is the immune system, initially represented by the innate immune response. Upon recognition of invading pathogens through binding of pathogen associated molecular patterns (PAMPs) to pattern recognition receptors (PRRs), innate immune cells release pro-inflammatory mediators such as cytokines and chemokines, which recruit inflammatory cells to the site of infection and potentiate their capacity to clear pathogens by phagocytosis or production of reactive oxygen species. Furthermore, cells of the adaptive immune system are activated and instructed, signifying a critical bridging function of the innate immune system. Simultaneously, this pro-inflammatory reaction is balanced by generation of an anti-inflammatory response, primarily consisting of production of anti-inflammatory mediators, aimed at maintaining homeostasis and promoting tissue repair and healing. An adequate innate immune response is characterized by a sufficiently strong pro-inflammatory reaction to eradicate the invading pathogens while collateral damage to the host is avoided. Pathogens breach the first and second line of defense on a daily basis (for example, when brushing our teeth) so a well-functioning innate immune system is vital for survival.

SEPSIS

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection and it represents a major healthcare problem. In 2017, the WHO designated sepsis as a disease with worldwide health priority (1) due to an enormous contribution to global morbidity and mortality (2, 3). Up till the present day, only two major advances have been made in the treatment for sepsis that have significantly contributed to improved survival, namely the introduction of antibiotics and the advent of intensive care units where organ supportive treatments are provided. Over the last decades, many research efforts have been focused on targeting the dysregulated immune reaction in sepsis (4). Early studies focused solely on attenuation of the immune response, for instance through blocking pro-inflammatory cytokines. These attempts have, without exception, failed to improve the outcome of patients, which is probably related to the complexity and heterogeneity of the immune response in sepsis. As mentioned before, both pro- and anti-inflammatory reactions are activated during sepsis and excessive manifestations on both sides of this spectrum are associated with worse outcome. Because studies aimed at immunosuppression failed to improve outcome, attention has shifted towards the detrimental role of a too pronounced or

protracted anti-inflammatory response, termed sepsis-induced immunoparalysis. Recently, immunoparalysis has gained attention as a major contributor to mortality in sepsis patients (5, 6) and this has promoted interest in novel treatment strategies aimed at reconstitution of the immune response, which include targeting of immune checkpoint molecules such as programmed death-ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) as well as use of immunostimulatory compounds such as interferon-γ (IFN- γ) (7). Furthermore, it is increasingly recognized that immunotherapy needs to be personalized, an approach known as 'precision medicine'. Patients with immunoparalysis might benefit from immune reconstitution while patients with a hyperinflammatory response, for example those suffering from macrophage activation-like syndrome (8) might benefit from immunosuppressive therapy. Furthermore, these new insights also compel us to critically review the immunological effects of the supportive treatments in current clinical use, as these might contribute to dysregulation of the immune response as well.

VASOPRESSOR THERAPY IN SEPSIS: NORADRENALINE

Patients with septic shock, the most severe form of sepsis, suffer from circulatory failure which causes inadequate oxygen delivery to tissues and cells to fulfill metabolic needs. As such, shock often results in multiorgan failure and death. Clinically, patients with septic shock often present with hypotension, decreased urine output, and altered mental status (9). Upon recognition of shock, urgent treatment is required and, in addition to fluid resuscitation, vasopressors are the mainstay supportive therapy. Currently, noradrenaline is the cornerstone vasopressor agent for hemodynamic support used in virtually every patient with septic shock worldwide. It is a sympathetic neurotransmitter which signals via α- and β-adrenergic receptors present on most tissues in the human body including immune cells (10). Together with the related adrenaline, noradrenaline (with the prefix *'nor'* meaning *N* (*nitrogen) ohne radikal*, to indicate the absence of a methyl group) belongs to a group of chemicals containing a catechol group and an amine side-chain, hence termed catecholamines (11). Noradrenaline has been used as a vasopressor since the 1950's (12), after its initial isolation and identification as a neurotransmitter by Ulf von Euler in 1946 (13). In recognition of this achievement, he received the Nobel prize in physiology and medicine in 1970, in conjunction with Julius Axelrod and Bernard Katz. Research into the application of noradrenaline quickly expanded, after it's favorable vasopressor characteristics compared to adrenaline were recognized (14). Von Euler noted: '*Although the condition may often appear alarming the chances of restoring the blood-pressure and satisfactory blood circulation with noradrenaline are generally good'* (12). Noradrenaline was found to be effective in patients suffering from several shock-etiologies*,* including *'shock associated with overwhelming states of infection'* (15). Currently, it is recommended as the first-line vasopressor by the surviving sepsis guideline (16). As a result millions of patients worldwide are treated

with this drug annually. However, it has become clear that use of noradrenaline and high systemic catecholamine levels in general are not without drawbacks. For example, noradrenaline levels are an independent risk factor for mortality in sepsis patients (17) and recently, a strategy of lowering catecholamine exposure in elderly patients was shown to result in reduced mortality, also after correction for prespecified baseline confounders (18). An explanation for these untoward effects could be immunologic effects exerted by noradrenaline, although they have hitherto not been properly investigated and consequently are not taken into account when administering this drug in daily clinical practice. *In vitro* experiments have demonstrated that noradrenaline exerts immunosuppressive effects. For instance, it was shown to attenuate the production of pro-inflammatory cytokines, diminish natural killer cell cytotoxicity, skew the development of T-helper cells towards an anti-inflammatory T-helper 2 phenotype, and reduces expression of HLA-DR, a well-known marker of immune function, on antigen-presenting cells (19-22). Unlike noradrenaline's vasopressor effects, which are mediated through the α-adrenergic receptor, these effects appear to be predominantly mediated via the β-adrenergic receptor (21). Through these immunosuppressive actions, noradrenaline may dysregulate the immune response in sepsis patients, and could therefore contribute to the development, sustainment, and aggravation of sepsis-induced immunoparalysis, and thus to impaired outcome.

This immunomodulatory role of the β-adrenergic receptor potentially has broad implications. For instance, beneficial effects of the β-adrenergic blocker agent esmolol were demonstrated in patients with septic shock and high noradrenaline requirements (23). Genetic variation in (β-)adrenergic receptors, caused by differences in base pairs, called single nucleotide polymorphisms (SNPs) may be of relevance as well, as this could influence the extent of or susceptibility towards noradrenaline's immunomodulatory effects. Along these lines, a combination of single nucleotide polymorphisms (a haplotype) in the β2-adrenergic receptor gene (*ADRB2*) has been associated with increased mortality in septic patients (24). Whether immunologic effects are involved nevertheless remained to be investigated.

VASOPRESSOR THERAPY IN SURGICAL PATIENTS: PHENYLEPHRINE

Although sepsis represents the archetypical condition associated with a dysregulated immune response, many other critically ill patients on the ICU suffer from it as well. These include burn and trauma victims, but also patients undergoing major surgery (25). During surgery, danger associated molecular patterns (DAMPs) are released, which, like PAMPs, induce an inflammatory response. This pro-inflammatory phase is often followed by a period of profound immunosuppression, which puts the surgical patient at increased risk for post-operative infections and possibly also leads to long-term adverse effects such as increased metastatic spread of cancer (25, 26). Albeit to a lesser extent

than in sepsis, hemodynamic instability is frequently encountered in anaesthetized patients in the operating room. Therefore many patients require vasopressor therapy in the peri-operative period, for which phenylephrine is a predominant agent of choice (27). Phenylephrine was originally developed in the 1930's and 1940's as an alternative for adrenaline and ephedrine to counteract peri-operative hypotension. The goal was to introduce a drug with a similar capability to stabilize blood pressure as adrenaline, but without the disadvantage of tachycardia and nervous symptoms (such as anxiety), which are associated with use of the latter (28). Phenylephrine is widely regarded as a pure α-adrenergic agonist, but there are indications for β-affinity as well (29, 30). If phenylephrine poses significant β-adrenergic affinity, it could be hypothesized that peri-operative administration contributes to post-operative immune suppression and thereby impairs outcome for patients. However, research on the immunologic effects of phenylephrine was hitherto largely lacking.

ALTERNATIVE VASOPRESSOR: VASOPRESSIN

Vasopressin is a synthetic analog of the endogenous antidiuretic hormone. It's vasoconstrictive effect was already established in the 1920's (31). In current clinical practice, vasopressin is mainly used as an adjunctive treatment for patients in the ICU with a high need for hemodynamic support, for which only noradrenaline does not suffice (so-called 'catecholamine resistant shock'). Immunologic effects of vasopressin were hardly studied up to this point.

MODULATION OF THE CEREBRAL, SYSTEMIC AND MICROCIRCULATION BY VASOPRESSORS

Shock results in impaired tissue oxygenation. The aim of vasopressor therapy is to restore tissue oxygenation, however no direct measurement of tissue oxygenation is available in current clinical practice. As a substitute, clinicians rely on surrogate markers such as lactate clearance or systemic hemodynamic parameters like mean arterial pressure (MAP) to guide vasopressor therapy. However, these measurements do not tell the complete story of the effects of vasopressor administration. Vasopressors exert effects on all vascular compartments, including the cerebral circulation. This is especially important for patients with inflammatory conditions such as sepsis, as they often suffer from disruptions in vascular reactivity. In the brain, this can result in sepsis-associated encephalopathy (SAE), which is characterized by diffuse brain dysfunction (32, 33). In addition to a disrupted cerebral perfusion, the microvascular compartment is severely affected by systemic inflammation as well. Under physiological circumstances, the microvasculature is a dense, well perfused network of capillaries. Sepsis causes endothelial dysfunction and is therefore associated with inappropriate vasodilation and vasoconstriction, which ultimately results in decreased delivery of oxygen in the tissues and organ dysfunction (34). Both dysfunction of the cerebral circulation and

microcirculatory dysfunction have been associated with poor outcomes in sepsis (33, 35). Human *in vivo* data regarding effects of vasopressors in these compartments were lacking.

THE EXPERIMENTAL HUMAN ENDOTOXEMIA MODEL

Both pre-clinical immunological data on vasopressors as well as their effects on cerebral perfusion and the microvasculature stem from *in vitro* experiments or (limited) animal models. Although mechanistically invaluable, the results of these models unfortunately have poor clinical translatability. Studying effects of vasopressors in patients however is cumbersome as well. There are marked inter-individual differences in sepsis patients in factors like age, co-morbidities, co-medication, causative pathogen, duration of the inflammatory hit and disease severity (36).

The experimental human endotoxemia model, where purified *E.Coli*-derived endotoxin (lipopolysaccharide, LPS) is administered to healthy volunteers, is a safe model of systemic inflammation in humans *in vivo.* Endotoxin administration results in a transient, controlled, and reproducible acute systemic inflammatory response, characterized by the development of fever and flu-like symptoms, changes in circulating leukocytes and increased plasma levels of cytokines and chemokines. Thereby, it captures many of the immunologic hallmarks of early sepsis. Other effects of endotoxemia include metabolic changes, increased renal flow and importantly, increases in cardiac index and a decrease in MAP, which can affect the cerebral perfusion. Furthermore, microvascular changes are also observed in this model (37, 38). Consequently the human endotoxemia model provides a much needed intermediary step between preclinical animal data and clinical patient studies (37).

OUTLINE OF THIS THESIS

In part I, an overview of the literature on sepsis-induced immunoparalysis, the immunomodulatory effects of vasopressors, and possible clinical consequences of vasopressor-induced immunosuppression is provided. **Chapter 2** comprises an extensive review on these topics, describing among others the available data on *in vitro,* animal and clinical studies into immunomodulation by noradrenaline and other vasopressors. A more concise opinionating piece is provided in **chapter 3**, focusing on noradrenaline's role in driving immunosuppression and its detrimental sequelae in sepsis patients**.**

Translational studies into the immunological effects of vasopressors are provided in part II. **Chapter 4** focuses on noradrenaline in comparison with vasopressin, comprising *in vitro, in vivo* animal and human experiments, and observational patient data. Whether SNPs and haplotypes in the *ADRB2* gene influence the immunosuppressive effects of noradrenaline or systemic inflammation *per se* is evaluated in **chapter 5**, making use of *ex vivo* stimulated leukocytes and the experimental human endotoxemia model in a large cohort of healthy volunteers. In **chapter 6,** phenylephrine's immunological effects are systematically evaluated using a similar translational approach as employed in chapter 4.

The final part of this thesis focuses on circulatory effects of noradrenaline, vasopressin and phenylephrine in the experimental human endotoxemia model. In **chapter 7,** the effects of these vasopressors on the cerebral circulation are examined using transcranial doppler measurements. **Chapter 8** entails a comparison of the systemic hemodynamic and microcirculatory effects of the three vasopressors.

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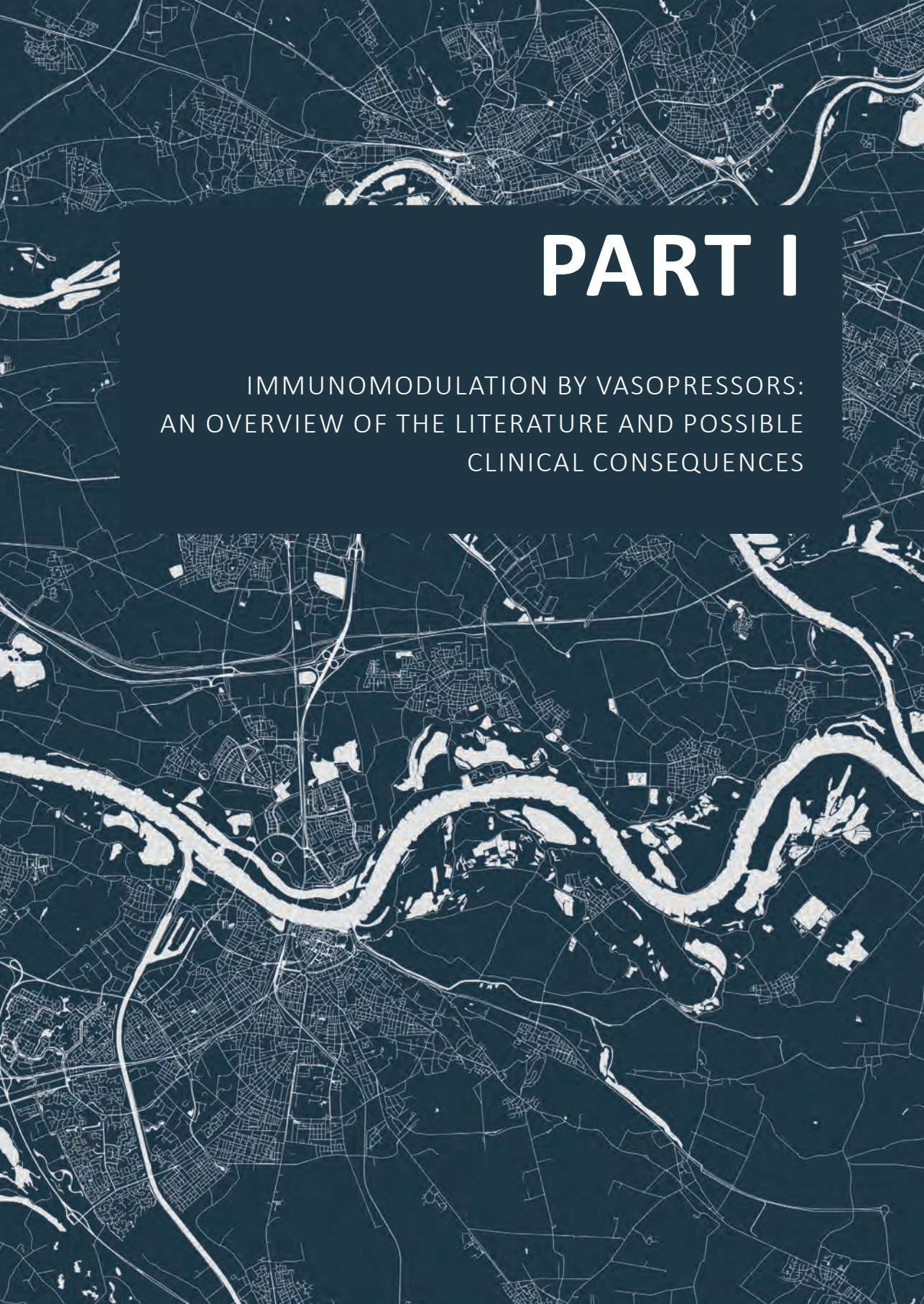

PART I

IMMUNOMODULATION BY VASOPRESSORS: AN OVERVIEW OF THE LITERATURE AND POSSIBLE CLINICAL CONSEQUENCES

CHAPTER 2

POTENTIALLY INADVERTENT IMMUNOMODULATION: NORADRENALINE USE IN SEPSIS

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ABSTRACT

Septic shock is a major cause of death worldwide and a considerable healthcare burden in the 21st century. Recently, attention shifted from damaging effects of the pro-inflammatory response to the detrimental role of anti-inflammation, a phenomenon known as sepsis-induced immunoparalysis. Sepsis-induced immunoparalysis may render patients vulnerable to secondary infections and is associated with impaired outcome. The immunoparalysis hypothesis compels us to re-evaluate the current management of septic shock and assess whether we are inadvertently compromising or altering the host immune response. In this perspective, we discuss the potential detrimental role of noradrenaline, the cornerstone treatment for septic shock, in sepsis-induced immunoparalysis. We provide a short overview of the current understanding of the immunologic pathophysiology of sepsis, followed by a detailed description of the immunomodulatory effects of noradrenaline and alternative vasopressors. We conclude that although novel therapies aimed to reverse immunoparalysis are underway, the use of noradrenaline may aggravate the development, extent, and duration of sepsis-induced immunoparalysis. Current *in vitro* and animal data indicate that noradrenaline treatment exerts immunosuppressive and bacterial growth-promoting effects, and may increase susceptibility towards infections. However, evidence in humans is circumstantial, as immunologic effects of noradrenaline have not properly been investigated in experimental or clinical studies. Alternatives such as vasopressin/selepressin, angiotensin II, and phenylephrine could have a fundamental advantage over noradrenaline with respect to their immunologic properties. However, also for these agents, *in vivo* immunologic data in humans are largely lacking. As such, human studies on the immunomodulatory properties of noradrenaline and viable alternatives are highly warranted.

INTRODUCTION

Septic shock continues to be a considerable healthcare burden in the 21st century and a major cause of death on Intensive Care Units (ICUs) worldwide (1). Septic shock is defined as hypotension and compromised organ perfusion with loss of peripheral vascular resistance and vascular leakage secondary to the host response to infection that is refractory to intravenous fluid resuscitation. Treatment necessitates vasopressor therapy, for which noradrenaline has been the primary agent of choice since the birth of modern day critical care medicine in the 1950s. In the past decades, adjunctive strategies have aimed to treat sepsis by targeting pro-inflammatory mediators; however they have failed to improve outcome in numerous clinical trials. Recently, attention shifted from inhibition of the pro-inflammatory response to the detrimental role of the anti-inflammatory phase, termed albeit somewhat simplistically as sepsis-induced 'immunoparalysis'. Immunoparalysis may render patients unable to clear their primary infection and could increase their vulnerability to secondary/opportunistic infections (2, 3). The possible involvement of sepsis-induced immunoparalysis as a contributing factor to poor outcome of sepsis patients has promoted interest in novel therapeutic targets and strategies designed to reverse or prevent immunoparalysis, including programmed death-ligand 1 (PD-L1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), interferon-gamma (IFN-γ), granulocyte-macrophage colony stimulating factor (GM-CSF), and interleukin-7 (IL-7) therapy (4-8). However, the immunoparalysis hypothesis also compels us to re-evaluate the current management of septic shock and assess whether we are inadvertently compromising or altering the host immune response. As the catecholamine and sympathetic neurotransmitter noradrenaline is the cornerstone treatment for septic shock in ICUs worldwide and exerts profound immunomodulatory effects (9), the current treatment of patients in shock might aggravate the development, extent, and duration of sepsis-induced immunoparalysis.

In this perspective, we provide a short overview of the current understanding of the immunologic pathophysiology of sepsis, followed by a detailed description of the immunomodulatory and bacterial growth-promoting effects of catecholamines, thereby focusing on noradrenaline. We include both preclinical and clinical studies that contribute to our understanding of how noradrenaline may contribute to sepsis-induced immunoparalysis. Finally, we will discuss alternative vasopressors that may not contribute to sepsis-induced immunoparalysis.

IMMUNOLOGIC PATHOPHYSIOLOGY OF SEPSIS

The immune response to infectious agents in sepsis is mediated via pattern recognition receptors (PRRs), present on immune cells such as monocytes, dendritic cells and macrophages, of which the Toll-like Receptor (TLR) family is the most studied. PRRs

recognize specific components of pathogens, so-called pathogen-associated molecular patterns (PAMPs). Activation of PRRs results in production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β and IL-6, as well as recruitment of other immune cells such as granulocytes and lymphocytes to the infected tissue. This cascade is aimed at elimination of pathogens by inducing cytotoxic and phagocytic properties of neutrophils, T-helper 1 (Th1) cells, and macrophages as well as activation of the complement and coagulation systems. Strong activation of the pro-inflammatory response (a so called "cytokine storm") may result in an excessive and injurious reaction, ultimately resulting in septic shock. Anti-inflammatory mechanisms (for example production of the key anti-inflammatory cytokine IL-10, maturation of T-regulatory cells, and lymphocyte apoptosis) are also initiated to limit collateral tissue damage. However, a too pronounced and/or sustained anti-inflammatory phenotype may result in severe impairment of the immune response, known as sepsis-induced immunoparalysis (3). Important hallmarks of sepsis-induced immunoparalysis are deactivation of monocytes and macrophages, exemplified by decreased HLA-DR expression, and impaired *ex vivo*-stimulated pro-inflammatory cytokine production, upregulation of negative regulatory molecules on immune cells, apoptosis of various immune cell populations, and dysregulated cytokine production, favoring the production of anti-inflammatory over pro-inflammatory cytokines (3). It has become clear that these immunosuppressive mechanisms are present from the onset of sepsis (3).

Several observational studies indicate that immunoparalysis may contribute to sepsis mortality. This is exemplified by the fact that markers of immunoparalysis such as reduced expression of human leukocyte antigen (HLA)-DR on monocytes and impaired *ex vivo*-stimulated cytokine production, correlate with outcome of septic patients (10, 11). Furthermore, in patients that died from sepsis, a continuous septic focus in 89% of patients who received antibiotic treatment for more than 7 days was observed (12), and multiple signs of immunosuppression, including reduced cytokine production by *ex vivo*-stimulated splenocytes, increased expression of inhibitory receptors such as PD-1, and expansion of suppressive T-cells were demonstrated (2). In addition, frequent infections with e.g. C*andida* species, *Aspergillus* species, and *Acinetobacter* or reactivations of low-virulent micro-organisms (dormant viruses such as cytomegalovirus, Epstein-Barr Virus, Herpes simplex virus-1 and Human Herpes virus-6) (13, 14) are encountered in sepsis patients, to an extent comparable with that observed in transplant patients on high dose immunosuppressive medication (13). Collectively, these findings indicate that the immune suppression associated with sepsis may contribute to the increased susceptibility of these patients for infections caused by pathogens that are not virulent in otherwise healthy hosts. A detailed overview of the pathogenesis of sepsis and sepsis-induced immunoparalysis is beyond the scope of this perspective and reviewed elsewhere (3, 15). Next to sepsis itself, pharmacological compounds routinely used in

the treatment of sepsis patients might also importantly contribute to immunoparalysis, although relatively little attention has been paid to this.

IMMUNOLOGIC AND BACTERIAL GROWTH-PROMOTING EFFECTS OF THE SYMPATHETIC NERVOUS SYSTEM AND CATECHOLAMINES

Sympathetic autonomic pathways are widespread in the human body, with the *'fight or flight*' reaction being the classic example of sympathetic activation. Noradrenaline is the main neurotransmitter of most autonomic sympathetic post-ganglionic fibers, whereas the related catecholamine adrenaline is predominantly produced in the adrenal chromaffin cells and secreted into the blood. Interestingly, next to the adrenal chromaffin cells, leukocytes are an ample source of catecholamines, signaling in an autocrine/paracrine manner (16). Noradrenaline and adrenaline exert their effects through α and β adrenoceptors (α-and β-ARs), each divided in several subtypes. These 7-transmembrane G-protein-coupled receptors are present on virtually all human tissues, including the lymphoid organs (bone marrow, thymus, spleen, and lymph nodes) and most immune cells. Noradrenaline has predominant α-AR affinity, with increasing β-AR affinity in higher concentrations, whereas adrenaline has predominant β-AR affinity. Noradrenaline has been associated with pro-, but mainly with anti-inflammatory effects, dependent on the specific receptor activated as detailed below.

In vitro studies

Activation of α-ARs by noradrenaline has been shown to augment TNF-α production by macrophages *in vitro* (17). These effects are mediated by activation of NF-κB through protein kinase C-induced IκB phosphorylation, in turn resulting in transcription of pro-inflammatory cytokine genes such as *TNFα, IL1β and IL6* (18). However, another *in vitro* study demonstrated anti-inflammatory effects of α-AR stimulation by noradrenaline (19), marking considerable ambiguity in immunologic effects of α-AR activation. Moreover, already at very low concentrations noradrenaline's immunologic effects appear mainly β-AR-dependent (9, 20), and activation of β-ARs exerts potent anti-inflammatory effects. Ligation of β-ARs enhances intracellular cAMP levels, which induce activation of protein kinase A (PKA), an inhibitor of NF-κB, ultimately resulting in reduced expression of pro-inflammatory cytokine genes (18). Furthermore, synthesis of the anti-inflammatory cytokine IL-10 is enhanced through cAMP- and PKA-dependent pathways (21). In accordance with these β-AR-dependent effects, noradrenaline has been shown to attenuate TNF-α and IL-6 and increase IL-10 production in human whole blood, effects that were diminished by β-blockers metoprolol and propranolol (9, 20). A schematic overview of the intracellular mechanisms behind the effects of α- and β-AR stimulation on cytokine production is provided in **Figure 1**. In addition to effects on cytokine production, other *in vitro* studies have shown that noradrenaline diminishes NK-cell cytotoxicity and downregulates IL-2 production by Th1 cells in a β2-AR-dependent

manner (22, 23). Th2 function was shown to be unaffected, presumably due to the lack of β2-AR expression on these cells (23). Via this mechanism, noradrenaline may skew the immune response from a Th1 towards a Th2 cell phenotype (23), one of the hallmarks of sepsis-induced immunoparalysis. Next to immunologic effects, noradrenaline directly promotes bacterial growth *in vitro*, which has been shown for both Gram-positive and Gram-negative bacteria (24).

In general, the effects of noradrenaline *in vitro* can be characterized as being generally anti-inflammatory, mediated through β-ARs, as well as directly bacterial growth-promoting. This may compromise the ability of the host to combat infection and thus contribute to sepsis-induced immunoparalysis, although this cannot be deduced from these *in vitro* studies.

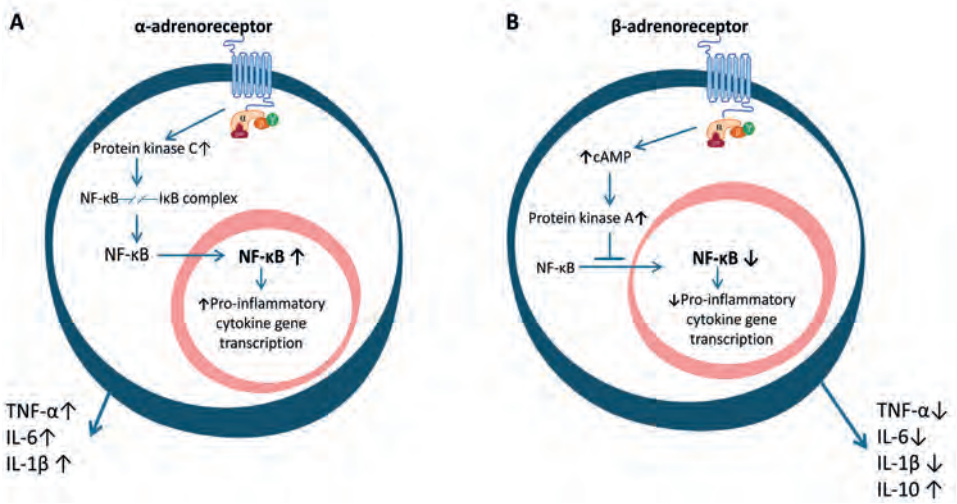

Figure 1. Intracellular mechanisms behind the effects of α- and β-AR stimulation on cytokine production. Panel A: Stimulation of the α-AR results in activation of PKC, which induces IκB phosphorylation, in turn resulting in translocation of NF-κB to the nucleus.
NF-κB facilitates pro-inflammatory cytokine transcription and this ultimately leads to enhanced pro-inflammatory cytokine production. Panel B: Stimulation of the β-AR increases intracellular cAMP levels, which activate PKA. PKA prevents NF-κB from entering the nucleus, resulting in reduced pro-inflammatory cytokine transcription and production and increased production of anti-inflammatory IL-10. AR= adrenoceptor, cAMP=cyclic adenosine monophospate, IκB= Inhibitor nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB= nuclear factor kappa-light-chain-enhancer of activated B cells, PKA=Protein Kinase A, PKC= Protein Kinase C, IL= interleukin, TNF=Tumor necrosis factor.

Animal studies

There are animal studies that confirm and extend the *in vitro* findings by demonstrating that noradrenaline exerts anti-inflammatory effects *in vivo*. For instance, administration of exogenous noradrenaline in the portal vein of rats resulted in increased IL-10 serum

levels, although concentrations of IL-1β were also elevated (25). Along these lines, acute brain injury in rats, associated with high endogenous catecholamine release, triggered systemic release of IL-10 which was completely prevented by propranolol (26), and atenolol increased lipopolysaccharide (LPS)-induced TNF-α production in mice, suggested to be mediated by blocking the effects of endogenous noradrenaline (27). Animal studies have also demonstrated that noradrenaline indeed compromises the host's ability to combat infection. For instance, neutrophils incubated with noradrenaline display an immunosuppressive phenotype, and inoculation of mice with these neutrophils increased susceptibility for sepsis induced by cecal ligation and puncture and resulted in increased mortality (28). Interestingly, in the same study it was demonstrated that the non-infectious inflammatory conditions pancreatitis or burn injury result in generation of neutrophils with the same suppressive phenotype *in vivo*, suggesting a significant role of endogenous release of noradrenaline (28). In line with these observations, destruction of noradrenergic nerve endings renders mice more resistant against *Listeria monocytogenes* infection (29). Furthermore, there is also *in vivo* evidence for noradrenaline's bacterial growth-promoting properties; a murine study revealed that endogenous release of noradrenaline during trauma directly increases the growth of bacteria in the gastro-intestinal system (30). This phenomenon could be an explanation for the high incidence of infectious complications after trauma.

Taken together, *in vivo* animal data on the immunologic and bacterial growth-promoting effects of noradrenaline corroborates the previously described *in vitro* findings. However, the translation of animal data to humans is naturally hampered by species differences. In addition, noradrenaline's profound effects on organ perfusion and thus tissue ischemia can be a confounding factor, as tissue ischemia may induce and/or propagate inflammation.

Clinical studies

To date, no clinical studies have specifically investigated the immunologic effects of noradrenaline in humans. Circumstantial evidence in support of noradrenaline's immunosuppressive effects can however be deduced from several studies, although clear interpretation is cumbersome, as outlined below. Sepsis and septic shock are associated with high levels of endogenously- and exogenously-derived circulating catecholamines. For instance, in septic patients without shock, endogenous noradrenaline plasma concentrations up to 0.9 ng/ml have been reported, compared with levels up to 2.9 ng/ml in septic shock patients (31). If septic shock persists, endogenous production of catecholamines is exhausted (32) and high circulating levels result from exogenous administration. After 5 days of exogenous noradrenaline infusion in non-surviving critically ill patients concentrations up to 10 ng/ml were found (33). There are observational studies indicating that high arterial noradrenaline levels are associated with mortality

in septic shock patients independent of disease severity score and hemodynamic parameters (34, 35); however, these findings are at high risk of confounding by indication and no immunological parameters were assessed. Recent findings indicate that blocking β-adrenergic effects may exert beneficial effects in septic shock patients (36). A retrospective study showed that septic patients treated with β-blockers before ICU admission displayed lower mortality, although causes of mortality were not specified (37). Furthermore, in a randomized controlled trial, esmolol treatment decreased mortality by 30% in patients with high noradrenaline load, although the mortality in the control group was very high (36). Naturally, cardiac effects of esmolol, such as a mildly reduced volume load and increased stroke volume were observed, but its beneficial effects might be beyond modulation of these hemodynamic parameters (36). Again, unfortunately no immunologic markers were assessed. Currently, the putative beneficial effects of esmolol are further evaluated in a larger clinical trial (NCT02068287), in which immunologic markers are assessed as well. Along these lines, blocking the actions of endogenous catecholamines with propranolol in pediatric burn patients, another condition accompanied by high catecholamine release, was associated with a lower rate of secondary infections (38). It could be contended that these beneficial effects are due to intrinsic immune-stimulatory effects of β-blockers, however, these have not been reported *in vitro* (9) and there are no indications that β-blockade exerts advantageous immune effects in diseases not associated with increased endogenous catecholamine levels. Finally, in traumatic brain injury patients, it was shown that profound sympathetic activation induces systemic release of IL-10 (26). This IL-10 release was shown to be associated with an immunosuppressive monocyte phenotype (decreased cell-surface HLA-DR expression), and an increased infection rate, although the relative contribution of noradrenaline and adrenaline was not assessed (26).

Collectively, these clinical studies suggest that high noradrenaline levels encountered in septic patients may contribute to sepsis-induced immunoparalysis, although to date, studies into sepsis-induced immunoparalysis have not assessed the potential role of noradrenaline. Furthermore, clear assessment of these effects in studies in septic shock patients will always be difficult for several reasons. First, until alternative vasopressors become standard of practice there is an absolute requirement of noradrenaline administration in patients with septic shock to achieve hemodynamic stabilization (31), as such, it would be unethical to withhold this treatment. Second, similar to animal studies, differences in hemodynamic effects of noradrenaline compared with a fluid heavy approach or alternative vasopressors could induce differences in organ perfusion and/or tissue ischemia, which, as alluded to before, hampers interpretation of immunologic effects as well. Therefore, the most promising approach to increase our understanding of intrinsic immunologic effects of noradrenaline is the use of experimental human *in vivo* models of inflammation or clinical conditions which are

not associated with compromised organ perfusion and/or tissue ischemia. As of yet, there is only such data on the highly related catecholamine adrenaline. Exogenous administration of this catecholamine was shown to potently attenuate plasma TNF-α levels, whereas it increased circulating anti-inflammatory IL-10 concentrations during experimental human endotoxemia (20). Adrenaline infusion also reduced the capacity of blood leukocytes to release IL-1β upon stimulation with LPS *ex vivo* (39). These findings of adrenaline-induced immunosuppression were recently extended by another human endotoxemia study which showed that increasing endogenous adrenaline levels through a behavioral intervention also resulted in markedly reduced plasma concentrations of pro-inflammatory cytokines and increased IL-10 levels (40). Interestingly, using the same human endotoxemia model, it was demonstrated that the specific β1-AR agonist dobutamine does not influence cytokine release, suggesting that the anti-inflammatory effects of adrenaline and other β-AR agonists in humans *in vivo* are mediated via β2-ARs (41). There are strong indications that immunologic effects of noradrenaline resemble the anti-inflammatory effects demonstrated for adrenaline, as noradrenaline and adrenaline exert equally potent immunosuppressive effects in LPS-stimulated human whole blood or isolated monocytes *in vitro* (9, 20, 42).

Studying adrenoceptor gene polymorphisms could represent an alternative approach to shed light on the contribution of noradrenaline to sepsis-induced immunoparalysis. Interestingly, in human lymphoblastoid cell lines carrying the CysGlyGln haplotype variant of the β2-AR, noradrenaline suppressed IL-6 production to a lesser degree compared with other haplotypes (43). Furthermore, in two large cohorts of septic shock patients, this haplotype was associated with decreased noradrenaline sensitivity, higher lactate levels, more organ dysfunction, and increased mortality (43). It appears plausible that the increased mortality was probably due to hemodynamic rather than immunologic effects, however no immunologic parameters in patients or incidence of secondary infections were reported (43).

Taken together, evidence obtained from *in vitro* and animal studies indicate that noradrenaline exerts anti-inflammatory and bacterial growth-promoting effects, which lead to increased susceptibility towards infections. This is supported by experimental human studies that show potent immunosuppressive effects of the highly related catecholamine adrenaline. As such, although clinical evidence is as of yet circumstantial, noradrenaline could importantly contribute to sepsis-induced immunoparalysis and use of alternative vasopressors may be superior in this respect.

Figure 2 provides an overview of receptor- and cell-specific immunologic and bacterial-growth promoting effects of noradrenaline derived from *in vitro* and animal studies and how these might contribute to sepsis-induced immunoparalysis.

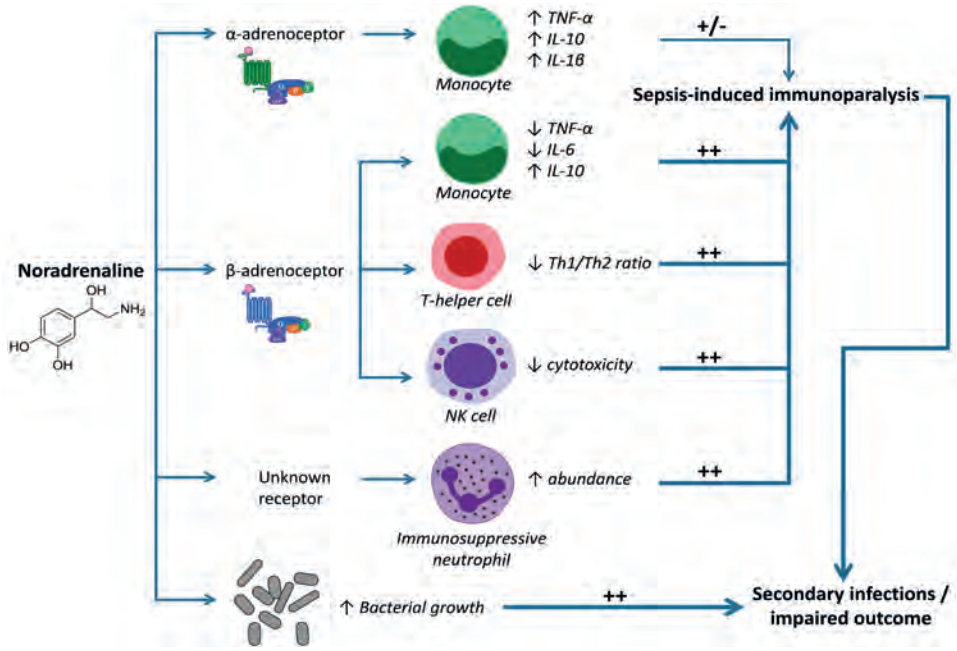

Figure 2. Several mechanisms by which noradrenaline may contribute to sepsis-induced immunoparalysis. Activation of the α-AR has been associated with both pro-and anti-inflammatory effects. Activation of the β-AR exerts anti-inflammatory effects, including decreased production of pro-inflammatory cytokines, increased production of anti-inflammatory cytokines, skewing of the immune response from a Th1 towards a Th2 cell phenotype, and decreased NK cell cytotoxicity. Furthermore, noradrenaline induces the generation of immunosuppressive neutrophils and directly promotes bacterial growth. AR= adrenoceptor, IL= interleukin, TNF=Tumor necrosis factor, Th= T-helper, NK= natural killer.

ALTERNATIVE VASOPRESSORS

Three alternative vasopressors for the treatment of septic shock that may not, or to a lesser extent contribute to immunoparalysis are vasopressin/ selepressin, angiotensin II (ATII), and phenylephrine. In this perspective, we focus on direct vasopressors and not blockers of endogenous vasodilators. Therefore we will not discuss the nitric oxide inhibitors methylene blue and L-NMMA. Furthermore, it might be argued that dopamine is a viable alternative. Nevertheless, a large RCT in shock patients slanted in favor of noradrenaline, although a significantly decreased mortality was only found in a subgroup of cardiogenic shock patients (44). This advantage was mainly explained by a lower number of adverse events (44). In addition, dopamine exerts potent anti-inflammatory effects, both through stimulation of dopaminergic receptors and β-ARs (45), so it appears plausible that it confers no immunologic advantages. In accordance, the incidence of new infectious episodes in the aforementioned RCT was similar between groups (44). Similar to dopamine, epinephrine exerts immunosuppressive effects, as described earlier, and did not show benefit over adrenaline plus dobutamine in a large

RCT (46).

Vasopressin is 8-arginine-vasopressin, a synthetic analogue of the endogenous anti-diuretic hormone (ADH). To date, vasopressin is mostly used as an adjunctive treatment to noradrenaline in severe septic shock, and reduction of the noradrenaline infusion rate by addition of vasopressin reduced mortality in a subgroup of patients with relatively mild septic shock (47). However, in this trial vasopressin was added as an adjunctive treatment to existing vasopressor treatment to allow tapering of vasopressor dosages, and was started after several hours of this existing treatment, which consisted of noradrenaline in the great majority of patients. Therefore, most patients in the vasopressin group initially received noradrenaline and many of them continued to receive it, albeit in lower dosages. This hampers clear interpretation and assessment of (immunological) effects, as noradrenaline already exerts immunosuppressive effects at low concentrations (9). Small studies did however show that vasopressin is a viable alternative for noradrenaline as first-line treatment in septic shock (48), which is evaluated in a currently ongoing larger clinical trial (ISRCTN 20769191). As of yet, little is known about the immunomodulatory properties of vasopressin in septic shock. In a post-hoc assessment of the previously mentioned trial (47), vasopressin was associated with a more pronounced decrease of plasma cytokine levels in the first 24 hours of treatment (49). Unfortunately, as mentioned before, sustained noradrenaline administration in the patients in the vasopressin group in this study impedes clear interpretation of these results. Furthermore, the difference reported failed to retain statistical significance following correction for baseline characteristics such as corticosteroids, APACHE II score, shock severity, age, and sex (49). Therefore, from this study no clear conclusions can be drawn on immunologic properties of vasopressin. Small animal studies, which are more easily interpretable, may suggest anti-inflammatory properties. Vasopressin treatment resulted in slightly decreased intestinal IL-6 levels compared with noradrenaline in a porcine sepsis model, while levels of circulating TNF-α, IL-6, and IL-10 were similar (50). In mice, vasopressin attenuated LPS-induced pulmonary but not systemic IL-6 levels, which was abolished by a V2-receptor antagonist (51). Likewise, recent data concerning early administration of a selective V1 receptor agonist (selepressin) in an ovine sepsis model showed reduced systemic IL-6 levels compared with noradrenaline (52). However, organ perfusion in general was better with selepressin compared to noradrenaline, which may indirectly affect systemic cytokine production due to attenuation of tissue ischemia and resulting inflammation (52). At present, selepressin is being evaluated in a multi-center trial for clinical efficacy and safety (NCT02508649). One might argue that terlipressin, another vasopressinergic agent with V1-receptor selectivity, represents a suitable alternative vasopressor as well. This agent is frequently used as rescue therapy in addition to noradrenaline, however its use as a first-line vasopressor for septic shock is hampered by its long half-life, which compromises exact titration (53).

Angiotensin II has no reported immunosuppressive effects. In a pilot study in patients with high-output shock, angiotensin II was well-tolerated and markedly reduced noradrenaline requirement, albeit with considerable interindividual variation (54). Angiotensin II is currently further investigated in an ongoing phase 3 trial in sepsis patients (NCT02338843).

Phenylephrine is a pure α-adrenergic agonist. Its use in septic shock patients has thus far been limited due to concerns for splanchnic blood flow. However, no difference was found in any regional hemodynamic parameters when compared with noradrenaline in septic shock (55). In addition, a recent meta-analysis failed to show a survival benefit for noradrenaline over phenylephrine (56). As described before, *in vitro* studies have shown that α-AR stimulation is mainly associated with pro-inflammatory effects. In accordance, LPS-stimulated monocytes exhibited increased IL-1β production after incubation with phenylephrine (57), however these results are not supported by work showing that phenylephrine does not affect TNF-α or IL-6 production in LPS-stimulated whole blood (9). *In vivo* data is limited to a canine endotoxemia model revealing that phenylephrine treatment resulted in a very modest increase in circulating TNF-α levels (58). Given the limited and pre-clinical nature of the current evidence, no definitive conclusions can be drawn on the immunological properties of vasopressin/selepressin, angiotensin II, and phenylephrine in humans *in vivo*, and studies into these effects are highly warranted as these compounds might represent viable less- or no immunosuppressive alternatives to noradrenaline. Lastly, administration of non-immunosuppressive alternative vasopressors would be most advantageous in the later phases of sepsis, when immunoparalysis is the overriding immune dysfunction (59) and endogenous catecholamine release is exhausted (32). Nevertheless, it is increasingly recognized that anti-inflammatory mechanisms are activated simultaneous with the pro-inflammatory response early on in sepsis (3). Furthermore immunologic changes induced by early noradrenaline treatment, for instance a skewed Th1/Th2 balance (23), can be long-lasting and thus have a detrimental influence on the entire course of the disease. In this respect, restriction of noradrenaline use should be pursued already early on in sepsis.

An overview of the different classes of vasopressors described in this perspective and their immunologic effects is provided in **Table 1**.

Table 1. Overview of different vasopressors, their classes, their receptors and immunologic effects.

Drug	Class	Receptor affinity	Immunologic effects
Adrenaline	Catecholamines	$\alpha_1, \alpha_2, \beta_1, \beta_2$	*In vitro* TNF-α↓ , IL-6↓, IL-8↓, IL-10↑ (20, 42) *In vivo* Human endotoxemia: circulating TNF-α↓, IL-10↑ (20)
Noradrenaline	Catecholamines	$\alpha_1, \alpha_2, \beta_1 > \beta_2$	*In vitro* TNF-α↓, IL-6↓, IL-8↓, IL-10↑, Th1/Th2 ratio↓, NK cell cytotoxicity↓, bacterial growth↑ (9, 20, 22-24, 42) *In vivo* Rats (noradrenaline injection in portal vein): circulating IL-1β↑, IL-10↑ (25)
Phenylephrine	Phentylamines	α_1	*In vitro* IL-1β↑, TNF-α–, IL-6– (9, 57) *In vivo* Canine endotoxemia: circulating TNF-α↑(58)
Vasopressin	Synthetic vasopressin-analogue	V_1, V_2	*In vivo* Pig sepsis (fecal peritonitis): circulating TNF-α–, IL-6–, IL-10– , intestinal TNF-α–, IL-6↓, IL-10–, compared with noradrenaline (50) Murine endotoxemia: circulating IL-6–, pulmonary IL-6↓(51)
Selepressin	Synthetic vasopressin-analogue	V_1	*In vivo* Sheep sepsis (fecal peritonitis): IL-6↓ compared with noradrenaline (52)
Angiotensin II	Angiotensin	AT_1, AT_2	Not investigated

TNF= Tumor necrosis factor, IL= interleukin, Th= T-helper, NK= natural killer, ↑= increased, ↓= decreased, –= not affected.

CONCLUSION

Sepsis-induced immunoparalysis is a common phenomenon in septic shock patients and may contribute to the development of secondary infections and late mortality. Next to sepsis itself, our current treatment approach may substantially contribute to the development of immunoparalysis. Furthermore, as many of these therapies are also administered in non-sepsis critically ill patients, such as those suffering from major trauma or traumatic brain injury, it might also be an explanation for the high risk of nosocomial infections in these populations. While novel therapies aimed to reverse immunoparalysis are underway, we believe that a reassessment of our old therapies is indicated. Current *in vitro* and animal data indicate that noradrenaline treatment exerts immunosuppressive effects and may increase infection rates. Furthermore, restricted use of noradrenaline is propagated due to its multitude of adverse effects (60). However,

to date, immunologic effects of noradrenaline and markers of immunoparalysis have not properly been investigated in experimental or clinical studies in humans. Alternatives such as vasopressin/selepressin, angiotensin II, and phenylephrine could have a fundamental advantage over noradrenaline with respect to these immunologic properties. However, also for these agents, *in vivo* immunologic data in humans are largely lacking. As such, human studies on the immunomodulatory properties of noradrenaline and viable alternatives are highly warranted.

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CHAPTER 3

NORADRENALINE DRIVES IMMUNOSUPPRESSION IN SEPSIS: CLINICAL CONSEQUENCES

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THE RELATIONSHIP BETWEEN DISEASE SEVERITY, IMMUNOSUPPRESSION, AND OUTCOME

The immunological response in sepsis patients is complex, comprising concurrent pro- and anti-inflammatory responses. It has become increasingly clear that most sepsis patients do not succumb to an early, overwhelming pro-inflammatory response, but rather to complications related to immunosuppression occurring later on in their disease trajectory (1, 2). This severely suppressed state of the immune system renders patients unable to clear their primary infection and increases susceptibility towards secondary infections, often with opportunistic pathogens (3, 4). Recently, a seminal observational study questioned the contribution of secondary infections to sepsis mortality (5). This study linked disease severity in sepsis patients to increased susceptibility towards secondary infections and higher mortality. Analysis of the transcriptome of circulating leukocytes revealed distinct hallmarks of immune suppression at the onset of secondary infections, consistent with an important role for sepsis-induced immunosuppression as a causative factor. However, after adjustment for age and, crucially, disease severity the attributable mortality from secondary infections was low (2%) (5). This may appear to indicate that sepsis-induced immunosuppression is of limited importance for outcome, and that interventions aimed to mitigate or reverse it will therefore have little impact. We believe this is not the case, because immunosuppression is an intermediary factor in sepsis patients. Disease severity drives immunosuppression, eventually leading to mortality related to secondary infections. It is therefore not surprising that correcting for disease severity abolishes the contribution of secondary infections to mortality. This does, however, not mean that therapeutic interventions aimed at an intermediary factor, in this case immunosuppression, may not affect outcome. We would like to illustrate this using diabetes as an example. In diabetic patients, poor glycemic control (indicated by increased HbA1c) induces vascular damage, resulting in an increased rate of myocardial infarctions. In this example, HbA1c reflects disease severity, vascular damage the intermediary factor, and myocardial infarction the outcome. If the attributable myocardial infarctions of vascular damage would be corrected for HbA1c in diabetic patients, the effect would be modest as well. However, it is widely accepted that vascular damage is causative for myocardial infarctions. More importantly, interventions targeting vascular damage (e.g. percutaneous coronary intervention) are highly effective, also in patients with diabetes. Analogously, strategies aimed at mitigating sepsis-induced immunosuppression should not be written off.

NORADRENALINE AS AN INTERMEDIATE FACTOR LINKING DISEASE SEVERITY TO IMMUNOSUPPRESSION

Although new therapies to reconstitute immune function in sepsis patients are being developed, current standard of care practices should also be reevaluated in light of sepsis-induced immunosuppression, as some interventions may significantly dysregulate the immune response. In sepsis patients, the use of noradrenaline reflects disease severity and we hypothesize that this cornerstone vasopressor treatment is an important driver of sepsis-induced immunosuppression. In this capacity, it acts as an intermediate factor as well, linking disease severity to immunosuppression and impaired outcome, as outlined below and in **Figure 1**.

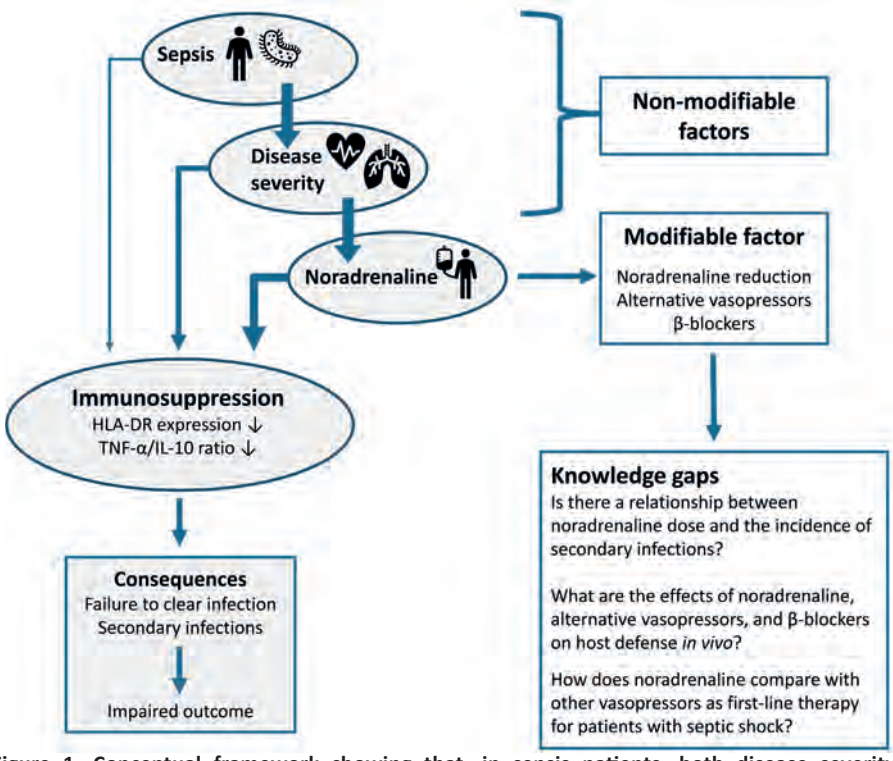

Figure 1. Conceptual framework showing that, in sepsis patients, both disease severity and noradrenaline administration are important drivers of sepsis-induced immunosuppression. The development of immunosuppression is an intermediary factor, linking disease severity to adverse clinical outcomes. Several features of sepsis-induced immunosuppression are listed, as well as the consequences of immunosuppression, ultimately leading to impaired clinical outcome. As more severely ill sepsis patients are more likely to suffer from hemodynamic instability, anti-inflammatory effects of noradrenaline, mediated through the β-adrenoceptor, link disease severity to the development of immunosuppression. Therefore, noradrenaline represents an intermediary factor as well. This is of clinical relevance, because noradrenaline is a modifiable factor, which can be exploited for the prevention or treatment of sepsis-induced immunosuppression. Different approaches can be pursued, such as use of alternative vasopressors or concurrent administration of β-blockers. The current knowledge gaps that need to be bridged to explore this new concept are highlighted.

There are several arguments for this. First, disease severity and circulating noradrenaline levels are highly intertwined, as more severely ill septic patients generally require more vasopressor support. These phenomena are also correlated by default, as blood pressure and/or vasopressor requirement is commonly part of the disease severity scores used in sepsis. Second, noradrenaline exerts profound anti-inflammatory effects, predominantly mediated via the β-adrenergic receptor (6). For instance, it attenuates production of pro-inflammatory mediators tumor necrosis factor (TNF)-α and interleukin (IL)-6, while enhancing the release of the anti-inflammatory cytokine IL-10 by lipopolysaccharide-stimulated leukocytes (7). Furthermore, noradrenaline was shown to inhibit natural killer cell cytotoxicity (8) and, conversely, destruction of noradrenergic nerve endings increased bacterial resistance in mice (9). To date, clinical evidence is circumstantial. In the aforementioned 'attributable mortality study', the prevalence of shock (defined as noradrenaline requirement in a dose >0.1 μg/kg/min) was significantly higher in patients who developed a secondary infection compared with those who did not (5). Furthermore, improved outcomes using a catecholamine-sparing 'permissive hypotension' strategy (10) or the use of a beta-blocker (11) in septic shock patients have been reported. Finally, previous studies have linked a higher vasopressor (i.e. noradrenaline) load to increased mortality (12), although none have assessed its relationship with secondary infections. This remains an important aspect for future studies.

BREAKING THE LINK: ALTERNATIVE VASOPRESSORS

Next to the use of β-blockers, alternative non-catecholaminergic vasopressors with less or no untoward immunological effects might be instrumental to break the putative link between disease severity and sepsis-induced immunosuppression, thereby improving outcome. Several clinical trials have compared noradrenaline with vasopressin (analogues). In the most recent, the selective V1-receptor agonist selepressin conferred no clear clinical advantage over noradrenaline (13), although development of secondary infections was not assessed. Angiotensin II is another vasopressor with no reported immunosuppressive effects that significantly reduces noradrenaline requirements (14). Importantly, in all previous trials investigating alternative vasopressors, the new vasopressor was used as add-on therapy to noradrenaline instead of a first-line vasopressor. Therefore, patients were treated with noradrenaline for hours already, which could have masked any immunological benefits of alternative vasopressors. To allow a beneficial immunological effect of an alternative vasopressor to emerge, they should be investigated as initial monotherapy against noradrenaline and development of secondary infections should be a predefined endpoint.

CONCLUSION

We argue that immunosuppression is an intermediary factor linking disease severity to adverse outcome in sepsis patients. Therefore, the contribution of immunosuppression to outcome will be severely underestimated when it is corrected for disease severity, while interventions aimed at restoring immunocompetence may still have a significant impact on outcome. Furthermore, we identify noradrenaline as an important driver of immunosuppression in sepsis, as it exerts profound immunosuppressive effects in preclinical studies. Because the need for noradrenaline is related to disease severity, this vasopressor may represent a crucial intermediary factor between disease severity and immunosuppression. Nevertheless, use of more clinically relevant models of inflammation and sepsis as well as translation to the human setting is highly warranted to properly assess the consequences of noradrenaline therapy for host defense. Furthermore, unlike disease severity, noradrenaline represents a modifiable intermediary factor. Therefore, the use of alternative vasopressors as first-line therapy should be further explored.

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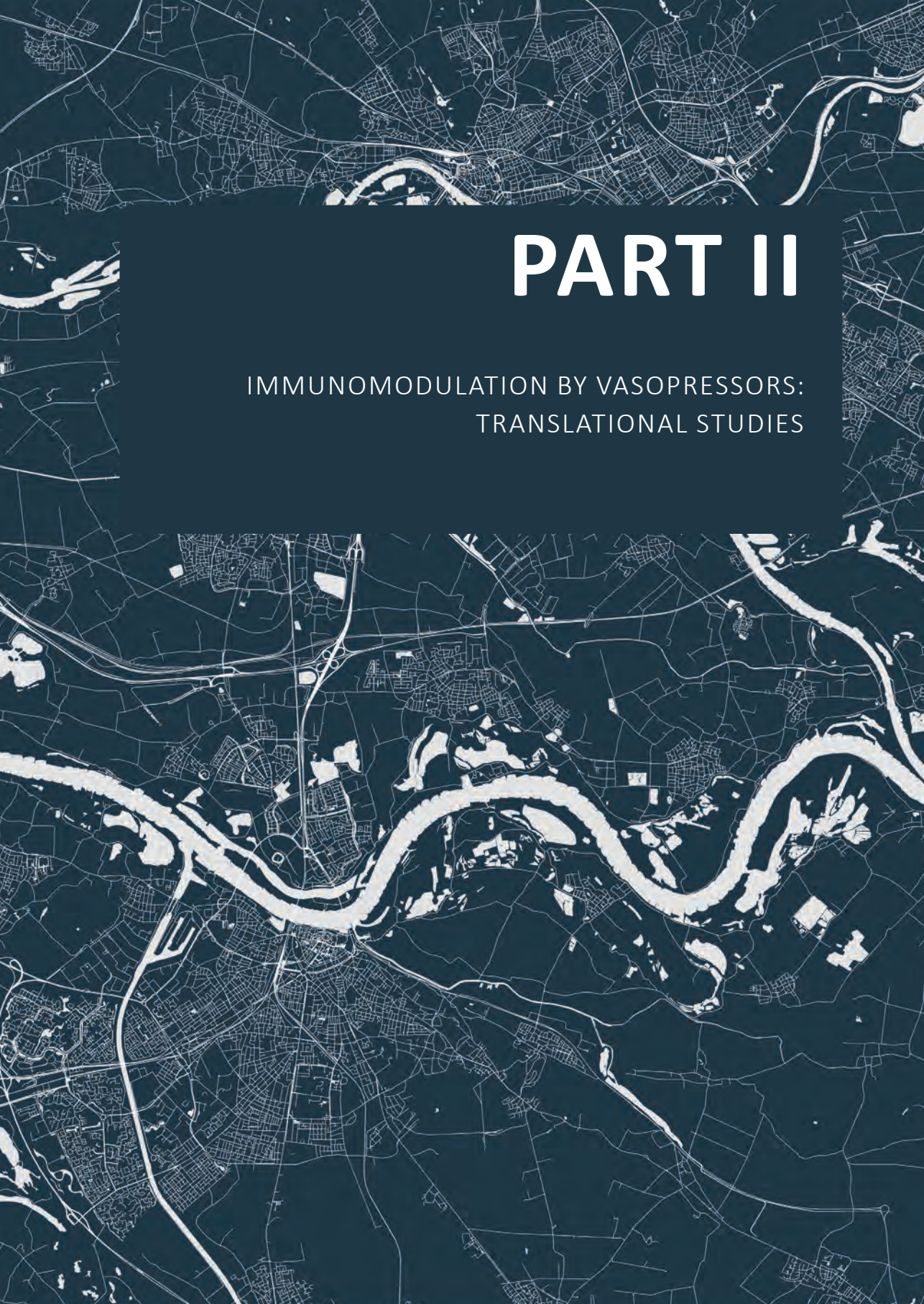

PART II

IMMUNOMODULATION BY VASOPRESSORS: TRANSLATIONAL STUDIES

CHAPTER 4

NORADRENALINE DYSREGULATES THE IMMUNE RESPONSE AND COMPROMISES HOST DEFENSE DURING SEPSIS

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ABSTRACT

RATIONALE

Sepsis is characterized by a dysregulated immune response to infection. Noradrenaline, the cornerstone vasopressor used in septic shock, may contribute to immune dysregulation and impact host defense.

OBJECTIVES

To investigate effects of noradrenaline and the alternative vasopressor vasopressin on the immune response and host defense.

MEASUREMENTS AND MAIN RESULTS

Leukocytes from 6-9 donors were stimulated in the presence or absence of noradrenaline and vasopressin. One-hundred-and-ninety C57BL/6J mice received continuous infusion of noradrenaline or vasopressin via micro-osmotic pumps and were challenged with lipopolysaccharide or underwent cecal ligation and puncture (CLP). Thirty healthy volunteers were randomized to a 5-hour infusion of noradrenaline, vasopressin or saline, and intravenously challenged with lipopolysaccharide. The relationship between noradrenaline infusion rate and the use of β-blockers, and plasma cytokines was assessed in 195 septic shock patients.

RESULTS

Noradrenaline attenuated the production of pro-inflammatory mediators and reactive oxygen species, while augmenting anti-inflammatory interleukin-10 production both in vitro and in lipopolysaccharide-challenged mice. Noradrenaline infusion during CLP resulted in increased bacterial dissemination to spleen, liver and blood. In lipopolysaccharide-challenged volunteers, noradrenaline enhanced plasma interleukin-10 concentrations and attenuated release of the pro-inflammatory cytokine interferon gamma-induced protein-10. Vasopressin exerted no immunomodulatory effects across these experimental setups. In patients, higher noradrenaline infusion rates were correlated with a more anti-inflammatory cytokine balance, whereas β-blocker use was associated with a more pro-inflammatory cytokine balance.

CONCLUSION

Noradrenaline dysregulates the immune response in mice and humans, and compromises host defense. Therefore, it may significantly contribute to sepsis-induced immunoparalysis, whereas vasopressin does not have untoward immunologic effects.

INTRODUCTION

Sepsis, defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (1), is the leading cause of death worldwide, accounting for 20% of global mortality (2). This has prompted the WHO to designate sepsis as a global health priority (3). Sepsis can comprise both hyperinflammatory and immunosuppressive traits (4). Although hyperinflammation, for instance observed in patients with the macrophage activation syndrome (5), can have clear detrimental effects, immunosuppressive interventions have failed to improve outcome in sepsis. Therefore, attention has shifted towards the detrimental role of sepsis-induced immunosuppression (6, 7). This "immunoparalysis" is characterized by various aberrations in the immune system, including decreased HLA-DR expression on antigen-presenting cells, diminished pro-inflammatory cytokine production by *ex vivo*-stimulated leukocytes, and imbalanced cytokine profiles, with higher levels of the archetypal anti-inflammatory cytokine IL-10 and lower levels of pro-inflammatory mediators such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 (6, 8). This results in ineffective clearance of infectious foci and increased susceptibility towards secondary infections, often with opportunistic pathogens, and accounts for high mortality and morbidity (7, 9). The importance of sepsis-induced immunoparalysis is further illustrated by recent reports showing that sepsis patients can be classified to different endotypes based on their leukocyte transcriptome, with immunosuppressed endotypes consistently showing the worst clinical outcome (10, 11). Therefore, many current efforts are focused on reconstitution of immunocompetence with immunostimulatory treatments (6, 8). However, supportive treatments, which are currently indiscriminately applied, could significantly contribute to dysregulation of the host response, and may require reevaluation in light of the emerging concept of immunoparalysis.

Noradrenaline is the current mainstay therapy for hemodynamic stabilization administered to practically all patients with septic shock and other severe systemic inflammatory conditions worldwide (1). *In vitro* evidence points towards overriding anti-inflammatory effects of noradrenaline (12-14). However, animal *in vivo* data on the putative immunomodulatory effects of noradrenaline are very scarce, and human *in vivo* data nonexistent.

We previously hypothesized that noradrenaline may contribute to dysregulation of the host response and sepsis-induced immunoparalysis, whereas other vasopressors, such as vasopressin, may not (15). In the present work, we first evaluated the immunological effects of noradrenaline and vasopressin *in vitro,* using primary human leukocytes. Subsequent experiments in murine models of inflammation and sepsis were performed to assess the functional relevance of these findings. Furthermore, we made the translation

to the human setting using an *in vivo* model of systemic inflammation (experimental human endotoxemia) and observations in sepsis patients.

MATERIALS & METHODS

Detailed study procedures and analysis methods are described in the supplement.

IN VITRO STUDIES

After approval from the local ethics committee of the Radboud university medical center (CMO 2010/10), blood was obtained from healthy adult volunteers (n=6-9 donors per experiment) who provided written informed consent. Whole blood and isolated monocytes were used in various experiments to assess immunological and metabolic effects of noradrenaline and vasopressin.

MURINE STUDIES

All procedures were approved by the Dutch Council for Animal Care (AVD103002016447). A total of 190 C57Bl/6J male mice (Charles Rivers, Cologne, Germany) aged 6-9 weeks were used for all experiments. For the endotoxemia experiments (n=29-41 per experiment), micro-osmotic pumps with connected jugular vein catheters were filled with noradrenaline, vasopressin, or phosphate-buffered saline (PBS), implanted 3 or 24 hours before LPS challenge, and animals were sacrificed 90 minutes post-LPS. For the sepsis experiments, cecal ligation and puncture (60% ligation, 21G needle, n=30) or a sham operation (n=3) was performed, followed by placement of micro-osmotic pumps with connected jugular vein catheters, filled with noradrenaline, vasopressin, or PBS, and animals were sacrificed 48 hours later.

HUMAN STUDIES

Thirty healthy male volunteers provided written informed consent to participate in a randomized, double blind, placebo-controlled study carried out in accordance with the declaration of Helsinki. The study was approved by the local ethics committee (CMO 2015-2079) and was registered at Clinicaltrials.gov (identifier NCT02675868). Under continuous monitoring of vital parameters, subjects (n=10 per group) received a 5-hour infusion of either low-dose noradrenaline (0.05 µg/kg/min), vasopressin (0.04 IU/min) or placebo (NaCl 0.9%), starting one hour before intravenous LPS challenge (2 ng/kg). Patient data were collected from a prospective observational cohort study performed between April 2012 and January 2017, in which 195 adults patients with newly developed septic shock admitted to the Intensive Care were included. The study was carried out in accordance with the applicable rules concerning the review of research ethics committees and informed consent in the Netherlands. All patients

or legal representatives were informed about the study details and could refuse to participate. Medication use was retrieved from the electronic patient data management system. Patients assigned to the noradrenaline + β-blocker group were chronic users who received a β-blocker on the day of study inclusion and before blood withdrawal, which was performed within 24 hours after inclusion.

RESULTS

NORADRENALINE EXERTS ANTI-INFLAMMATORY EFFECTS IN PRIMARY HUMAN LEUKOCYTES

In initial experiments, whole blood was stimulated with the Toll-like receptor (TLR)4 ligand LPS to elicit an inflammatory response, in the presence or absence of increasing concentrations of noradrenaline and vasopressin. Noradrenaline dose-dependently attenuated production of pro-inflammatory mediators TNF-α ($p<0.0001$), interferon gamma-induced protein (IP)-10 ($p<0.0001$) and IL-1β ($p=0.01$), whereas release of the anti-inflammatory cytokine IL-10 was enhanced ($p<0.0001$, **Figure 1A**). Vasopressin did not influence cytokine production ($p=0.58$, $p=0.98$, $p=0.84$, and $p=0.87$ for TNF-α, IP-10, IL-1β, and IL-10, respectively, **Figure 1A**). No effects of noradrenaline on cell survival were observed (**Supplementary Figure E1**). To assess whether noradrenaline's effects are confined to TLR4 stimulation, whole blood was stimulated with other TLR ligands, namely Pam3Cys (TLR1/2), Poly I:C (TLR3), Flagellin (TLR5), R848 (TLR7/8), and CpG (TLR9) as well as heat killed pathogens *E.coli*, *S.aureus*, *C.albicans*, *P.aeruginosa*, and *A.fumigatus* in the presence or absence of noradrenaline. In general, noradrenaline caused a dose-dependent shift towards an anti-inflammatory phenotype, exemplified by attenuated production of pro-inflammatory cytokines IP-10 and TNFα, and enhanced IL-10 responses (**Figure 1B**). LPS stimulation experiments using isolated primary human monocytes yielded identical effects of noradrenaline and vasopressin on TNF-α and IL-10 production as observed in the whole blood stimulation experiments (Supplementary **Figure E2**). We subsequently assessed the effects of both vasopressors on the production of radical oxygen species (ROS) by monocytes. As depicted in **Figure 2A-B**, noradrenaline inhibited ROS production by PMA-stimulated monocytes ($p=0.004$), whereas vasopressin exerted no such effect ($p=0.10$) (**Figure 2C-D**).

ANTI-INFLAMMATORY EFFECTS OF NORADRENALINE INVOLVE B2-ADRENERGIC STIMULATION AND PROTEIN KINASE A

Because noradrenaline has both α- and β-adrenergic receptor (AR) affinity, we set out to determine which receptor is involved in the observed immunomodulatory effects. Pre-incubation with the non-selective β-AR antagonist propranolol nullified the effects of noradrenaline on both LPS-induced TNF-α ($p=0.001$) and IL-10 ($p=0.006$) production

in whole blood cultures (**Supplementary Figure E3A**). The selective α1-AR antagonist prasozin and the α2-AR antagonists yohimbine exerted no effects on the noradrenaline-mediated attenuated TNF-α release (p=0.12 and p=0.13 respectively), although yohimbine reversed noradrenaline's IL-10-enhancing effects (p=0.01, **Supplementary Figure E3A**). These findings indicate predominant involvement of β-ARs, and we therefore examined the effects of selective β1-AR (atenolol) and β2-AR (ICI-1158,551) antagonists in a follow-up whole blood LPS stimulation experiment. ICI-118,551 reversed the effects of noradrenaline on TNF-α (p=0.04), but not IL-10 production (p=0.22), whereas atenolol did not modulate LPS-induced cytokine responses (p=0.75 and p=0.31 for TNFα and IL-10, respectively, **Supplementary Figure E3B**). In isolated primary human monocytes, ICI-118,551 nullified noradrenaline's effects on both TNF-α (p=0.003) and IL-10 (p=0.005) (**Supplementary Figure E3C**), supporting an important role for β2-ARs. β-ARs are G-protein-coupled receptors and signal intracellularly via protein kinase A (PKA) (14). As illustrated in **Supplementary Figure E3D**, pre-incubation with PKA inhibitor H89 dose-dependently reversed the effects of noradrenaline on both TNF-α (p=0.02, p=0.06, and p=0.007 for 300 nM, 1 μM, and 3 μM H89, respectively) and IL-10 production (p=0.04, p=0.007, and p=0.001) in LPS-stimulated monocytes.

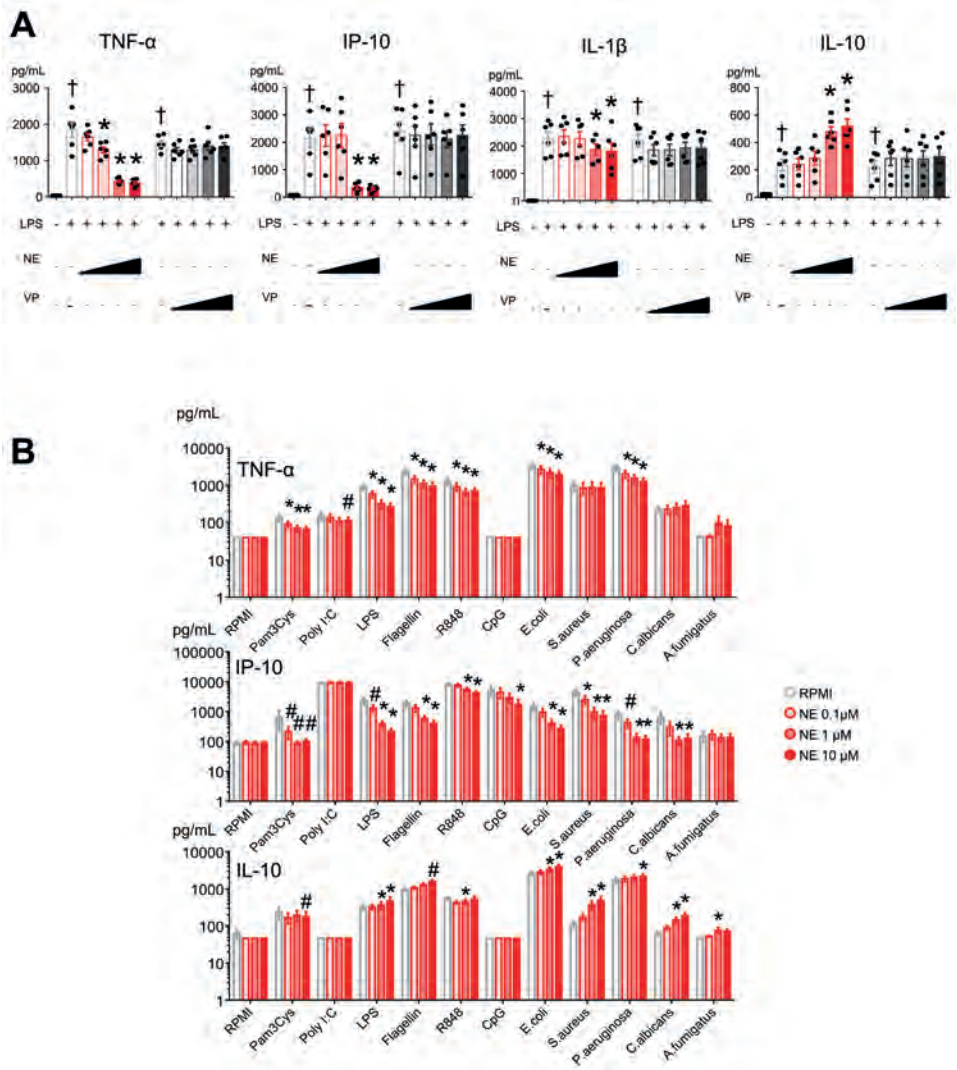

Figure 1. Noradrenaline modulates cytokine production by human whole blood (A) Concentrations of TNF-α, IP-10, IL-1β, and IL-10 in supernatants of human whole blood cultures preincubated with either RPMI (medium control), noradrenaline (NE; 0.01, 0.1, 1, and 10 µM) or vasopressin (VP; 0.01, 0.1, 1, and 10 µM) for 1 h and subsequently stimulated with LPS (10 ng/mL) or RPMI for 24 h. (B) Concentrations of TNF-α, IP-10, and IL-10 in supernatants of human whole blood cultures that were preincubated with either RPMI or NE (0.1, 1, and 10 µM) for 1 h and subsequently stimulated with several TLR ligands and heat-killed pathogens (Pam3Cys 10 µg/mL, Poly I:C 50 µg/ml, LPS 10 ng/mL, flagellin 10 µg/mL, R848 1 µM, CpG 10 µg/mL, *E.coli* 1x10^7/mL, *S.aureus* 1x10^7/mL, *P.aeruginosa* 1x10^7/mL, *C.albicans* 1x10^6/mL, *A.fumigatus* 1x10^7/mL), or RPMI for 24 h. Data are expressed as individual data points and/or mean ± SEM of 6 individual donors. †p<0.05 compared to RPMI; *p<0.05, #p=0.05-0.10 compared to LPS or other stimuli, calculated using one-way analysis of variance with Dunnett's post hoc tests.

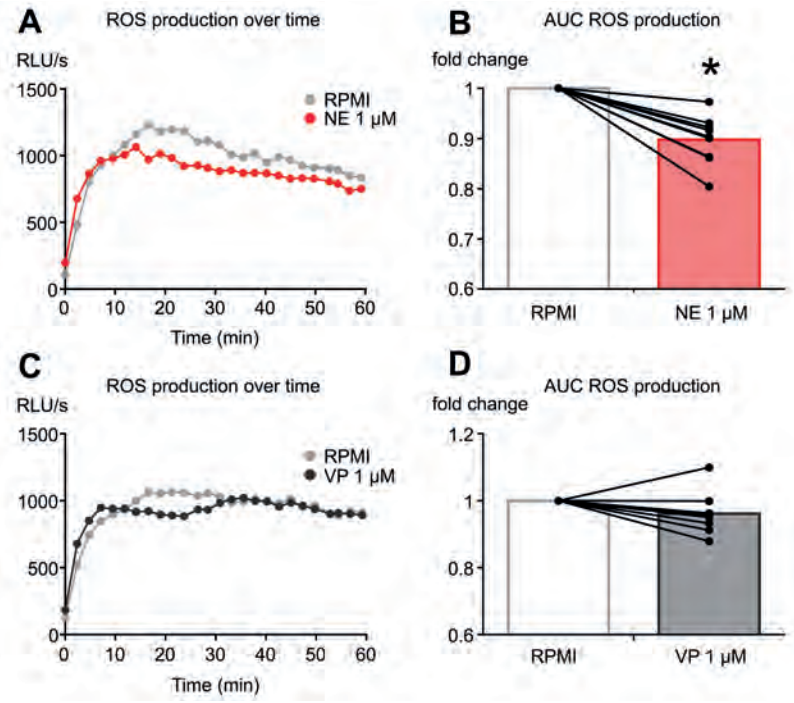

Figure 2. Noradrenaline attenuates PMA-induced reactive oxygen species production by primary human monocytes. (A-D) Primary human monocytes were incubated with RPMI (medium control), noradrenaline (NE; 1 µM) or vasopressin (VP; 1 µM) for 1 h and subsequently stimulated with PMA (50 ng/mL) in the presence of luminol, and luminescence was measured for 1 h. (A-B) Modulation of reactive oxygen species (ROS) production by noradrenaline expressed as (A) relative light units (RLU) per second and (B) area under the curve (AUC) depicted under A. (C-D) Modulation of ROS production by vasopressin expressed as (C) relative light units (RLU) per second and (D) AUC depicted under C. Data are expressed as means (panels A and C), and individual data points and mean fold change compared with RPMI-incubated cells (panels B and D) of 6 individual donors; *p<0.05 compared to RPMI calculated using a t-test.

NORADRENALINE SUPPRESSES IMMUNOMETABOLISM

Because metabolic defects were recently shown to play a crucial role in impaired cytokine responses in general, and in sepsis-induced immunoparalysis in particular (16), this could represent a mechanism through which noradrenaline exerts its immunosuppressive effects. Therefore, we evaluated the effects of noradrenaline on energy metabolism in isolated monocytes. Noradrenaline attenuated the LPS-induced increase in lactate production (p=0.003, **Figure 3A),** indicating inhibition of the glycolytic shift, and this effect was mitigated by pretreatment with either ICI-118,551 (p=0.06) or H89 (p=0.03, **Figure 3B-C**). Next, the extracellular acidification rate (ECAR, reflecting glycolytic capacity) and oxygen consumption rate (OCR, reflecting oxidative phosphorylation capacity) of monocytes was assessed in a live-cell metabolic assay. As depicted in **Figure 3D-G**, noradrenaline, attenuated both OCR (expressed by spare respiratory capacity [SRC], p=0.002), and ECAR (p=0.004) in these experiments.

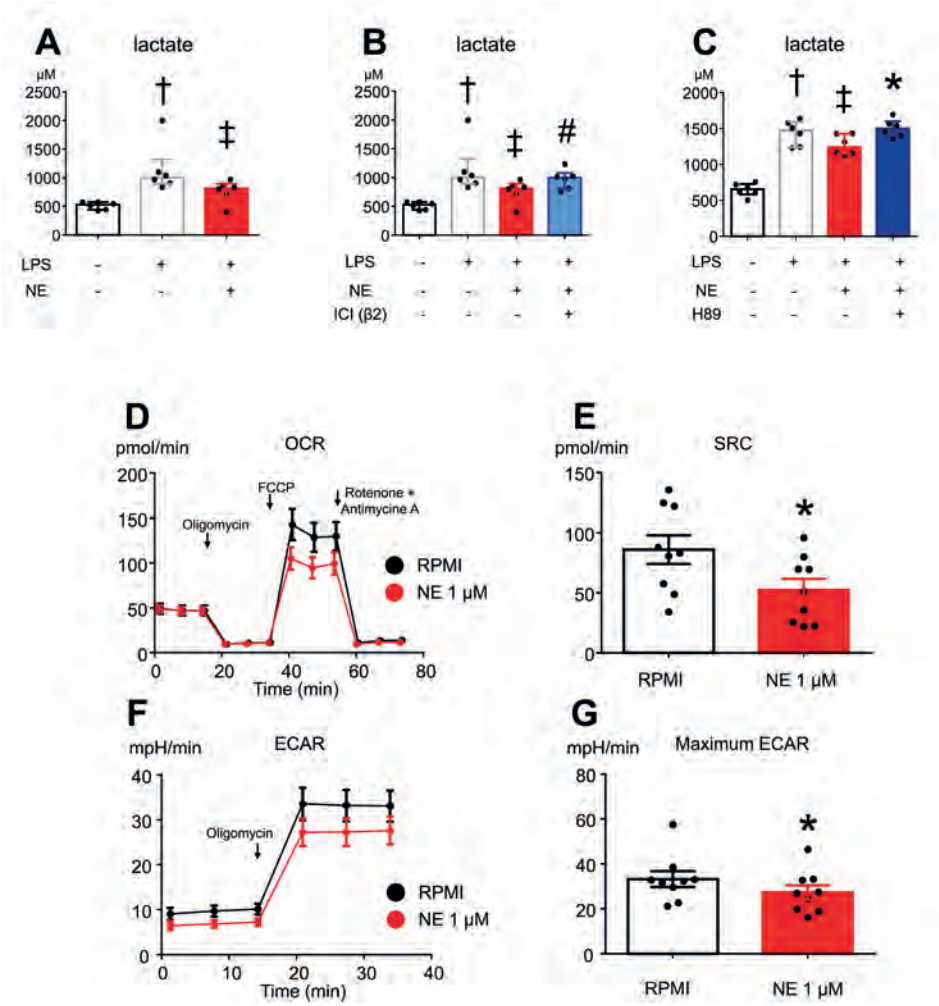

Figure 3. Noradrenaline suppresses immunometabolism in primary human monocytes. (A-C) Lactate concentrations in supernatants of primary human monocyte cultures that were preincubated with either RPMI or the β2-antagonist ICI-118,551 (ICI; 0.1 µM) or the PKA inhibitor H89 (3 µM) for 30 min, followed by incubation with noradrenaline (NE; 1 µM) for 1h and subsequent stimulation with LPS (10 ng/mL) or RPMI for 24 h. (D-G) Oxygen consumption rate (OCR, panels D) and spare respiratory capacity (SRC, calculated as the difference between basal and maximal OCR, panel E), extracellular acidification rate (ECAR, panels F-G) of primary human monocytes in the absence (RPMI) or presence of NE (1 µM). Panels D and F depict real-time changes in OCR and ECAR during the 'Mito-stress test', consisting of sequential treatment with oligomycin (ATPase inhibitor) or for panel D only, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP; proton uncoupler), and rotenone together with antimycin A (electron transport chain complex I and III inhibitors). Data are expressed as median and IQR (panels A-C) or mean ± SEM (panels D-G) of 6-9 individual donors. †p<0.05 compared to RPMI; ‡p<0.05 compared to LPS; *p<0.05, #p=0.05–0.10 compared to NE + LPS calculated using Wilcoxon matched pairs tests (panels A-C) or t-tests (panels E and G).

NORADRENALINE INFUSION EXERTS ANTI-INFLAMMATORY EFFECTS IN MICE

To investigate whether the immunosuppressive effects of noradrenaline also apply *in vivo*, we investigated cytokine levels in endotoxemic mice that received continuous intravenous (i.v.) noradrenaline treatment delivered by micro-osmotic pumps. First, mice were infused with noradrenaline or PBS for 3 hours followed by an i.v. injection with LPS or saline. Continuous delivery by the micro-osmotic pumps was verified by dose-dependent increases in plasma noradrenaline concentrations (**Supplementary Figure E4A**). Furthermore, noradrenaline infusion dose-dependently attenuated LPS-induced TNF-α (p=0.33 and p=0.04 for 1 and 5 μg/kg/min, respectively), IP-10 (p=0.34 and p=0.0004), macrophage inflammatory protein (MIP)-1α (p=0.12 and p=0.01) and macrophage chemoattractant protein (MCP)-1 (p=0.33 and p=0.001) responses, while plasma levels of IL-10 were enhanced (p=0.34 and p=0.0004, **Figure 4A**), indicating broad anti-inflammatory effects. A similar experiment using 24- instead of 3-hour infusion yielded similar immunosuppressive effects of noradrenaline infusion (**Figure 4B, Supplementary Figure E4B and E5A**). Furthermore, noradrenaline infusion dose-dependently attenuated basal neutrophilic ROS production in LPS-challenged mice (p=0.38, p=0.03, and p=0.02 for 1, 3 and 5 μg/kg/min, respectively) and suppressed the maximal neutrophilic respiratory burst in non LPS-challenged mice (p=0.06, p=0.006, and p=0.02, **Figure 4C)**. Finally, we compared the effects of 3-hour infusion of noradrenaline and vasopressin **(Figure 5)**. As expected, increased plasma noradrenaline levels were only observed in noradrenaline-infused animals (**Supplementary Figure E4C)** and no effects of either vasopressor were found in non LPS-challenged mice (**Supplementary Figure E5B**). Noradrenaline infusion again significantly suppressed LPS-induced pro-inflammatory cytokine responses (p=0.03 for TNFα, IP-10, MIP-1α, and MCP-1), while enhancing IL-10 levels (p=0.03). Vasopressin infusion exerted no immunomodulatory effects (all p-values >0.30), **Figure 5**). Furthermore, pulmonary MPO levels, reflecting neutrophil influx, were attenuated in noradrenaline- (p=0.02), but not vasopressin-infused (p=0.65) mice challenged with LPS (**Supplementary Figure E6**).

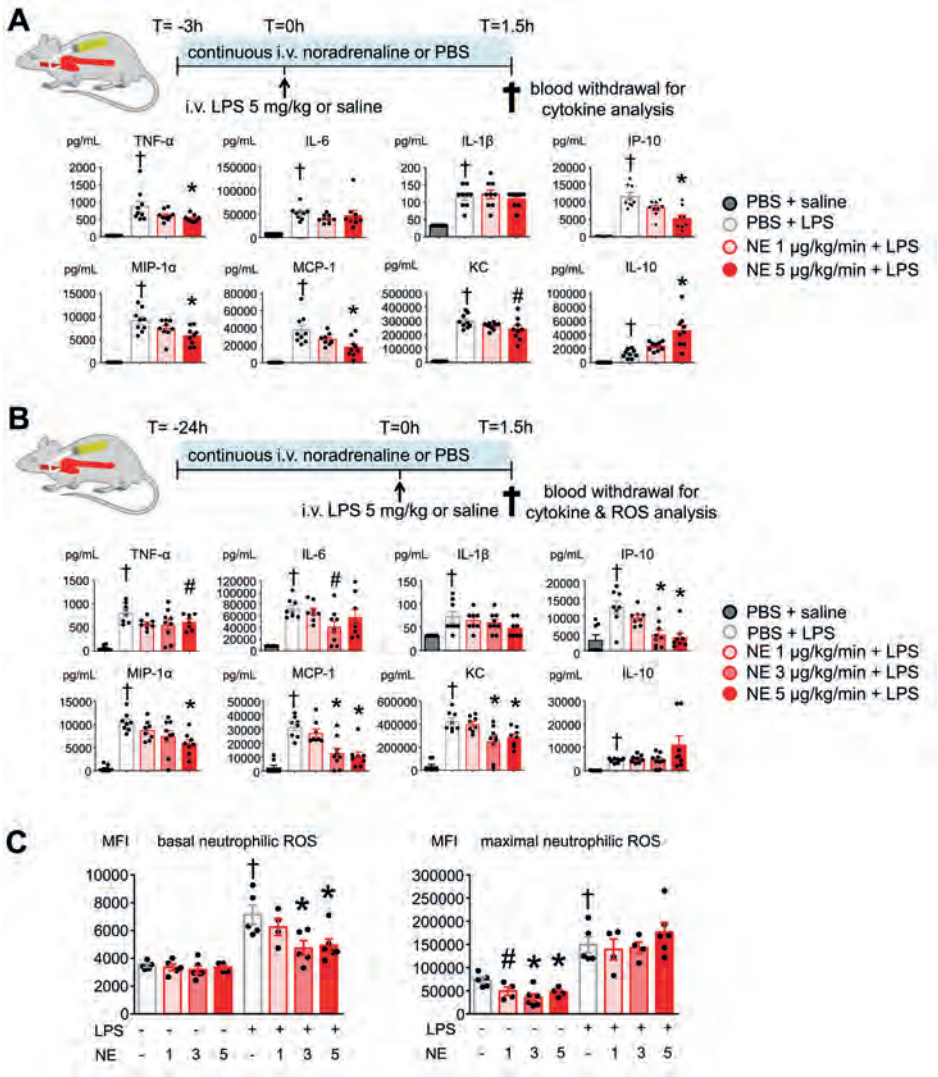

Figure 4. Noradrenaline infusion modulates *in vivo* cytokine responses and *ex vivo* reactive oxygen species production in mice. (A-B) Plasma concentrations of TNF-α, IL-6, IL-β, IP-10, MIP-1α, MCP-1, KC, and IL-10 in mice intravenously infused with noradrenaline (NE; 1, 3 or 5 µg/kg/min) or phosphate-buffered saline (PBS) via micro-osmotic pumps connected to a jugular vein catheter for 4.5 (A) or 25.5 (B) h and challenged intravenously with LPS (5 mg/kg) or saline 3 (A) or 24 (B) h after start of infusion. (C) reactive oxygen species (ROS) content and PMA-induced maximal respiratory burst of neutrophils obtained at the end of the experiment depicted in panel B. Data are expressed as mean ± SEM of 7-9 (cytokines) or 4-6 (ROS) animals per group. †p <0.05 compared to PBS + saline; *p<0.05, #p=0.05-0.10 compared to PBS + LPS calculated using t-tests followed by FDR correction (Benjamini-Hochberg).

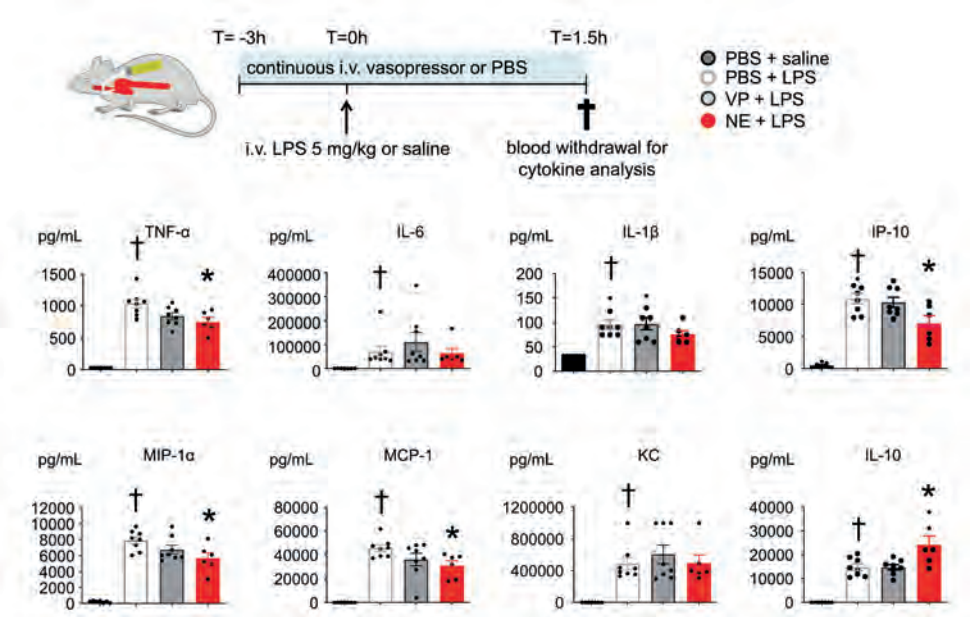

Figure 5. Vasopressin infusion does not exert immunomodulatory effects in mice. Plasma concentrations of TNF-α, IL-6, IL-β, IP-10, MIP-1α, MCP-1, KC, and IL-10 in mice intravenously infused with noradrenaline (NE; 5 μg/kg/min), vasopressin (VP; 0.00057 IU/kg/min, equivalent to 0.04 IU/min in a 70 kg human) or phosphate-buffered saline (PBS) via micro-osmotic pumps connected to a jugular vein catheter for 4.5 h and challenged intravenously with LPS (5 mg/kg) or saline 3 h after start of infusion. Data are expressed as mean ± SEM of 6-8 animals per group. †p<0.05 compared to PBS + saline; *p<0.05 compared to PBS + LPS calculated using t-tests followed by FDR correction (Benjamini-Hochberg).

NORADRENALINE INFUSION INCREASES BACTERIAL DISSEMINATION IN EXPERIMENTAL SEPSIS

To investigate if noradrenaline functionally impairs host defense, mice were implanted with micro-osmotic pumps containing noradrenaline, vasopressin, or PBS, followed by induction of experimental sepsis using cecal ligation and puncture (CLP). Sham-operated mice were used as a negative control. CLP led to profound bacterial dissemination, as evidenced by increased CFU counts in spleen (p=0.04), liver (p=0.04), and blood (p=0.046) of PBS-infused CLP mice compared to sham-operated animals **(Figure 6)**. Compared to PBS-infused CLP mice, CFU counts were significantly higher in spleen (p=0.04) and liver (p=0.03) of noradrenaline-, but not vasopressin-infused (p=0.37 and p=0.75) CLP mice (**Figure 6**). Furthermore, a trend towards higher blood CFUs was observed in noradrenaline-infused vs. PBS-infused CLP mice (p=0.06), whereas no difference was observed in vasopressin-infused CLP mice (p=0.95, **Figure 6**). Plasma cytokine levels, determined four hours after induction of CLP, did not reveal statistically significant differences between groups (**Supplementary Figure E7,** TNF-α concentrations were below the detection limit [3.2 pg/mL] in all animals). Nevertheless, several pro/

anti-inflammatory cytokine ratios, providing an indication of the pro/anti-inflammatory balance, were lower in noradrenaline-infused CLP mice compared with PBS-infused CLP mice (p=0.04 for KC/IL-10, p=0.06 for IP-10/IL-10, and p=0.03 for MIP-1α/IL-10, **Supplementary Figure E8**).

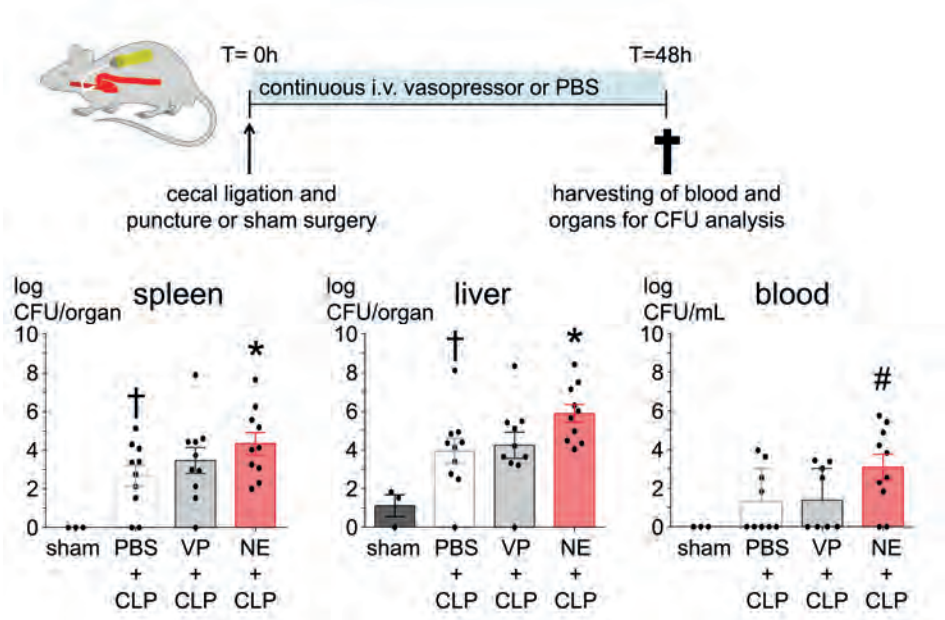

Figure 6. Noradrenaline infusion increases bacterial dissemination in experimental sepsis. Bacterial load (expressed as colony forming units [CFU]) in spleen, liver and blood of mice intravenously infused with noradrenaline (NE; 3 µg/kg/min), vasopressin (VP; 0.00057 IU/kg/min [equivalent to 0.04 IU/min in a 70 kg human]) or phosphate-buffered saline (PBS) for 2 days via micro-osmotic pumps connected to a jugular vein catheter and subjected to cecal ligation and puncture to induce sepsis or sham surgery. Data are expressed as mean ± SEM of 3 (sham group) or 10 (other groups) animals per group (it was not possible to obtain sufficient amounts of blood for CFU counts from three animals due to severity of illness resulting in inadequate blood flow.) †p<0.05 compared to sham surgery; *p<0.05, #p=0.05–0.10 compared to PBS + CLP calculated using t-tests.

NORADRENALINE EXERTS ANTI-INFLAMMATORY EFFECTS IN LPS-CHALLENGED HEALTHY VOLUNTEERS AND IN PATIENTS WITH SEPTIC SHOCK

In order to translate our findings to the human *in vivo* setting, we first performed an experimental endotoxemia study, in which healthy subjects were randomized to a five-hour i.v. infusion of either low-dose noradrenaline (0.05 µg/kg/min), vasopressin (0.04 IU/min), or placebo (saline), starting one hour before i.v. administration of 2 ng/kg LPS. Baseline characteristics of the three groups were comparable (**Supplementary Table E1**). Noradrenaline, but not vasopressin treatment resulted in a swift increase in mean arterial pressure (MAP, +13 ± 1 mmHg, **Supplementary Figure E9A**) and a reduction

in heart rate (-5 ± 2 bpm, **Supplementary Figure E9B**). None of the vasopressors influenced the LPS-induced increase in body temperature (p=0.64 and p=0.74 for noradrenaline and vasopressin, respectively, **Supplementary Figure E9C).** Plasma noradrenaline levels increased to a maximum of 10.2 ± 0.4 nmol/L in the noradrenaline group (**Supplementary Figure E9D).** Circulating numbers of monocytes, neutrophils, or lymphocytes were not affected by noradrenaline (p=0.27, p=0.21, and p=0.45, respectively) or vasopressin (p=0.78, p=0.08, and p=0.22) treatment (**Figure 7A**). Plasma concentrations of TNF-α, IL-6, IL-8, IP-10, MCP-1, granulocyte colony-stimulating factor (G-CSF), and IL-10 increased profoundly in all subjects following LPS administration (**Figure 7B)**. Low-dose noradrenaline infusion resulted in a significantly enhanced IL-10 response (p=0.007) compared to the placebo group. Furthermore, LPS-induced plasma levels of IP-10 were attenuated by noradrenaline (p=0.04, **Figure 7B**), while the other cytokines were not influenced. Vasopressin treatment did not modulate levels of any of the measured cytokines (all p-values >0.76, **Figure 7B**).

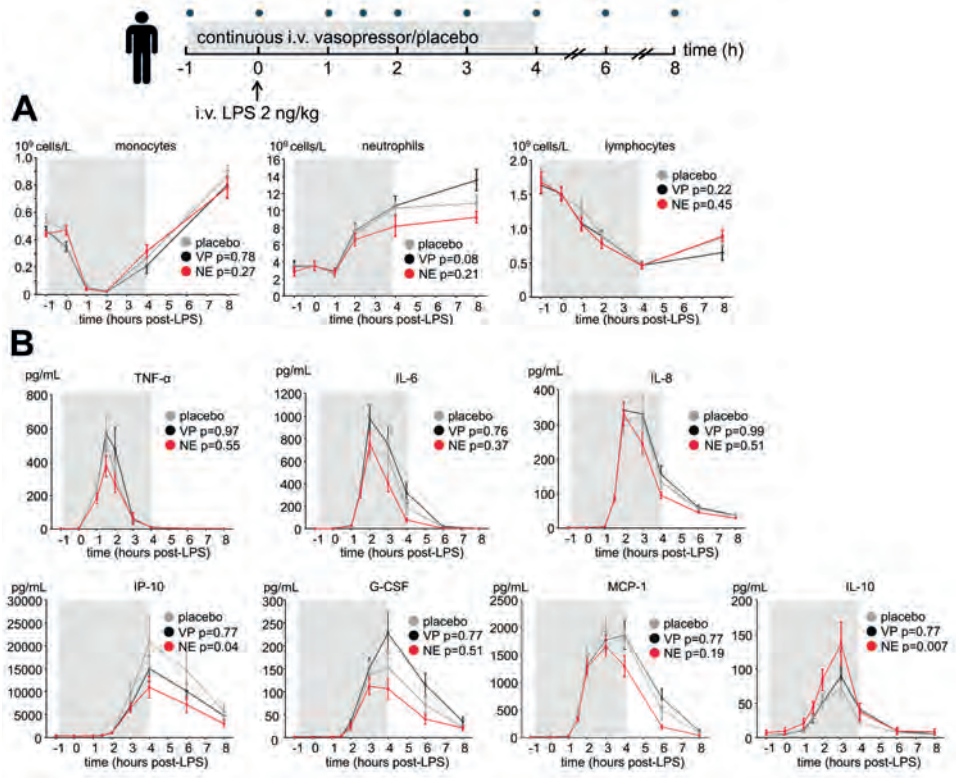

Figure 7. Low-dose noradrenaline infusion enhances the IL-10 response and attenuates IP-10 concentrations during experimental human endotoxemia. (A) Circulating monocyte, neutrophil and lymphocyte numbers, (B) plasma concentrations of TNF-α, IL-6, IL-8, IP-10, G-CSF, MCP-1, and IL-10, and (C) heatmap of mean area under the curve (AUC) cytokine responses in healthy volunteers randomized to a 5 h intravenous infusion with either placebo (saline), low-dose noradrenaline (NE; 0.05 μg/kg/min) or vasopressin (VP; 0.04 IU/min) and challenged intravenously with 2 ng/kg LPS 1 h after start of infusion. Data are expressed as mean ± SEM of 10 subjects per group. The grey area represents the period during which noradrenaline, vasopressin, or saline was infused. The blue dots in the study design indicate blood withdrawal timepoints for cytokine and leukocyte count analysis. p-values were calculated using repeated measures two-way analysis of variance (time*treatment interaction term) vs. the placebo group followed by FDR correction (Benjamini-Hochberg).

Finally, we explored the immunomodulatory effects of noradrenaline in a cohort of 195 patients admitted to the Intensive Care with septic shock, who were all treated with noradrenaline, and 66 of whom chronically used β-blockers. Blood for TNF-α and IL-10 analysis was obtained at study inclusion, which took place within 24 hours after start of noradrenaline infusion (median of 8.8 [4.2–14.2] and 7.6 [4.4–15.9] hours in the noradrenaline-only and noradrenaline + β-blocker groups, respectively, p=0.91). In the 129 patients not treated with β-blockers, noradrenaline infusion rates at inclusion correlated with lower TNF-α/IL-10 ratios (r=-0.38, p<0.0001, **Figure 8A**), illustrating immunosuppressive effects of noradrenaline. We hypothesized that concomitant use

of β-blockers would mitigate noradrenaline's anti-inflammatory effects, which we previously showed to be primarily mediated via the β2-AR. Although most of the clinically used β-blockers, such as metoprolol and atenolol, are regarded as β1-selective, they actually have significant β2-affinity (17)**.** Patients in the noradrenaline + β-blocker group used metoprolol (n=57), atenolol (n=3), bisoprolol (n=3), labetalol (n=1), sotalol (n=1), and propranolol (n=1), and had received a dose on the day of study inclusion before blood was obtained for cytokine analysis. There were no differences in sex, age, disease severity (APACHE II score), use of corticosteroids, requirement of mechanical ventilation, or noradrenaline infusion rate between patients with or without concomitant β-blocker use (**Supplementary Table E2**). The plasma TNF-α/IL-10 ratio, reflecting the pro/anti-inflammatory balance, was significantly higher in patients with β-blockers (0.74 [0.24-1.97]) than in those without (0.51 [0.16 – 1.18], p=0.03, **Figure 8B**).

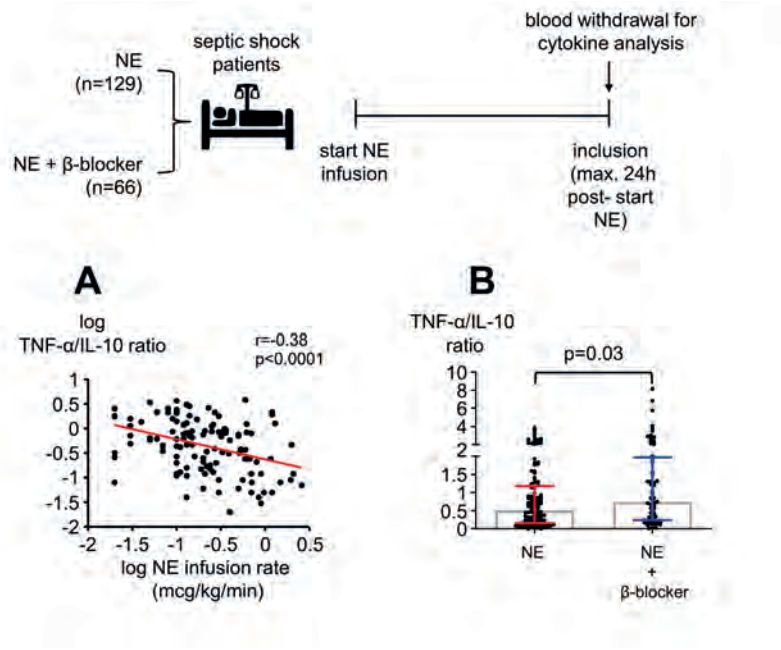

Figure 8. Relationship between noradrenaline infusion rate, β-blocker use, and the pro/anti-inflammatory balance in septic shock patients. (A) Correlation between noradrenaline (NE) infusion rate at the time of inclusion and the plasma TNF-α/IL-10 ratio at the same timepoint in septic shock patients. (B) Plasma TNF-α/IL-10 ratio at the time of inclusion in septic shock patients treated with NE with or without concomitant use of β-blockers. Data are expressed as median ± IQR (panel B). p values were calculated using Pearson correlation (panel A) and a Mann-Whitney U test (panel B).

DISCUSSION

We demonstrate that noradrenaline enhances anti-inflammatory cytokine production while attenuating pro-inflammatory cytokine release and ROS generation by human leukocytes in response to stimulation with a wide variety of inflammatory ligands. The underlying mechanism involves activation of the β2 adrenergic receptor, while immunometabolism is suppressed as well. In endotoxemic mice, continuous noradrenaline infusion caused a dose-dependent shift towards an anti-inflammatory cytokine response pattern and compromised neutrophilic ROS production. Furthermore, noradrenaline infusion resulted in increased bacterial dissemination during experimental sepsis. In healthy volunteers, noradrenaline infused at a very low dosage exhibited anti-inflammatory effects during experimental endotoxemia. In sharp contrast, vasopressin exerted no immunomodulatory effects across all our experimental setups. Finally, higher noradrenaline infusion rates were related to a more pronounced anti-inflammatory cytokine balance in septic shock patients, whereas use of β-blockers was associated with a more pro-inflammatory cytokine balance.

Noradrenaline's immunomodulatory effects were counteracted by the nonselective β-blocker propranolol and the selective β2-blocker ICI-118,551. These findings are in line with earlier reports showing that β-blockers reverse (nor)epinephrine-induced inhibition of pro-inflammatory cytokine release after LPS stimulation (13, 18, 19), and with previous work demonstrating that selective β2-agonists attenuate pro-inflammatory cytokine production (20). In this respect, it is also noteworthy that dobutamine, which primarily has β1-affinity, did not influence the innate immune response during human endotoxemia (21), while adrenaline infusion enhanced the IL-10 response and attenuated TNF-α concentrations in the same model (18). Our data further reveal that the effects of noradrenaline are nullified by the PKA-inhibitor H89, supporting the notion that β2-receptor-mediated immunologic actions are cAMP-dependent (22, 23).

Disturbances in energy metabolism have been associated with worse outcomes in sepsis patients (24) and recently, defective immune cell metabolism was identified as a mechanism underlying sepsis-induced immunoparalysis (16). More specifically, suppressed glycolysis and oxidative phosphorylation (OXPHOS) in leukocytes of septic patients was associated with impaired pro-inflammatory cytokine production (16). Regarding the specific roles of glycolysis and oxidative phosphorylation, previous work showed that inhibition of glycolysis with 2-deoxyglucose decreased production of both pro- and anti-inflammatory mediators in LPS-stimulated primary human monocytes (25). Data on inhibition of oxidative phosphorylation are ambiguous: while LPS-induced production of pro-inflammatory cytokines was shown to be attenuated by the oxidative phosphorylation inhibitor rotenone in both human monocytes and murine

bone marrow-derived macrophages (BMDMs) (25, 26), LPS-induced IL-10 release was enhanced in murine BMDMs (26) but decreased in human monocytes (25). We show that noradrenaline suppresses both glycolysis and oxidative phosphorylation in human monocytes, which potentially contributes to its modulatory effects on cytokine production. Noradrenaline also impaired ROS production by monocytes and neutrophils. The generation of ROS is an essential mechanism for bacterial killing, for instance exemplified by the fact that genetic disorders characterized by defective ROS production are associated with greatly increased susceptibility towards bacterial and fungal infections (27, 28). Furthermore, increased ROS generation by macrophages was shown to confer resistance towards *Listeria monocytogenes* infection (29).

A distinctive feature of the present work is the use of micro-osmotic pumps connected to a jugular vein catheter to allow for continuous intravenous administration of vasopressors in conscious mice. This set-up was chosen to mimic the human situation as closely as possible and to minimize possible confounding effects of sedatives (30). For vasopressin, we used a clinically relevant dosage which has been used in various septic shock trials (31-33). It could be argued that the noradrenaline dosages used are relatively high compared to humans. Nevertheless, previous murine experiments employed similar dosages, which were infused subcutaneously 14 days, suggesting that mice are less sensitive to noradrenaline (34). Moreover, the plasma noradrenaline levels attained in our murine experiments were comparable to those in septic shock and trauma patients treated with noradrenaline, where median concentrations of approximately 100 nmol/L were found (range 6-1000 nmol/L) (35). Noradrenaline infusion resulted in increased bacterial dissemination in liver, spleen, and blood in mice subjected to CLP, indicating functional impairment of host defense. No statistically significant effects of noradrenaline infusion on cytokine levels were observed in CLP mice, which may be related to the early sampling timepoint on which cytokine concentrations were still relatively low. Due to the limited amount of blood which can be obtained from mice, we did not assess cytokine profiles at other timepoints, which represents a limitation of our work. Nevertheless, in line with the immunomodulatory effects observed in our other experiments and patient data, noradrenaline infusion resulted in significantly lower pro/anti-inflammatory cytokine ratios in CLP mice.

In order to assess immunomodulatory effects of noradrenaline and vasopressin in humans, healthy volunteers were challenged with LPS. Similar to our *in vitro* and murine endotoxemia results, noradrenaline infusion resulted in an enhanced IL-10 response, whereas concentrations of the pro-inflammatory cytokine IP-10 were attenuated. The fact that significant effects were not attained for other pro-inflammatory cytokines may be explained by the low dose of noradrenaline used (for safety reasons), and the subsequently low plasma levels reached (~10 nmol/L). Because our data illustrate

that the immunosuppressive effects of noradrenaline are clearly dose-dependent, stronger anti-inflammatory effects are expected in septic patients, who exhibit much higher noradrenaline concentrations (35). Of note, we used a clinically relevant dose of vasopressin (which was also similar to that used in our murine experiments), because this compound does not exert vasopressor effects in healthy volunteers at this infusion rate.

Clinical studies have established that high noradrenaline levels are an independent risk factor for mortality in patients with sepsis (36). However, to date, no clinical studies have specifically investigated whether or not these detrimental effects may be due to noradrenaline-induced immunosuppression. Clear assessment of immunologic effects of noradrenaline in sepsis patients is precluded by the fact that it is the recommended vasopressor specified by the surviving sepsis guidelines, and is therefore used in virtually all patients. In order to gain insight into possible immunomodulatory effects of noradrenaline in the clinical setting, we therefore analyzed the relationship between noradrenaline infusion rates and inflammatory parameters as well as the influence of β-blockers in a relatively large cohort of patients with septic shock treated with noradrenaline. Our data reveal that the noradrenaline infusion rate is associated with lower plasma TNF-α/IL-10 ratios, indicating a shift towards a more anti-inflammatory phenotype and use of β-blockers was associated with an increased plasma TNF-α/IL-10 ratio, signifying a more pro-inflammatory phenotype. Collectively, these results are indicative of anti-inflammatory effects of noradrenaline in septic shock patients. Therefore, clinical use of noradrenaline appears to dysregulate the host response and β-blockers might have beneficial immunological effects in sepsis patients. In this context, it is noteworthy that a higher TNF-α/IL-10 ratio was previously shown to be associated with improved survival in sepsis patients (37). Furthermore, the β-blocker esmolol dramatically increased survival in a randomized trial in patients with septic shock treated with noradrenaline (38). Although these promising results were ascribed to esmolol's cardiac effects and no immunological parameters were assessed, immunomodulation may have played an important role as well. In addition, our findings may shed new light on recent studies that identified different sepsis endotypes based on transcriptomic signatures already alluded to in the introduction. Interestingly, compared with the other endotypes, a higher proportion of patients classified as having an immune-suppressed endotype were in shock and required (high dosages of) noradrenaline (10, 11). As such, it may be speculated that the endotypes are not only driven by pathogen and host factors, but also by iatrogenic factors such as noradrenaline administration.

In all large clinical trials to date, vasopressin and more recently, the selective V1 receptor agonist selepressin, was investigated as an adjunctive therapy to noradrenaline (32, 33, 39). Therefore, virtually all patients in the vasopressin/selepressin treatment groups

also received noradrenaline, and in most cases in much higher dosages than those we used in our human endotoxemia study, which already significantly dysregulated the host response. As an add-on therapy, vasopressin (analogues) exerted no overall effects on mortality (32, 33, 39), although a survival benefit was observed in a subset of vasopressin-treated patients (33) and vasopressin reduced the need for renal replacement therapy (32). Differences in infectious complications were unfortunately not assessed in these trials. Nevertheless, a secondary analysis of the VASST trial assessed cytokine levels, which were similar between the vasopressin and noradrenaline groups after correction for baseline characteristics such as sex, age and shock severity (40). This might appear to contradict our findings, but its use as an adjunctive therapy to noradrenaline in this trial precludes clear interpretation. As compounds like vasopressin and selepressin appear to be suitable as first-line vasopressors as well (31, 41), it would be interesting to evaluate immunologic effects in a noradrenaline versus alternative vasopressor monotherapy study. If our results could be recapitulated in such a setting, reappraisal of the current vasopressor therapy for sepsis is warranted. With the emergence of the era of 'precision medicine' for sepsis, future use of different vasopressors may be tailored to specific subgroups of septic patients based on their (molecular) immunological endotype.

CONCLUSION

Noradrenaline, the first choice vasopressor administered to patients with septic shock worldwide, significantly dysregulates the host response. Due to its potent anti-inflammatory and host defense-compromising effects, it may therefore importantly contribute to the development and propagation of sepsis-induced immunoparalysis. Vasopressin, a viable alternative vasopressor, does not exert these untoward side effects. These findings may prompt reappraisal of the current clinical management of sepsis patients.

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SUPPLEMENTARY MATERIAL

IN VITRO STUDIES

Cytokine production by whole blood and isolated monocytes

For whole blood experiments, lithium heparin-anticoagulated venous blood was diluted five times in RPMI 1640 Dutch modification (Invitrogen, Carlsbad, USA) supplemented with 10 µg/mL gentamicin, 10 mM Glutamax and 10 mM pyruvate (this supplemented RPMI was used for all *in vitro* experiments). For monocyte stimulation experiments, peripheral blood mononuclear cells (PBMCs) were isolated from ethylenediaminetetraacetic acid (EDTA)-anticoagulated venous blood using Ficoll (Ficoll-Paque Plus, GE healthcare, Chicago, USA) density gradient centrifugation (10 min, 1200 g at room temperature [RT]) in SepMate™ tubes (STEMCELL Technologies, Vancouver, Canada). The PBMC fraction was washed thrice in cold phosphate-buffered saline (PBS, 10 min, 1700 rpm at 4°C). Subsequently, monocytes were isolated from PBMCs using magnetic separation (negative selection using the Pan Monocyte Isolation Kit [Miltenyi Biotec, Bergisch Gladbach, Germany]) according to the manufacturer's instructions and resuspended in RPMI. All experiments were performed in duplicate. Diluted blood or monocytes (1 x 10^5 per well) were incubated in 96-well plates (Eppendorf) with noradrenaline (Centrafarm BV, Etten-Leur, The Netherlands) or vasopressin (V9879, Sigma-Aldrich, Saint-Louis, Missouri, USA). Sixty minutes later, stimuli were added: Pam_3CysSK_4 (Pam3Cys, TLR2, EMC microcollections, Tübingen, Germany), Poly I:C (TLR3, Invivogen, San Diego, USA), *Escherichia coli* (*E.coli*) lipopolysaccharide (LPS, serotype 055:B5, TLR4, Sigma-Aldrich), flagellin (TLR5, Invivogen), R848 (TLR7 – 8, Invivogen), CpG (TLR9, Invivogen), heat-killed (HK) *E.coli*, *Staphylococcus aureus* (*S.aureus*), *Pseudomonas aeruginosa* (*P.aeruginosa*), *Candida albicans* conidia (*C.albicans*), and *Aspergillus fumigatus* conidia (*A.fumigatus*). After 24 h of incubation at 37°C, 5% CO_2, plates were centrifuged (8 min, 1400 rpm, RT) and supernatants were stored at -80°C until analysis. In additional whole blood and monocyte experiments, adrenergic receptor antagonists prazosin hydrochloride (α1-antagonist, P791, Sigma-Aldrich), yohimbine hydrochloride (α2-antagonist, Y3125, Sigma-Aldrich), propranolol hydrochloride (non-selective β-antagonist, P0884, Sigma-Aldrich), atenolol (β1-antagonist A7655, Sigma-Aldrich) ICI-118,551 hydrochloride (I127, β2-antagonist Sigma-Aldrich)), and the PKA inhibitor H89 (2910, Tocris, Bristol, UK) were added to blood or monocyte cultures 30 min prior to incubation with noradrenaline. Cytokines in cell culture supernatants were measured using enzyme-linked immunosorbent assays (ELISA, Duosets, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Lactate measurements

Lactate was measured in stimulated whole blood and monocyte culture supernatants (see description above) as described before (1). Shortly, after perchloric acid precipitation (only for whole blood samples), the supernatants were neutralized with NaOH (0.25 M).

Thereafter, lactate levels were determined by oxidizing and coupling the resulting H_2O_2 by horseradish peroxidase to Amplex Red reagent resulting in fluorescent resorufin for 20 min at room temperature, and the fluorescence of resorufin (excitation/emission maxima of 570/585 nm) was measured on a 96-well plate reader (Biotek).

Cytotoxicity assay

Levels of lactate dehydrogenase (LDH) were measured using the Cytotox96 colorimetric assay (Promega, Madison, USA) according to the manufacturer's instructions. Supernatants were collected from stimulated whole blood cultures (see description above). Untreated cells (RPMI only) served as background controls while positive controls consisted of otherwise untreated samples incubated with lysis solution. The amount of LDH measured in the positive controls was set at 100% and LDH levels in the samples were calculated accordingly.

Monocytic reactive oxygen species production

Reactive oxygen species (ROS) production by monocytes was determined using a luminol-based luminescence assay. Monocytes were isolated as described before and quadruplicate wells containing 1 x 10^5 monocytes were incubated with noradrenaline, vasopressin or RPMI in flat-bottom 96-well plates (Eppendorf) for 60 min at 37°C, 5% CO_2. Subsequently, luminol was added, followed by phorbol 12-myristate 13-acetate (PMA; 50 ng/mL, P1585, Sigma-Aldrich). Chemiluminescence was measured every 142 s at 37°C during 1 h on a 96-well plate reader (Biotek, Winooski, USA).

Live-cell metabolism

Monocytes were isolated as described before and 2 x 10^5 cells per well were incubated with noradrenaline (1 µM) or RPMI on a Cell-Tak and Tissue Adhesive (both Corning, Corning, USA) pre-treated Seahorse XF96 Cell Culture Microplate (Agilent Technologies Santa Clara, USA) at 37°C, 5% CO_2 for 1 h. Subsequently, medium was changed to unbuffered Seahorse medium (8.3 g DMEM powder, 0.016 g phenol red and 1.85 g NaCl in 1 L Milli-Q water, pH 7.4; sterile filtered), supplemented with 11 mM glucose, 2 mM L-glutamine, 1 mM pyruvate, and 1 µM noradrenaline or RPMI. Cells were subsequently kept in a non-CO_2 incubator for 60 min prior to starting measurements. Real-time oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) was determined using an XF-96 Extracellular Flux Analyzer (Agilent Technologies). All conditions were performed in quintuplicate. Three basal measurements were obtained, followed by three measurements after the sequential addition of 1 µM oligomycin (ATPase inhibitor), 1 µM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; proton uncoupler), and 1.3 µM rotenone together with 2.5 µM antimycin A (mitochondrial electron transport chain complex I and III inhibitors). Data were analyzed using Wave 2.6.0 software.

MURINE STUDIES

Housing, randomization and blinding

Mice were housed under specific-pathogen-free conditions, with water and standard rodent chow available ad libitum. Mice were randomized using a random number generator (https://www.sealedenvelope.com) and biotechnicians performing the surgical procedures were blinded for experimental groups.

Endotoxemia experiments

Mice were weighed immediately before placement of micro-osmotic pumps; mean ± standard error of the mean (SEM) weight was 25.3 ± 0.1 g. Micro-osmotic pumps (model 2001, Alzet Corporation Cupertino, USA) with connected jugular vein catheters (0007701, Alzet Corporation) were filled with noradrenaline (74500, Sigma-Aldrich; for delivery of 1, 3, or 5 µg/kg/min), vasopressin (V9879, Sigma-Aldrich; for delivery of 0.00057 IU/kg/min [equivalent of 0.04 IU/min in a 70 kg human]) or PBS. Filled pumps were placed in sterile NaCl 0.9% at 37°C in the dark 3-5 days prior to implantation to ensure priming of the pump and complete filling of the catheter. Dosing of the vasopressors was based on literature (2, 3). Under isoflurane anesthesia, the catheter connected to the pre-filled pump was inserted in the right jugular vein for intravenous delivery and the pump and remaining part of the catheter were placed in a subcutaneous pocket created on the back of the animal. Afterwards, mice recovered in a 37°C incubator for 30 min before being returned to their cages. To induce endotoxemia, LPS (5 mg/kg, serotype 0111:B4, L2630, Sigma-Aldrich,) or saline was injected in the tail vein 3 or 24 h after pump implantation. Animals were sacrificed 90 minutes later by exsanguination under deep isoflurane anesthesia. Lithium heparin-anticoagulated blood was collected and kept on ice for analysis of *ex vivo* neutrophilic ROS production. EDTA-anticoagulated blood was centrifuged (10 min, 2000 g, RT) after which plasma was stored at -80°C until analysis of cytokine and noradrenaline concentrations. The right lung was snap frozen in liquid nitrogen, and stored at -80°C until myeloperoxidase analysis.

Cecal ligation and puncture experiments

Mice were weighed immediately before the induction of experimental sepsis and placement of micro-osmotic pumps; mean ± SEM weight was 24.6 ± 0.3 g. Experimental sepsis was induced by cecal ligation and puncture (CLP). Under isoflurane anesthesia, a 1 cm incision was made in the shaved and disinfected lower abdomen. This was followed by incision of the muscular and peritoneal wall. The cecum was located and exteriorized, after which it was ligated at 60% and punctured once (“through and through”) with a 21 gauge needle. After puncture, a small amount of feces was extruded and the cecum was placed back in the abdominal cavity. The peritoneal wall was closed with running sutures and the skin was closed with clips. Sham mice underwent the exact same procedures with the exception of cecal ligation and puncture. Directly following CLP, micro-

osmotic pumps containing noradrenaline (3 µg/kg/min), vasopressin (0.00057 IU/kg/min [equivalent of 0.04 IU/min in a 70 kg human]) or PBS were implanted as described above. Following surgery, mice were injected with 1 mL of saline for fluid resuscitation. Analgesia was provided by subcutaneous injections with 0.05 mg/kg buprenorphine twice daily on the day of surgery (one injection 1 h before the procedure) and on the day afterwards. Four hours after surgery, 30 µL of lithium-heparin-anticoagulated blood was obtained via a small tail incision and centrifuged at 2000 g, RT, after which plasma was stored at-80°C until further analysis. Forty-eight hours after CLP, mice were sacrificed by exsanguination under deep isoflurane anesthesia. The presence of a cecal abscess was visually confirmed in all CLP mice following sacrifice. Lithium heparin-anticoagulated blood was divided over two tubes, one of which was kept on ice for determination of bacterial load, whereas the other was centrifuged (2000 g, RT) after which plasma was stored at -80°C for cytokine determination. Spleen and liver were harvested aseptically and kept on ice for the determination of bacterial load.

Plasma cytokine analysis

Concentrations of IL-1β, IL-6, IL-10, IP-10, keratinocyte chemoattractant (KC, murine homolog of IL-8), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 alpha (MIP-1α), and tumor necrosis factor-α (TNF-α) were determined using a Luminex assay (Milliplex, Millipore, Burlington USA) according to the manufacturer's instructions.

Plasma noradrenaline measurements

Plasma noradrenaline levels were determined using the ELISA Fast Track kit (LDN, Nordhorn, Germany) according to the manufacturer's instructions.

Myeloperoxidase assay

Lung tissue was homogenized for 5 min at 50 Hz in Tissue Protein Extraction Reagent (Life Technologies, Carlsbad, USA) containing protease inhibitors (complete EDTA-free tablets, Roche, Basel, Switzerland) using a Tissuelyzer LT instrument and 5 mm stainless steel beads (both from Qiagen, Hilden Germany). Myeloperoxidase (MPO) levels were measured as a proxy for pulmonary neutrophil infiltration using an ELISA (Hycult Biotech, Uden, the Netherlands) according to the manufacturer's instruction. MPO levels were normalized to total protein content in the homogenates determined by a bicinchoninic acid assay (BCA Protein Assay, Life Technologies).

Ex vivo neutrophilic ROS production

One hundred µL of lithium heparin-anticoagulated blood was added to preheated tubes (10 min, 37°C) containing dihydrorhodamine 123 (DHR; 0.5 µg/mL, Sigma-Aldrich) or DHR with PMA (50 ng/mL, Sigma-Aldrich), and incubated for 15 min at 37°C with gentle

vortexing every 5 min. After erythrocyte lysis using an isotonic NH4Cl solution and washing, cells were stained with an APC/Cy7 anti-mouse Ly-6G antibody (clone 1A8, Biolegend, San Diego, USA) for 5 min at RT. After washing, samples were measured on a CytoFLEX flow cytometer (Beckman Coulter, Brea, USA). Data were analyzed using Kaluza Analysis 1.5a (Beckman Coulter).

Bacterial counts

Liver samples were homogenized in M-tubes on a gentleMACS device (both from Miltenyi) using the "RNA" program. The homogenized samples were then filtered through a 70 µM cell strainer (BD Falcon, Franklin Lakes, USA). Spleen samples were mechanically homogenized using a 70 µM cell strainer. Subsequently, serial ten-fold dilutions from blood, liver and spleen in PBS were made and 10 µL triplicate spot-plating of each dilution was performed on BBL Columbia agar plates with 5% sheep blood (BD) and incubated overnight at 37°C in aerobic conditions, after which bacterial colonies were counted. Results are specified as colony forming units (CFU) per mL (blood) or per organ.

HUMAN STUDIES

Subjects

For the human endotoxemia study, subjects were screened before entry into the study and had normal physical examination, electrocardiography and routine laboratory results. Exclusion criteria were febrile illness within two weeks before the endotoxemia experiment day, use of prescription drugs, history of hypertension or spontaneous vagal collapse, and participation in earlier endotoxemia trials. Subjects were randomized using the sealed envelope method by an independent research nurse otherwise not involved in the study to the noradrenaline, vasopressin, or placebo group.

Patients were included in the cohort study in accordance to then applicable criteria for septic shock: a suspected or proven infection accompanied by two or more systemic inflammatory response syndrome (SIRS) criteria in addition to noradrenaline requirement to maintain blood pressure despite adequate fluid resuscitation. Patients were excluded if they were on immunosuppressive therapy at home or had an active malignancy. In the noradrenaline + β-blocker group, β-blockers were either administered at home (for patients that were admitted to the ICU with septic shock directly from home), on the ward, or, in rare cases, on the ICU.

Experimental human endotoxemia procedures

Endotoxemia experiments were conducted at the research unit of the intensive care department of the Radboud university medical center according to our standard protocol (4). Subjects were instructed to refrain from caffeine and alcohol in the 24 h before the experiment and from food and drinks 10 h before the experiment. Two venous canulae

were placed in the antecubital vein, one for fluid infusion and endotoxin administration and the other for study drug administration. Subjects were prehydrated in the hour before LPS administration (1.5 L 2.5% glucose/0.45% saline) followed by 150 mL/h for 6 h and 75 mL/h for the remainder of the experiment. Blood pressure monitoring and blood withdrawals were performed using an arterial cannula placed in the radial artery. In addition, there was continuous ECG and SaO_2 monitoring. Vital signs were recorded on a Phillips MP50 patient monitor using an in-house developed data capturing system. Body temperature was measured every 30 mins with infrared tympanic measurements (FirstTemp Genius 2, Covidien Geldermalsen, the Netherlands). Subjects received a 5 h infusion of low-dose noradrenaline (0.05 μg/kg/min, Centrafarm B.V.), vasopressin (0.04 IU/min, Argipressin, Mercury Pharmaceuticals Ltd. London, UK) or placebo (NaCL 0.9%) starting one hour before LPS administration. At T=0, 2 ng/kg U.S. Reference *Escherichia coli* endotoxin (serotype O:113, Clinical Center Reference Endotoxin, National Institute of Health, Bethesda, USA) was administered intravenously to elicit a systemic inflammatory response.

Plasma cytokines, circulating noradrenaline concentrations, and hemocytometry

For cytokine analysis, blood was collected in EDTA tubes (Vacutainer, BD) and immediately centrifuged at 2000 g at 4 °C for 10 min. Plasma was then stored at -80°C until levels of TNF-α, IL-6, IL-8, IP-10, G-CSF, MCP-1, and IL-10 (human endotoxemia study), or TNF-α and IL-10 (sepsis patients) were measured in one batch using a Luminex assay (Milliplex, Millipore). For noradrenaline measurements, blood was collected into lithium heparin tubes (Vacutainer, BD) that were immediately placed on ice and centrifuged at 2000 g at 4 °C for 10 min, after which plasma was stored at −80 °C until analysis using HPLC with fluorometric detection, as described previously (5). Hemocytometry was performed in EDTA-anticoagulated blood using routine analysis methods also used for patient samples (flow cytometric analysis on a Sysmex XE-5000).

STATISTICAL ANALYSIS

Data are presented as mean ± standard error of the mean (SEM), median and interquartile range [IQR], depending on distribution of data (determined using Shapiro-Wilk tests). Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnet's post-hoc tests, t-tests, Wilcoxon matched pairs tests, Mann-Whitney U tests, Chi-square tests or Pearson correlation. For the human endotoxemia data, differences between vasopressor and placebo groups over time were analyzed using repeated measures two-way ANOVA (interaction term: time*treatment). A p value < 0.05 was considered significant. If more than 4 parameters were analyzed within an experiment, Benjamini-Hochberg false discovery rate (FDR) adjustment was applied for each comparison across the multiple parameters (6), and the adjusted p-values are presented. Note that for the murine experiments, it was not always possible to obtain sufficient blood to perform

all measurements due to severity of illness resulting in inadequate blood flow, leading to lower n-numbers for some of the presented data. Analyses were performed using GraphPad Prism 6 (Graphpad software, San Diego, USA) and SPSS 25 (IBM statistics, NY, USA).

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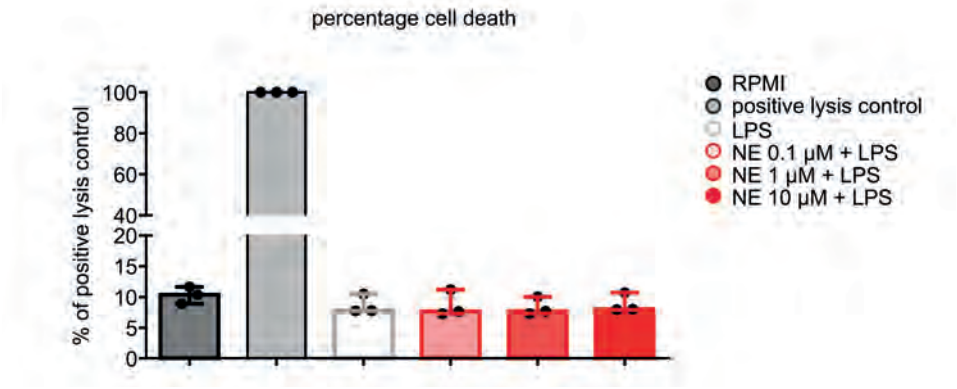
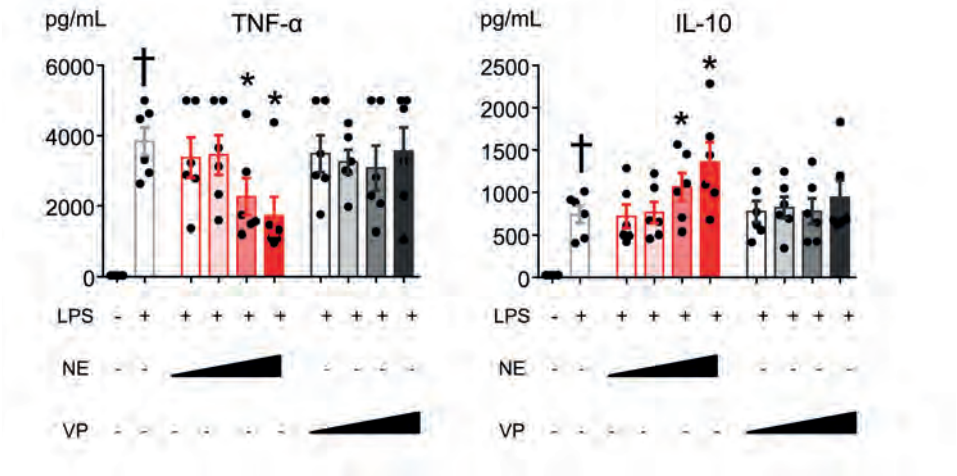
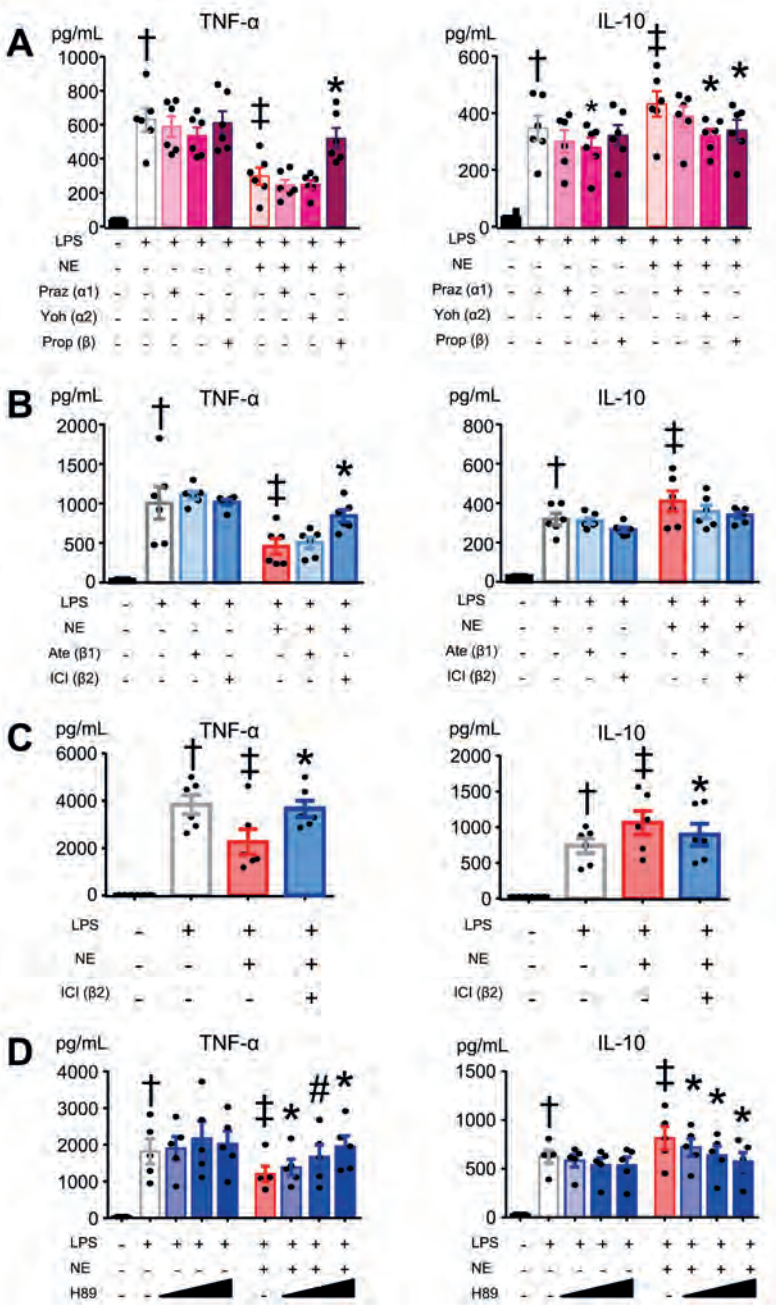


Supplementary Figure E1. Noradrenaline does not affect cell survival. Percentage cell death relative to positive lysis control measured by lactate dehydrogenase (LDH) release in human whole blood cultures preincubated with either RPMI (medium control), noradrenaline (NE; 0.1, 1, and 10 µM) for 1 h and subsequently stimulated with LPS (10 ng/mL) or RPMI for 24 h. Positive controls are otherwise untreated samples incubated with lysis solution. The amount of LDH measured in the positive controls was set at 100% and LDH levels in the samples were calculated accordingly. Data are expressed as individual data points and median and IQR of 3 individual donors.

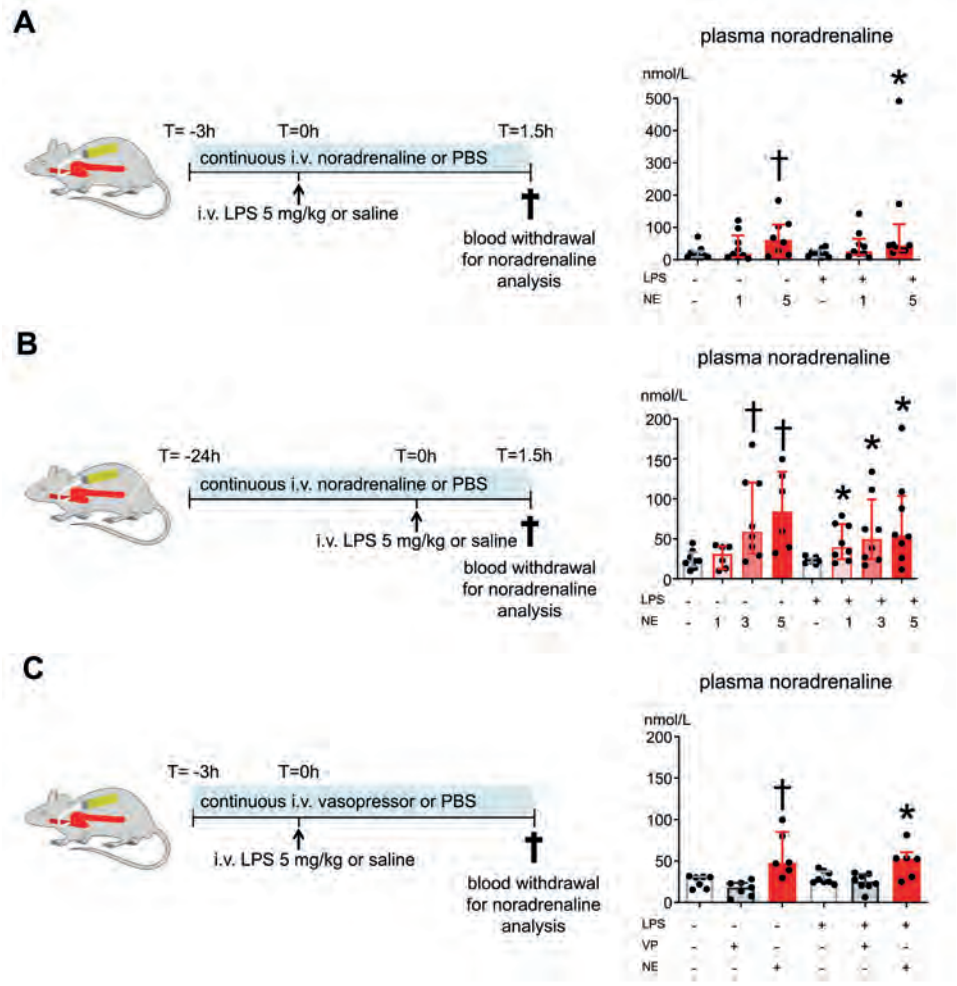


Supplementary Figure E2. **Noradrenaline modulates cytokine production by primary human monocytes.** Concentrations of TNF-α and IL-10 in supernatants of primary human monocyte cultures that were preincubated with either RPMI, noradrenaline (NE; 0.01, 0.1, 1, and 10 µM) or vasopressin (VP; 0.01, 0.1, 1, and 10 µM) for 1 h and subsequently stimulated with LPS (10 ng/mL) or RPMI for 24 h. Data are expressed as individual data points and mean ± SEM of 6 individual donors. †$p<0.05$ compared to RPMI; *$p<0.05$, compared to LPS, calculated using one-way analysis of variance with Dunnett's post hoc tests.

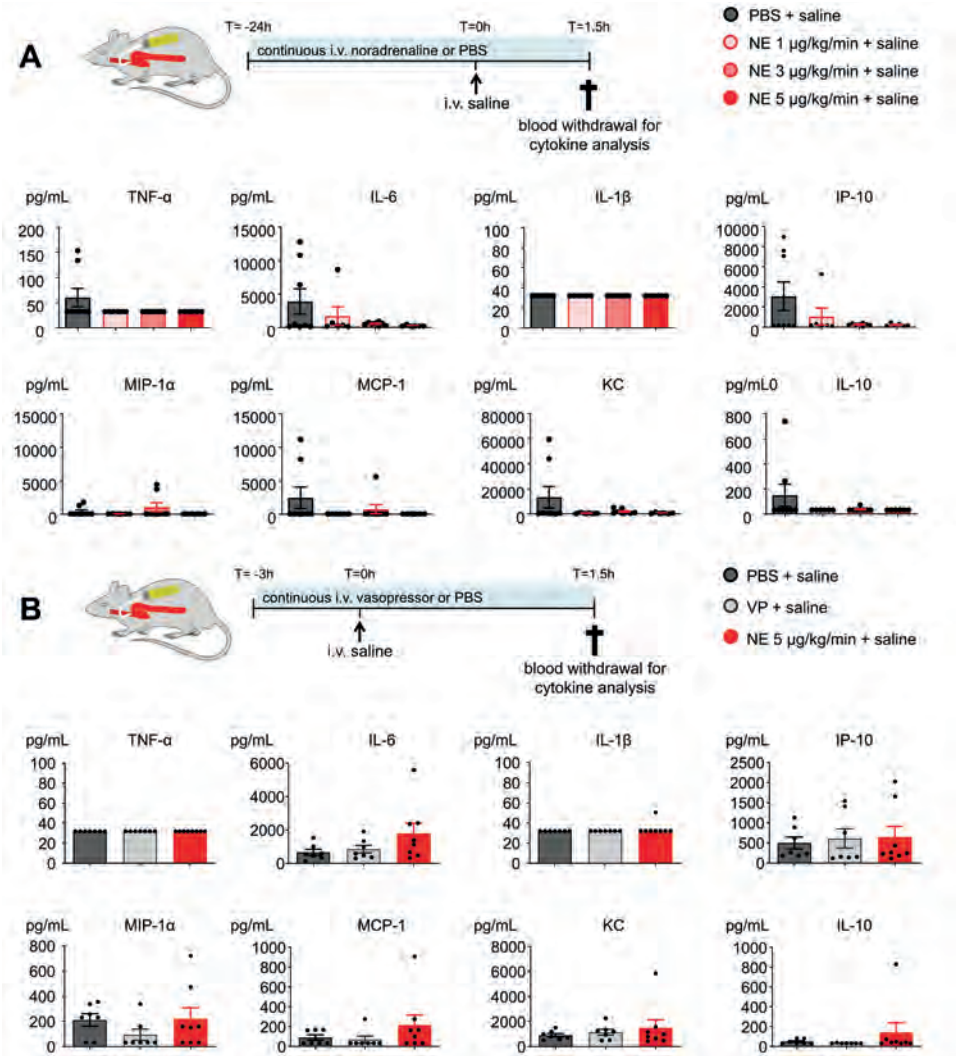


Supplementary Figure E3. Noradrenaline-induced modulation of cytokine production involves the β2 adrenoceptor and protein kinase A. (A-B) Concentrations of TNF-α, and IL-10 in supernatants of human whole blood cultures preincubated with either RPMI (medium control) or adrenoceptor antagonists prazosin (Praz, α1; 1 μM), yohimbine (Yoh, α2; 1 μM), propranolol (Prop, β; 1 μM), atenolol (Ate, β1; 0.1 μM) or ICI-118,551 (ICI, β2; 0.1 μM) for 30 min, followed by incubation with noradrenaline (NE; 1 μM) for 1 h and subsequent stimulation with LPS (10 ng/mL) or RPMI for 24 h. (C-D) Concentrations of

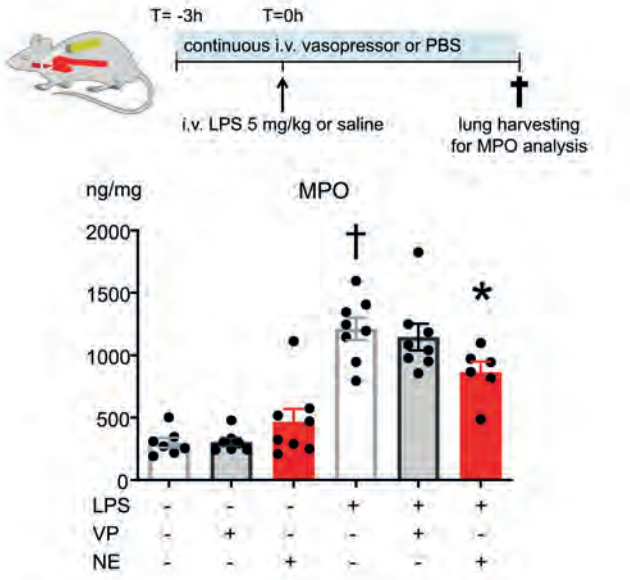
TNF-α, and IL-10 in supernatants of primary human monocyte cultures preincubated with either RPMI, ICI (0.1 μM) or the protein kinase A (PKA) inhibitor H89 (300 nM, 1 μM, and 3 μM) for 30 min, followed by incubation with NE (1 μM) for 1 h and subsequent stimulation with LPS (10 ng/mL) or RPMI for 24 h. Data are expressed as mean ± SEM of 6 individual donors. †p<0.05 compared to RPMI; ‡p<0.05 compared to LPS. *p<0.05, #p=0.05–0.10 compared to NE + LPS calculated using t-tests.



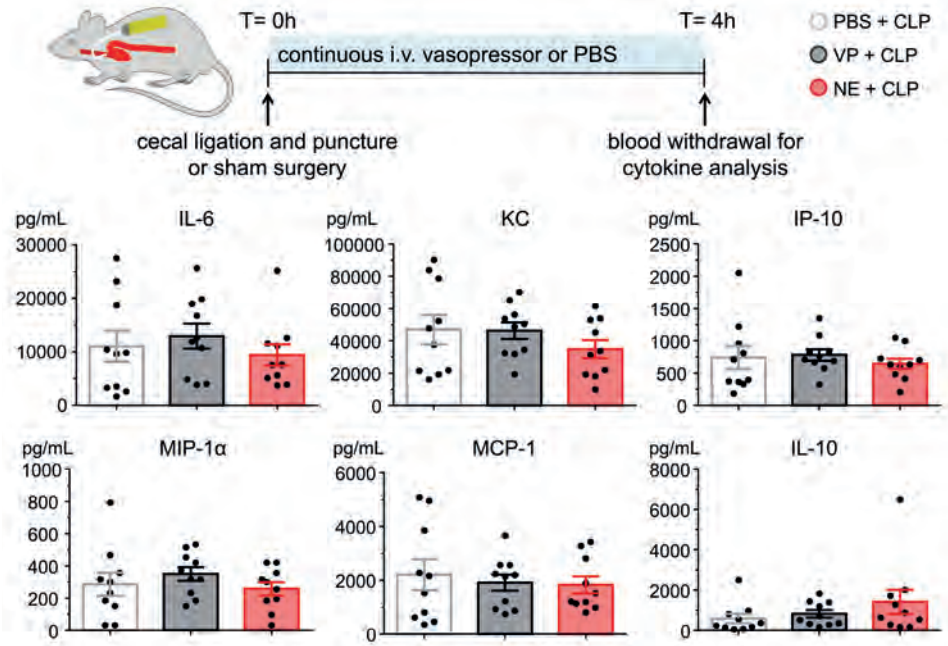
Supplementary Figure E4. Continuous intravenous noradrenaline infusion via micro-osmotic pumps results in increased plasma noradrenaline concentrations. (A-C) Plasma noradrenaline concentrations in mice intravenously infused with noradrenaline (NE; 1, 3 or 5 μg/kg/min), vasopressin (VP; 0.00057 IU/kg/min, equivalent to 0.04 IU/min in a 70 kg human) or phosphate-buffered saline (PBS) via a micro-osmotic pump connected to a jugular vein catheter for 4.5 (A,C) or 25.5 (B) h and challenged intravenously with LPS (5 mg/kg) or saline 3 (A-C) or 24 (B) h after start of infusion. Data are expressed as median and IQR of 6-8 animals per group. †p<0.05 compared to PBS + saline (LPS- / NE- / VP-); *p<0.05 compared to PBS + LPS (LPS+ / NE- / VP-) calculated using Mann-Whitney U tests.



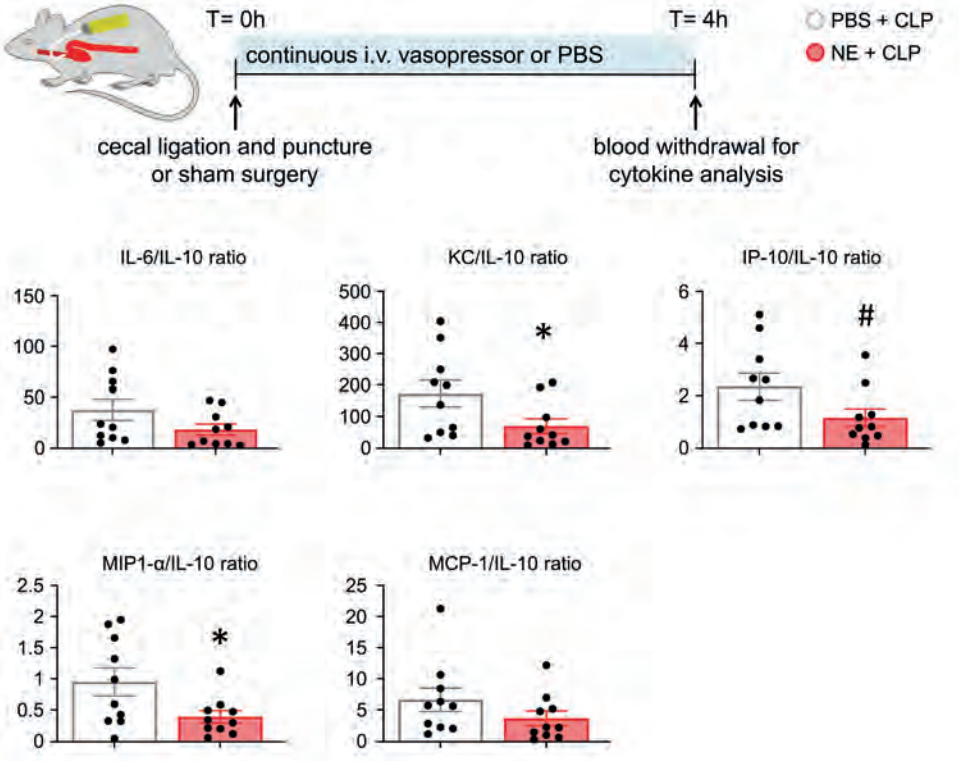
Supplementary Figure E5. Noradrenaline infusion does not affect plasma cytokine levels in non LPS-challenged mice. (A-B) Plasma concentrations of TNF-α, IL-6, IL-β, IP-10, MIP-1α, MCP-1, KC, and IL-10 in mice intravenously infused with noradrenaline (NE; 1, 3 or 5 µg/kg/min), vasopressin (VP; 0.00057 IU/kg/min, equivalent to 0.04 IU/min in a 70 kg human) or phosphate-buffered saline (PBS) via a micro-osmotic pump connected to a jugular vein catheter for 25.5 (A) or 4.5 (B) h and injected intravenously with saline 24 (A) or 3 (B) h after start of infusion. Data are expressed as mean ± SEM of 6-8 animals per group.



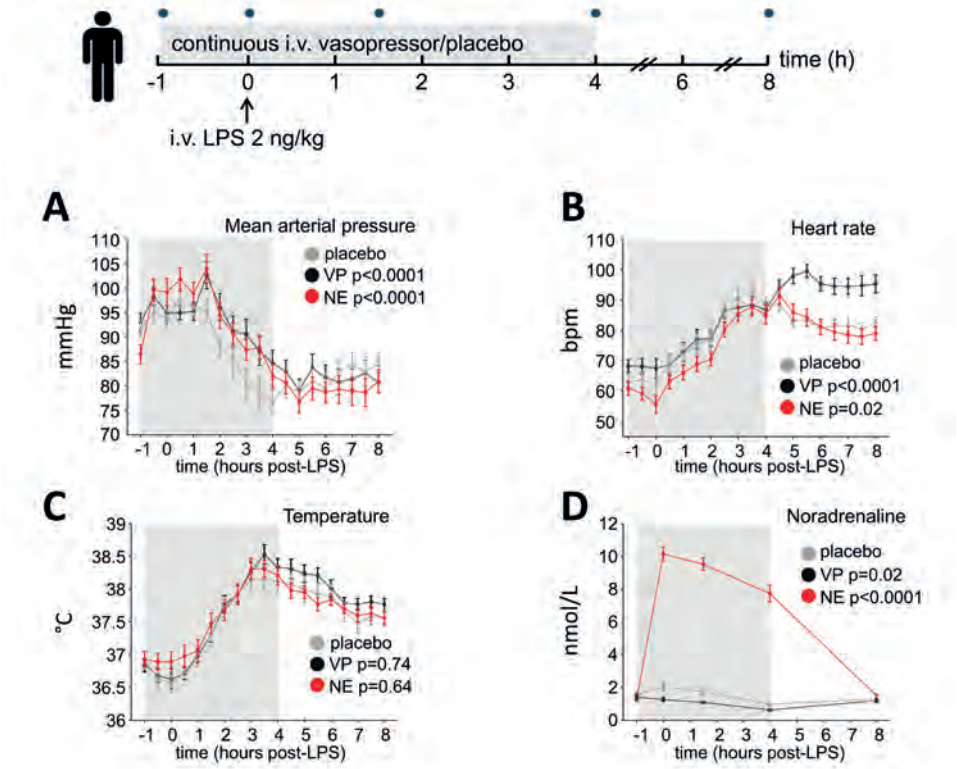
Supplementary Figure E6. Infusion of noradrenaline, but not vasopressin reduces pulmonary myeloperoxidase levels in LPS-challenged mice. Myeloperoxidase (MPO) levels in lung homogenates of mice infused with noradrenaline (NE; 5 µg/kg/min) vasopressin (VP; 0.00057 IU/kg/min, equivalent to 0.04 IU/min in a 70 kg human) or PBS via a micro-osmotic pump connected to a jugular vein catheter for 4.5 h and challenged intravenously with LPS (5 mg/kg) or saline 3 h after start of infusion. MPO levels were normalized to total protein content in the homogenates. Data are expressed as mean ± SEM for 6-8 animals per group. †p<0.05 compared to PBS + saline (LPS- / NE- / VP-); *p<0.05 compared to PBS + LPS (LPS+ / NE- / VP-) calculated using t-tests.



Supplementary Figure E7. Cytokine levels in CLP mice. Plasma concentrations of IL-6, KC, IP-10, MIP-1α, MCP-1, and IL-10 in mice intravenously infused with noradrenaline (NE; 3 µg/kg/min), vasopressin (VP; 0.00057 IU/kg/min, equivalent to 0.04 IU/min in a 70 kg human) or PBS via a micro-osmotic pump connected to a jugular vein catheter and subjected to cecal ligation and puncture to induce sepsis. Blood for cytokine analysis was obtained 4 h after induction of CLP. TNF-α concentrations were below the detection limit (3.2 pg/mL) in all animals. Data are expressed as mean ± SEM of 10 animals per group.



Supplementary Figure E8. Pro/anti-inflammatory cytokine balance in CLP mice. Plasma cytokine ratios (calculated using data presented in Supplementary Figure E7) in mice intravenously infused with noradrenaline (NE; 3 µg/kg/min), vasopressin (VP; 0.00057 IU/kg/min, equivalent to 0.04 IU/min in a 70 kg human) or PBS via a micro-osmotic pump connected to a jugular vein catheter and subjected to cecal ligation and puncture to induce sepsis. Blood for cytokine analysis was obtained 4 h after induction of CLP. Data are expressed as mean ± SEM of 10 animals per group. *p<0.05, #p=0.05-0.10 compared to PBS + CLP calculated using t-tests.



Supplementary Figure E9. Hemodynamics, body temperature and noradrenaline plasma concentrations during experimental human endotoxemia. (A) Mean arterial pressure, (B) heart rate (C) body temperature, and (D) noradrenaline plasma concentrations in healthy volunteers randomized to a 5 h intravenous infusion with either saline (placebo) low-dose noradrenaline (NE; 0.05 µg/kg/min) or vasopressin (VP; 0.04 IU/min) and challenged intravenously with 2 ng/kg LPS 1 h after start of infusion. Data are expressed as mean ± SEM of 10 subjects per group. The blue dots in the study design indicate blood withdrawal timepoints for noradrenaline analysis. The grey area represents the period during which NE, VP, or placebo was infused. p-values were calculated using repeated measures two-way analysis of variance (time*treatment interaction term) vs. the placebo groups.

Supplementary Table E1. Baseline demographic characteristics of healthy volunteers enrolled in the experimental endotoxemia study.

	Noradrenaline (n=10)	Vasopressin (n=10)	Placebo (n=10)	
Age (years)	21 [20-22]	23 [21-25]	23 [22-25]	p = 0.07
BMI (kg/m²)	23.7 [21.5-24.9]	23.6 [21.9-25.2]	20.0 [19-22]	p = 0.05
HR (bpm)	61 [56-64]	69 [62-73]	62 [56-68]	p = 0.13
MAP (mmHg)	86 [83-89]	92 [88-97]	92 [89-95]	p = 0.06

Parameters measured at the start of the endotoxemia experiment day (before start of vasopressor/placebo infusion). Data are presented as median [interquartile range], p-values calculated using Kruskall-Wallis tests. BMI body mass index; HR heart rate; MAP mean arterial pressure.

Supplementary Table E2. Characteristics of septic shock patients enrolled in the observational cohort study.

	Noradrenaline (n=129)	Noradrenaline + β-blocker (n=66)	
Sex (% male)	54%	60%	p = 0.40
Age (years)	64 [41.5 – 88.5]	68 [52-84]	p = 0.17
APACHE II score	22 [12 – 32]	23.5 [12.8 – 34]	p = 0.34
Use of corticosteroids*	41 (32%)	23 (35%)	p = 0.66
Source of infection			p = 0.80
Urinary	7 (5%)	5 (8%)	
Respiratoy	40 (31%)	20 (30%)	
Abdominal	26 (20%)	16 (24%)	
Other#	56 (43%)	25 (38%)	
Mechanical ventilation	97 (75%)	51 (77%)	p = 0.82
NE infusion rate at inclusion (mcg/kg/min)	0.20 [0.1 – 0.5]	0.16 [0.1 – 0.3]	p = 0.17

Parameters determined within the first 24 hours after ICU admission. Data are presented as median [interquartile range] or number (percentage). P values calculated using Mann-Whitney U-tests and Chi-square tests. *Corticosteroids were either administered as part of the shock treatment protocol (hydrocortisone 3x100 mg/day, n=29; 21 [16%] in the noradrenaline-only group and 8 [12%] in the noradrenaline + β-blocker group) or in a 'stress' dose if patients received chronic corticosteroid therapy, varying from 20 to 100 mg/day prednisone (equivalent) (n=35; 20 [16%] in the noradrenaline-only group and 15 [23%] in the noradrenaline +β-blocker group). The distribution of corticosteroids used (shock treatment and stress dose) was similar between the two groups (p=0.40). #Other sources of infection include, blood, skin, bones and joints and central nervous system.

CHAPTER 5

COMMON β2-ADRENERGIC RECEPTOR POLYMORPHISMS DO NOT AFFECT NORADRENALINE-INDUCED IMMUNOSUPPRESSION EX VIVO OR THE SYSTEMIC INFLAMMATORY RESPONSE IN VIVO

Submitted.

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ABSTRACT

BACKGROUND

Genetic variation is an important determinant of the host response in sepsis patients. Moreover, a specific genetic variant in the β2-adrenergic receptor was associated with increased mortality in sepsis. Recently, it was discovered that the vasopressor noradrenaline, ubiquitously used in septic shock patients, exerts profound anti-inflammatory effects and may contribute to sepsis-induced immunoparalysis via stimulation of the β2-adrenergic receptor. In the present study, we evaluated whether common non-synonymous variants (individual single nucleotide polymorphisms [SNPs] or SNP haplotypes) in the β2-adrenergic receptor render subjects more susceptible for noradrenaline-induced immunosuppression and whether they are associated with a dysregulated systemic inflammatory response in vivo.

METHODS

Peripheral blood mononuclear cells (PBMCs) of 109 healthy volunteers (52 female, 57 male) were ex vivo stimulated with 10 ng/mL lipopolysaccharide (LPS) in the presence and absence of 1 μM noradrenaline, after which production of cytokines TNF, IL-6 and IL-10 was assessed. The same cohort of volunteers subsequently underwent experimental endotoxemia (intravenous administration of 1 ng/kg LPS), during which plasma concentrations of TNF, IL-6, IL-8, IL-10, IP-10, G-CSF, MCP-1, IL1-RA, and MIP-1α were measured. Furthermore, mean arterial pressure, heart rate, and temperature were recorded. Subjects were genotyped and common SNPs in the ADRB2 gene were extracted (rs1042711, rs1042713 and rs1042714). Furthermore, the presence of common haplotypes of these SNPs was assessed (CysGlyGln, CysArgGln, and ArgGlyGlu). Linear regression models were used to evaluate associations between polymorphisms and ex vivo as well as in vivo cytokine production, and hemodynamic parameters and temperature.

RESULTS

Noradrenaline attenuated production of the pro-inflammatory cytokines TNF and IL-6 (-26% (-22% to-30%), and-14% (-9% to-18%), respectively, both $p<0.0001$), and enhanced release of the anti-inflammatory mediator IL-10 (+9% (+3% to +15%), $p=0.003$) by ex vivo-stimulated PBMCs, but this was not affected by the presence of ADRB2 SNPs or haplotypes (all p-values->0.37). In addition, no influence of SNPs or haplotypes on in vivo cytokine levels, hemodynamic parameters, and temperature were observed (all p-values->0.14).

CONCLUSION

Common non-synonymous variants in the ADRB2 gene do not influence noradrenaline-mediated immunosuppression ex vivo or the systemic inflammatory and hemodynamic response induced by LPS administration in healthy volunteers.

INTRODUCTION

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (1). Despite substantial improvements in healthcare over the past decades, sepsis mortality remains high (2, 3). It represents the number one cause of death on the intensive Care, accounting for 6 million casualties annually (4), which has prompted the WHO to designate sepsis as a global health priority (5). One of the greatest challenges in sepsis research is to gain more insight into the large heterogeneity observed among sepsis patients. It has become apparent that the nature of the dysregulated host response differs greatly between sepsis patients, varying from hyperinflammation to profound immunosuppression, a phenomenon also known as sepsis-induced immunoparalysis. Attempts to reduce mortality by modulating the host response have invariably been disappointing, in large part because these immunomodulatory interventions were applied to unselected patients, irrespective of their immunological status or phenotype (6). Genetic variation (in the form of single nucleotide polymorphisms, SNPs) has been shown to be an important determinant of the host response in healthy subjects (7) as well as in sepsis patients (8). Furthermore, SNPs in several genes were shown to be associated with outcome in sepsis (9).

Recently, we discovered that the catecholamine and vasopressor noradrenaline, the primordial supportive treatment for septic shock patients, exerts substantial anti-inflammatory effects and may thereby significantly contribute to the development of sepsis-induced immunoparalysis (10). Noradrenaline's immunosuppressive effects were shown to be primarily mediated via the β2-adrenergic receptor, which is encoded by the *ADRB2* gene located on chromosome 5q31/32. *ADRB2* is a small intronless gene, in which several non-synonymous common SNPs have been identified, namely *Arg19Cys (rs1042711)*, which is located at the 5' untranslated region as well as *Gly16Arg (rs1042713)* and *Glu27Gln (rs1042714),* located in the extracellular N-terminus of the receptor. Reports on the functional consequences of these SNPs are conflicting. *In vitro* studies have suggested that the *Arg19Cys* SNP reduces β2-adrenergic receptor expression compared to wildtype (11), that *Gly16Arg* is associated with enhanced agonist-induced desensitization (12), whereas *Glu27Gln* is associated with resistance to desensitization (13). A haplotype of the aforementioned SNPs (the CysGlyGln haplotype) was shown to be associated with a slight reduction of isoproterenol (a β-agonist)-induced inhibition of *ex vivo* CD3/CD28-stimulated lymphocytic IL-5 production (14). Conversely, in isoproterenol-stimulated lymphocytes (15) and peripheral blood mononuclear cells (PBMCs)(16), *ADRB2* polymorphisms *Gly16Arg* and *Glu27Gln* did not affect production of cAMP, the intracellular messenger molecule downstream of the β2-adrenergic receptor which was shown to be critical for its anti-inflammatory effects (10). *In vivo* data on these SNPs are also equivocal and not congruent with the functional consequences observed

in the *in vitro* studies. For instance, reduced vascular desensitization upon repeated isoproterenol infusion was observed in carriers of *Gly16Arg* (17), whereas subjects homozygous for *Glu27Gln* exhibited reduced maximal venodilation (18). In septic shock, a condition characterized by profound adrenergic stimulation through high endogenous catecholamine and exogenous noradrenaline levels (19), the presence of the CysGlyGln haplotype in *ADRB2* was associated with a higher 28 day mortality in two independent cohorts (20). Possibly, modulation of immunological effects exerted by endogenous or exogenous catecholamines could play a role in this association, because partial reversal of the noradrenaline-induced reduction of IL-6 production by a lymphoblastoid cell line upon mixed inflammatory stimulation was observed in carriers of this haplotype (20). Immunomodulatory effects of the aforementioned separate SNPs or other common haplotypes in *ADRB2* (CysArgGln and ArgGlyGlu) have not been investigated. Elucidating the possible immunologic influence of common *ADRB2* variants is important, as this knowledge could be used to identify patients more or less vulnerable towards anti-inflammatory effects of catecholamines and facilitate precision medicine.

In the present study, we primarily investigated whether the presence of common SNPs and haplotypes in the *ADRB2* gene influence the previously identified immunosuppressive effects of noradrenaline in lipopolysaccharide (LPS)-stimulated leukocytes obtained from a large cohort of healthy volunteers. Second, in the same cohort, we assessed whether these variants are associated with an altered *in vivo* immune, hemodynamic, and fever response during experimental human endotoxemia, a model of systemic inflammation induced by intravenous administration of LPS. This model captures various hallmarks of early sepsis (21) and leads to profoundly elevated endogenous catecholamine levels (22).

METHODS

SUBJECTS AND ETHICS

One-hundred-and-thirteen male (n=58) and female (n=55) healthy volunteers provided written informed consent to participate in this interventional cohort study (**Figure 1**). All procedures were approved by the local ethics committee of the Radboud University Medical Center (registration numbers NL68166.091.18 and 2018-4983) and the study was performed in accordance with the declaration of Helsinki, including latest revisions.

EX VIVO STIMULATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Before LPS administration (see next section), peripheral blood mononuclear cells (PBMCs) were isolated from ethylenediaminetetraacetic acid (EDTA)- anticoagulated venous blood using Ficoll (Ficoll-Paque Plus, GE healthcare, Chicago, USA) density gradient centrifugation (10 min, 1200 g at room temperature [RT]) in SepMateTM

tubes (STEMCELL Technologies, Vancouver, Canada). All experiments were performed in duplicate. PBMC's (5 x 10^5 per well) were incubated in 96-well plates (Eppendorf) with LPS (10 ng/mL, serotype 055:B5, Sigma-Aldrich) with or without noradrenaline (1 mM, Centrafarm BV, Etten-Leur, The Netherlands). The concentration of noradrenaline was based on our previous work(10). All stimuli were prepared in one batch before the study commenced and stored at -80°C until use. After 24 h of incubation at 37°C, 5% CO_2, plates were centrifuged (8 min, 1400 rpm, RT) and supernatants were stored at -80°C until analysis. TNF, IL-6, and IL-10 were measured in supernatants using enzyme-linked immunosorbent assays (ELISA, Duosets, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

IN VIVO EXPERIMENTAL HUMAN ENDOTOXEMIA

Human endotoxemia experiments were conducted at the research unit of the Intensive Care department of the Radboud university medical center according to our standard protocol (21). Subjects refrained from caffeine and alcohol in the 24 h before the experiment and from food and drinks 10 h before the experiment. One venous canulae was placed in the antecubital vein, for fluid infusion and endotoxin administration. Subjects were pre-hydrated in the hour before LPS administration (1.5 L 2.5% glucose/0.45% saline) followed by 150 mL/h for 6 h (until the end of the experiment). Blood pressure monitoring and blood withdrawals were performed using an arterial cannula placed in the radial artery. In addition, ECG was continuously monitored and vital signs were recorded every 5 seconds on a Phillips MP50 patient monitor using an in-house developed data capturing system. Body temperature was measured every 30 mins with infrared tympanic measurements (FirstTemp Genius 2, Covidien Geldermalsen, the Netherlands). At T=0, 1 ng/kg bodyweight LPS (*E. coli* type O113, Lot no. 94332B1; List Biological Laboratories, Campbell, USA) was administered intravenously to elicit a transient systemic inflammatory response.

PLASMA CYTOKINES

For cytokine analysis, blood was collected in EDTA tubes (Vacutainer, BD) one hour before LPS administration (T=-1 hours) as well as at T=0, T=0.5, T=1, T=1.5, T=2, T=3, T=4, and T=6 hours. Tubes were immediately centrifuged at 2000 g at 4 °C for 10 min. Plasma was then stored at -80°C until levels of TNF, IL-6, IL-8, IL-10, IP-10, G-CSF, MCP-1, IL1-RA, and MIP-1α were measured in one batch using a Luminex assay (Milliplex, Millipore, Burlington, USA).

GENOTYPING, QUALITY CONTROL AND IMPUTATION

All 113 participants were genotyped using the commercially available Illumina Infinium Global Screening Array MD version 3. The genotype calling was performed using Genome Studio 2.0. Quality control was performed in PLINK (v1.90b6.21) (23). Samples with a

call rate below 90% and variants missing in more than 10% of the samples or with a minor allele frequency (MAF) below 0.01 were discarded. Strands and variant-identifiers were aligned to the 1000Genome project (24) using the GenotypeHarmonizer (v1.4.23) (25). By combining our samples with the genotypes from the 1000Genome project and creating multi-dimensional scaling plots, 4 individuals were identified as ethnic outliers and excluded for analysis. This resulted in a dataset comprising 109 participants and 469.102 variants, from which genotypes for *rs1042711*, *rs1042713*, and *rs1042714* were extracted and used for analysis.

STATISTICAL ANALYSIS

Distribution of data was determined using Shapiro-Wilk tests. Data are presented as geometric mean with 95% confidence intervals (95% CI), or median and range for baseline characteristics. Baseline characteristics were analyzed using chi-square tests for categorical data and Kruskal-Wallis tests for continuous data. Noradrenaline-induced effects on *ex vivo* cytokine production were analyzed using paired t-tests on logarithmically transformed data. Associations between genotypes and outcome data were analyzed using linear regression models adjusted for sex, on logarithmically transformed data. To correct for multiple testing, p-values obtained from the regression models were adjusted using the false discovery rate (FDR) method according to Benjamini and Hochberg (26). For the analysis of *in vivo* cytokine responses, area under curve plasma cytokine levels were used as dependent variables in the regression models, as these represent an integral measure of the *in vivo* cytokine production over time in response to LPS administration. Three subjects were inadvertently administered a lower dose of LPS due to a faulty vial and were therefore excluded for the *in vivo* analyses. These subjects carried the TC/CG, TC/CG, and TT/CC genotype for the *rs1042711/rs1042714* SNPs (which were in complete linkage disequilibrium, see results section) and the GA, GA, and GG genotype for the *rs1042713* SNP, but were not carriers of any of the three haplotypes investigated in the present study. A p value < 0.05 was considered significant. Analyses were performed using GraphPad Prism 8 (Graphpad software, San Diego, USA), SPSS 25 (IBM statistics, NY, USA), and R (version 3.6.3).

RESULTS

BASELINE CHARACTERISTICS

A graphical overview of the study is provided in **Figure 1**. Demographic characteristics of the subjects are listed in **Table 1**. *rs1042711* and *rs1042714* were in complete linkage disequilibrium (r^2=1.0) in our population and will henceforth be described as the *rs1042711/14* SNP. Carriers of the TT/CC genotype of *rs1042711/14* were more likely male (69% vs 43% p=0.04) compared to the other genotypes, but no other differences

were observed between genotypes of *rs1042711/14* or *rs1042713*. Thirteen subjects (12%) were homozygous for Arg19/Glu27 (reference alleles), whereas 36 subjects (33%) were homozygous for Cys19/Gln27, and 60 (55%) were heterozygous. Forty (36%) subjects were homozygous for the reference allele encoding Gly16, 56 (51%) were heterozygous and 13 (12%) were homozygous for the allele encoding Arg16. In addition, three haplotypes were present within our cohort: ArgGlyGlu (13 subjects) and CysArgGln (13 subjects), and these haplotypes were mutually exclusive. Three subjects had the CysGlyGln haplotype. No differences in baseline characteristics were present between these haplotypes (**Table 2**).

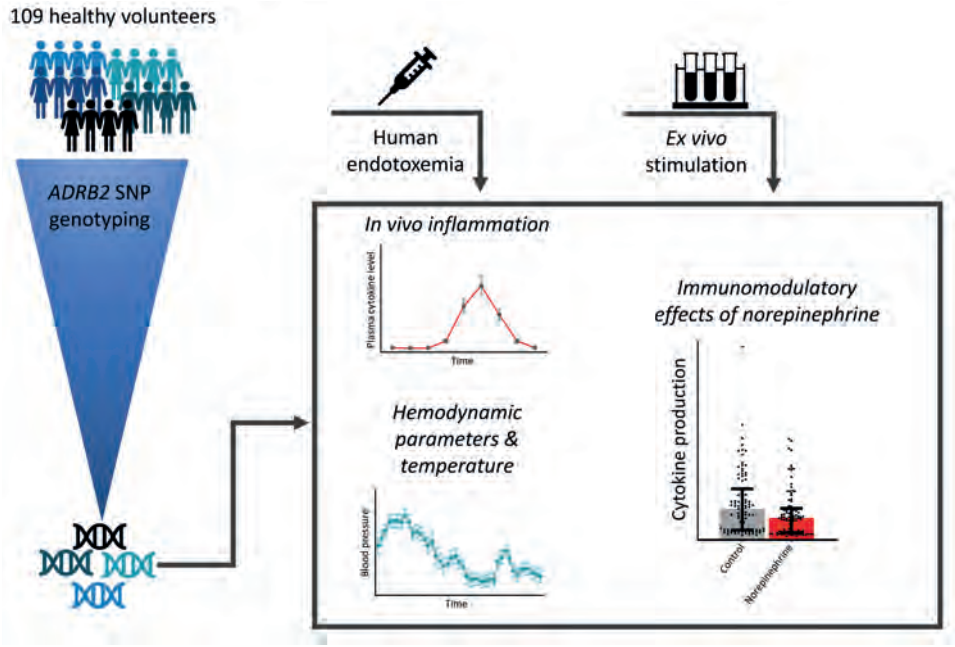

Figure 1. Schematic overview of this study from the 100LPS cohort. One-hundred-and-nine volunteers were genotyped for three SNPs in the *ADRB2* gene. We explored associations between these polymorphisms and *in vivo* cytokine levels as well as hemodynamic and temperature responses during experimental human endotoxemia, and noradrenaline-mediated modulation of cytokine production by peripheral blood mononuclear cells *ex vivo* stimulated with LPS.

Table 1.

	rs1042711/ rs1042714 (Arg19Cys [C>T] / Glu27Gln [G>C])				rs1042713 (Gly16Arg [G>A])			
	CC/GG (n=13)	TC/CG (n=60)	TT/CC (n=36)	P value	GG (n=40)	GA (n=56)	AA (n=13)	P value
Age, years	24 (19-29)	22 (18-33)	23 (20-30)	0.23	22 (18-30)	23 (19-33)	24 (19-26)	0.46
Male sex, n (%)	6 (46)	26 (43)	25 (69)	0.04	17 (43)	33 (59)	7 (54)	0.28
BMI, kg/m²	23.6 (19.3-31.9)	22.8 (17.7-31.5)	23.2 (19.5-31.3)	0.09	23 (19-31.5)	23.5 (17.7-30.3)	24.6 (20-31.9)	0.17

Parameters were measured during screening visit. Data are presented numbers (%) or median with range. P values were calculated using the Kruskall-Wallis test. BMI: Body Mass Index.

Table 2.

	CysGlyGln	ArgGlyGlu	CysArgGln	
	TTGGCC (n=3)	CCGGGG (n=13)	TTAACC (n=13)	P value
Age, years	23 (24-29)	24 (21-27)	23 (19-27)	0.28
Sex, %male	1 (33)	7 (54)	6 (46)	0.79
BMI, kg/m²	23.5 (21.5-27.7)	24.6 (19.35-29.9)	23.2 (17.7-28.7)	0.69

Parameters were measured during screening visit. Data are presented numbers (%) or median with range. P values were calculated using the Kruskall-Wallis test. BMI: Body Mass Index.

ADRB2 POLYMORPHISMS DO NOT INFLUENCE NORADRENALINE-INDUCED MODULATION OF LPS-INDUCED CYTOKINE PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR CELLS.

To explore whether presence of the *ADRB2* SNPs affected noradrenaline-induced modulation of cytokine production, PBMCs were stimulated with LPS in the presence and absence of noradrenaline for 24 hours, after which the production of the pro-inflammatory cytokines TNF and IL-6, and the anti-inflammatory cytokine IL-10 was assessed. In accordance with our previous study (10), noradrenaline attenuated LPS-induced TNF and IL-6 production (-26% (-22% to -30%), and -14% (-9% to -18%), respectively, both p<0.0001), while release of IL-10 was enhanced (+9% (+3% to +15%), p=0.003, **Figure 2A**). However, neither individual SNPs nor haplotypes in the *ADRB2* gene influenced the immunomodulatory effects of noradrenaline (**Figure 2B, Tables 3-4**). Furthermore, no influence of individual SNPs or haplotypes on the noradrenaline-induced decrease of the TNF/IL-10 or IL-6/IL-10 ratios was observed (data not shown).

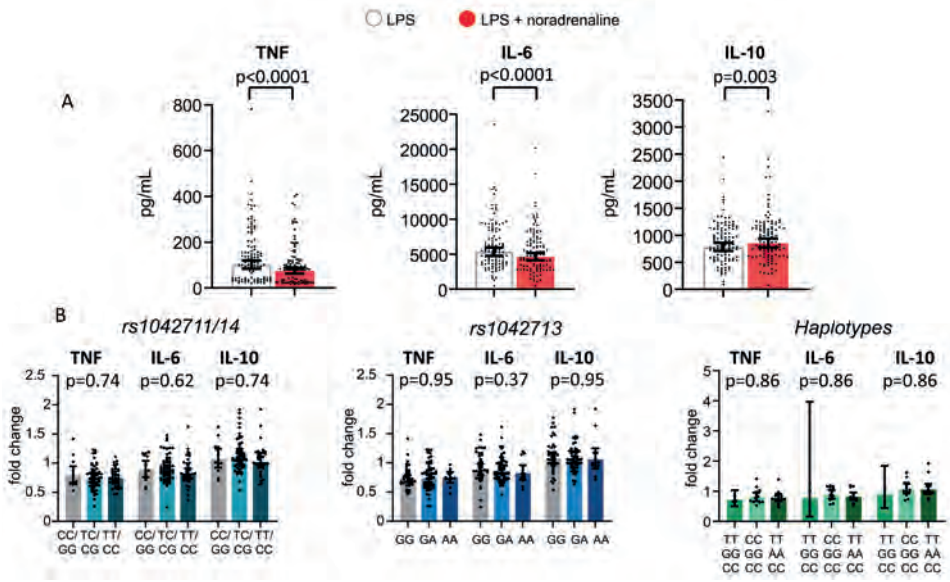

Figure 2. *ADRB2* polymorphisms do not influence noradrenaline-induced modulation of LPS-induced cytokine production by peripheral blood mononuclear cells. (A) Concentrations of TNF, IL-6 and IL-10 in supernatants of peripheral blood mononuclear cells of 109 subjects that were stimulated *ex vivo* with lipopolysaccharide (LPS 10 ng/mL) in the presence or absence of noradrenaline (NE, 1 µM). (B) Noradrenaline-induced fold changes in production of TNF, IL-6 and IL-10 in supernatants of peripheral blood mononuclear cell cultures according to SNP genotype or haplotype (rs1042711/rs1042714 CC/GG: n=13, TC/CG: n=60, TT/CC: n=36; rs1042713 GG: n=40, GA: n=56, AA: n=13; CysGlyGln [TTGGCC]: n=3, ArgGlyGlu [CCGGGG]: n=13, CysArgGln [TTAACC]: n=13). Data are expressed as individual datapoints with geometric mean and 95% CI. P-values were calculated using paired t-tests on log transformed data (A) or linear regression analysis on log transformed data with SNP or haplotype and sex as covariates and FDR correction was applied (B).

ADRB2 POLYMORPHISMS DO NOT AFFECT THE IN VIVO CYTOKINE RESPONSE INDUCED BY INTRAVENOUS LPS ADMINISTRATION

During endotoxemia endogenous catecholamine levels increase and subsequently can influence the immune response (22), an effect which may be modulated by the presence of *ADRB2* SNPs/haplotypes. Therefore, we investigated the effects of the presence of these SNPs and haplotypes on the systemic inflammatory response induced by intravenous LPS administration, quantified by plasma cytokine levels. LPS administration induced a marked increase in plasma concentrations of TNF, IL-6, IL-8, IP-10 and IL-10 and IL-1RA, MIP-1α, MCP-1 and G-CSF in all subjects (**Figure 3 A-E** and **supplemental Figure 1 A-E**, respectively). However, individual *ADRB2* SNPs or haplotypes did not affect any of these cytokine responses (**Figure 3 F-J**, **supplemental Figure 1 F-J**, **supplemental Figure 2**, **Tables 3-4**) .

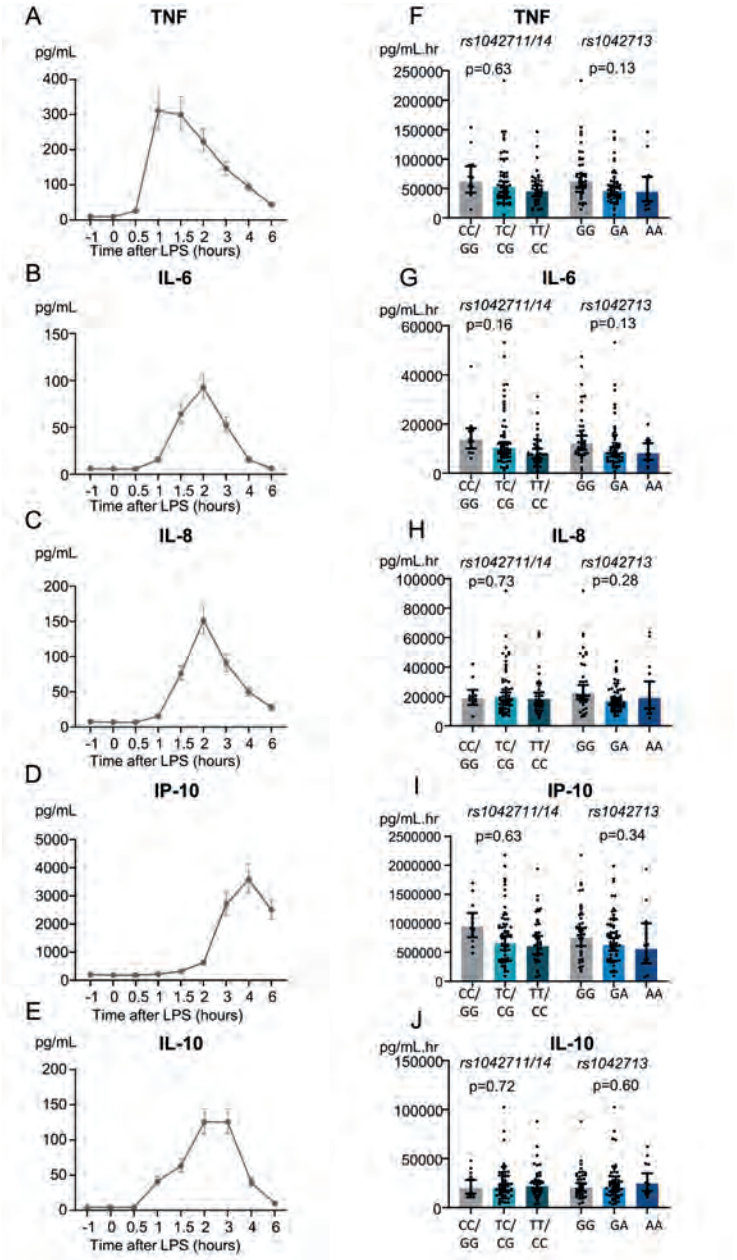

Figure 3. ADRB2 polymorphisms do not affect the in vivo cytokine response induced by intravenous LPS administration. Plasma concentrations of TNF, IL-6, IL-8, IP-10 and IL-10 during experimental human endotoxemia in 106 subjects (A-E) and area under curve cytokine responses (F-J) according to SNP genotype (rs1042711/rs1042714 CC/GG: n=13, TC/CG: n=58, TT/CC: n=35; rs1042713 GG: n=39, GA: n=54, AA: n=13). Data are expressed as geometric mean and 95% CI (A-E) or individual datapoints with geometric mean and 95% CI (F-J). P-values were calculated using linear regression analysis on log transformed data with SNP and sex as covariates and FDR correction was applied. Data of other cytokines (IL-1RA, MCP-1, MIP-1α and G-CSF) are provided in Supplementary Figure 1. All cytokine responses according to haplotype are provided in Supplementary Figure 2.

ADRB2 POLYMORPHISMS ARE NOT ASSOCIATED WITH ALTERATIONS IN THE HEMODYNAMIC OR TEMPERATURE RESPONSE INDUCED BY INTRAVENOUS LPS ADMINISTRATION

Heart rate and mean arterial blood pressure showed a typical pattern for human endotoxemia: a gradual decrease in MAP (maximum decrease of 19% (18% to 20%), **Figure 4A**) and a compensatory increase in heart rate (maximum increase of 37% (37% to 38%), **Figure 4B**). Furthermore, LPS administration resulted in an increase in body temperature (maximum increase of 1.0 ^{O}C (0.7 ^{O}C to 1.3 ^{O}C), **Figure 4C**). Again, no influence of individual SNPs or haplotypes in the *ADRB2* gene on these hemodynamic and temperature responses were observed **(Figure 4D-F**, **supplemental Figure 3**, **Tables 3-4**).

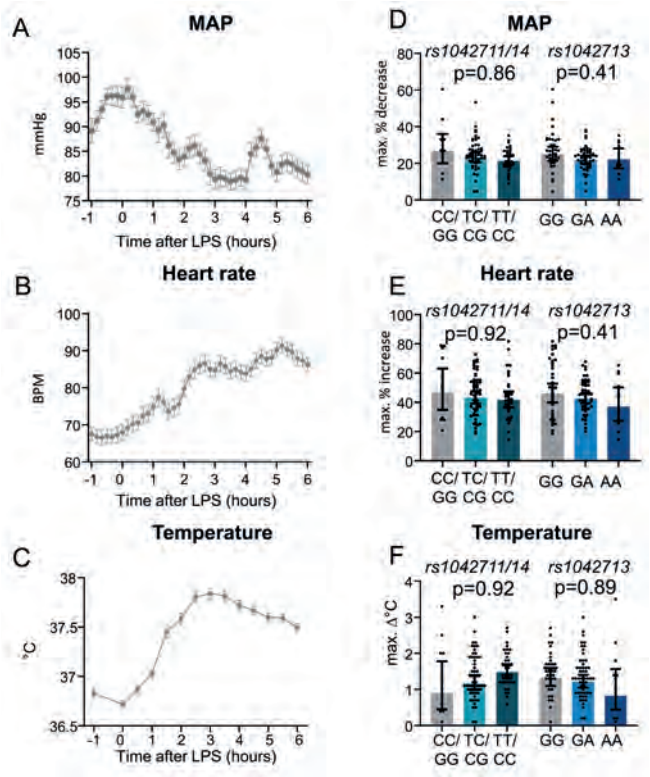

Figure 4. ADRB2 polymorphisms are not associated with alterations in the hemodynamic or temperature response induced by intravenous LPS administration. Mean arterial pressure (A), heart rate (B), and body temperature (C) during experimental human endotoxemia in 106 subjects, and percentage changes in mean arterial pressure (D) and heart rate (E), and changes in body temperature (F) according to SNP genotype (rs1042711/rs1042714 CC/GG: n=13, TC/CG: n=58, TT/CC: n=35; rs1042713 GG: n=39, GA: n=54, AA: n=13). Data are expressed as geometric mean and 95% CI (A-C) or individual datapoints with geometric mean and 95% CI (D-F). P-values were calculated using linear regression analysis on log transformed data with SNP and sex as covariates and FDR correction was applied. Hemodynamic and temperature responses according to haplotype are provided in Supplementary Figure 3.

Table 3.

	rs1042711/ rs1042714 (Arg19Cys [C>T] / Glu27Gln [G>C])		rs1042713 (Gly16Arg [G>A])	
Ex vivo cytokine production	**P-value**	**Standardized β**	**P-value**	**Standardized β**
TNF	0.74	-0.33	0.95	0.027
IL-6	0.62	0.134	0.37	-0.160
IL-10	0.74	-.071	0.95	0.006
In vivo plasma cytokine levels				
TNF	0.63	-0.103	0.13	-0.179
IL-6	0.16	-0.203	0.13	-0.184
IL-8	0.73	0.034	0.28	-0.126
IL-10	0.72	0.047	0.60	0.052
IL1-RA	0.11	-0.225	0.10	-0.224
MCP-1	0.69	-0.063	0.48	0.084
MIP-1a	0.69	-0.083	0.21	-0.155
IP-10	0.63	-0.112	0.34	-0.105
G-CSF	0.69	-0.064	0.12	-0.211
Hemodynamic parameters & temperature				
MAP maximal delta	0.86	-0.108	0.414	-0.111
Heart rate maximal delta	0.92	-0.049	0.414	-0.126
Change degrees Celcius	0.92	-0.03	0.89	-0.014

Data were analyzed using sex-adjusted linear regression models. P-values are FDR-corrected.

Table 4.

	Haplotypes (CysGlyGLn. ArgGlyGlu. CysArgGln) (TTGGCC. CCGGGG. CCAAGG)	
Ex vivo cytokine production	**P-value**	**Standardized β**
TNF	0.86	-0203
IL6	0.86	-0.107
IL10	0.86	-0.042
In vivo cytokine levels		
TNF	0.69	-0.110
IL6	0.49	-0.231
IL8	0.49	0.200
IL10	0.49	0.332
IL1RA	0.49	-0.205
MCP-1	0.49	-0.192
MIP-1α	0.92	0.042
IP-10	0.49	-0.272
G-CSF	0.92	0.019
Hemodynamic parameters & temperature		
MAP maximal delta	0.793	-0.079
Heart rate maximal delta	0.793	0.055
Change degrees Celcius	0.793	0.083

Data were analyzed using sex-adjusted linear regression models. P-values are FDR-corrected.

DISCUSSION

In the present study, we investigated whether non-synonymous polymorphisms in the β2-adrenergic receptor gene *ADRB2* affect noradrenaline's immunomodulatory effects in *ex vivo* LPS-stimulated leukocytes. Furthermore, we explored whether these polymorphisms were associated with altered cytokine, hemodynamic, and temperature responses following LPS administration *in vivo*. We demonstrate that neither is the case. There have been conflicting results from *in vitro* and *ex vivo* studies on the functionality of *ADRB2* polymorphisms. For example, it was reported that Gly16 and Glu27 variants in human lung mast cells were resistant to isoproterenol desensitization compared to the Arg16 and Gln27 variants, when examining histamine production after IgE stimulation (inhibition of histamine was demonstrated to be a β-adrenergically driven effect) (27), suggesting these variants are associated with reduced sensitivity for adrenergic stimulation. In contrast, when examining intracellular production of cAMP, the intracellular messenger molecule induced by β-adrenergic receptor stimulation, in lymphocytes or PBMCs of asthma patients upon *ex vivo* isoproterenol stimulation, no influence of the presence of these polymorphisms was found (15, 16). The results from our *ex vivo* stimulation experiments in PBMCs also reveal no effects of the presence of individual *ADRB2* SNPs on the previously established β2-adrenergic receptor-mediated immunomodulatory effects of noradrenaline, i.e. attenuation of pro-inflammatory cytokine production and enhanced production of the anti-inflammatory cytokine IL-10, which were shown to be cAMP-dependent (10).

Other *in vitro* and *ex vivo* investigations have focused on the effects of the presence of several haplotypes of *ADRB2*, with equivocal results as well. An examination of the three haplotypes which were also assessed in the present study in lymphocytes of non-asthmatic, nonallergic subjects showed no association with β2-adrenergic receptor expression, but found increased desensitization upon repeated isoproterenol-induced cAMP production for the CysGlyGln haplotype compared to the CysArgGln and ArgGlyGlu haplotypes, suggestive of enhanced signaling (14). However, isoproterenol-mediated inhibition of IL-5 production after CD3/CD28 stimulation was attenuated in individuals carrying the CysGlyGln haplotype (14), as was suppression of IL-6 release by noradrenaline in a lymphoblastoid cell line after mixed inflammatory stimulation (20), suggesting rather impaired adrenergic signaling in subjects bearing the CysGlyGln haplotype. A similar examination in PBMCs derived from several asthmatic patient cohorts demonstrated no influence of several haplotypes, including CysGlyGln, on β2-adrenergic receptor expression and cAMP production after isoprenaline stimulation (16). In our *ex vivo* experiments, no functional effects on noradrenaline-induced dysregulation of cytokine production were found for the ArgGlyGlu and CysArgGln haplotypes. As only three of our participants carried the CysGlyGln haplotype, we cannot draw meaningful

conclusions on the functional effects of this haplotype in our experimental setup.

We also observed no influence of the presence of individual *ADRB2* SNPs or haplotypes of these SNPs on the *in vivo* inflammatory response in subjects challenged with LPS. The experimental human endotoxemia model used in the present study is a reproducible standardized and controlled model of systemic inflammation and shares many hallmarks of early sepsis, among which an increase in circulating catecholamine concentrations (21). Strikingly, genetic variants in *hTLR4*, the gene encoding for the LPS receptor TLR4, were also shown not to influence the inflammatory response during experimental human endotoxemia (28), illustrating the possibility that compensatory mechanisms are in play even if apparently essential pathways are affected. Marked anti-inflammatory effects by catecholamines such as adrenaline, noradrenaline, and the synthetic sympaticomimetic agent phenylephrine have been demonstrated previously by our group and others using this model (10, 22, 29, 30). Furthermore, noradrenaline and phenylephrine were shown to facilitate bacterial dissemination in experimental sepsis, and noradrenaline use was related to dysregulated cytokine profiles in patients with septic shock (10). These immunosuppressive effects of noradrenaline could be reversed by selective β2-adrenergic receptor blockers in *in vitro* experiments and were attenuated in sepsis patients who were treated with a β-adrenergic receptor blocker for cardiovascular reasons prior to ICU admission (10), highlighting a critical role for the β2-adrenergic receptor. As adrenergic agents, and especially noradrenaline, are routinely used in daily practice on the intensive care for hemodynamic support of patients in shock, their unfavorable immunologic effects could be of major clinical relevance (31, 32). Based on our findings however, variations in the *ADRB2* gene do not render subjects more or less susceptible for catecholamine-induced immunological dysregulation. Presence of the CysGlyGln haplotype was associated with increased mortality in two separate cohorts of septic shock patients (20). Furthermore, this haplotype was associated with an increased heart rate and higher noradrenaline requirements, which the authors contribute to increased systemic inflammation because of attenuation of catecholamine-induced anti-inflammatory effects (20). We did not find signs of increased systemic inflammation during experimental endotoxemia in carriers of this haplotype. However, as mentioned before, CysGlyGln was only present in very few subjects of our cohort, so definitive conclusions cannot be drawn.

Most research into *in vivo* effects of *ADRB2* polymorphisms concerns vascular studies. For instance, It was demonstrated that subjects homozygous for Gly16 exhibited a more pronounced isoproterenol (a non-selective β-adrenergic receptor agonist)-induced increase in forearm blood flow (i.e. mediated by resistance arteries) compared to homozygotes for Arg16, whereas subjects with Gln27 demonstrated an attenuated increase in forearm blood flow compared with Glu27 homozygotes (18). Furthermore,

homozygous carriers of Gly16 exhibited reduced desensitization for hand vein dilation upon repeated isoproterenol infusion compared to homozygous Arg16 carriers, irrespective of their Glu27Gln status (17). Finally, increased systolic and diastolic blood pressure upon terbutaline infusion for Gly16-bearing subjects compared to Arg16 carriers was shown under non-inflammatory conditions (33). However, again, other work reported no hemodynamic effects associated with the presence of *ADRB2* polymorphisms (34). We did not observe functional hemodynamic effects of the polymorphisms during experimental endotoxemia. Of course, while this model is characterized by substantial increases in endogenous catecholamines (21, 22), no exogenous adrenergic stimulation was applied. Therefore, we conclude that possible functional effects of individual SNPs and haplotypes are too small to impact on macrocirculatory changes induced by increased endogenous catecholamine levels.

Our study is limited by the fact that we did not genotype and select subjects before study inclusion, leading to low numbers of subjects carrying the relatively rare CysGlyGln haplotype. Nevertheless, we were able to properly assess possible functional effects of individual *ADRB2* SNPs and of two other common haplotypes, namely ArgGlyGlu and CysArgGln. Considering the common linkage disequilibrium encountered in *ADRB2* polymorphisms, it is essential to include the functionality of haplotypes in addition to the functionality of individual SNPs in an analysis (35).

In conclusion, we report no consequences of common non-synonymous variants in the *ADRB2* gene on noradrenaline-mediated immunosuppression *ex vivo* or on the systemic *in vivo* inflammatory and hemodynamic response induced by LPS administration. As a consequence, these genetic variants do not influence the susceptibility towards catecholamine-induced dysregulation of the host response.

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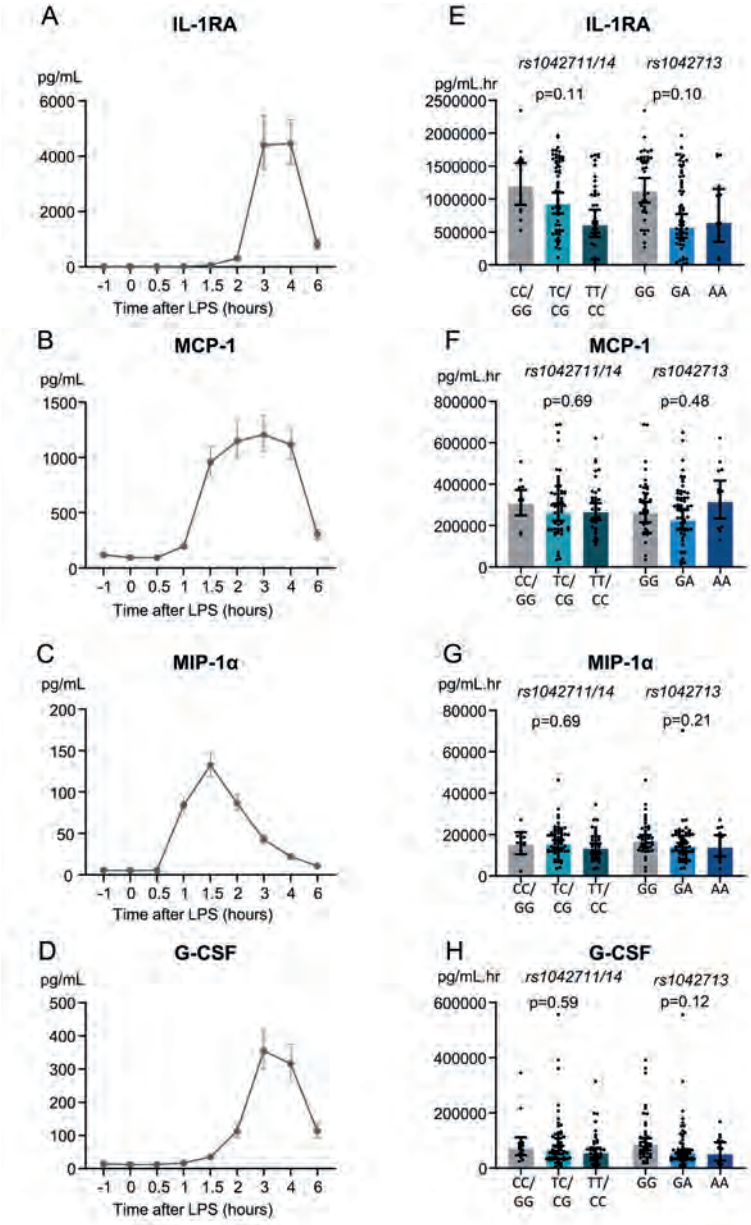
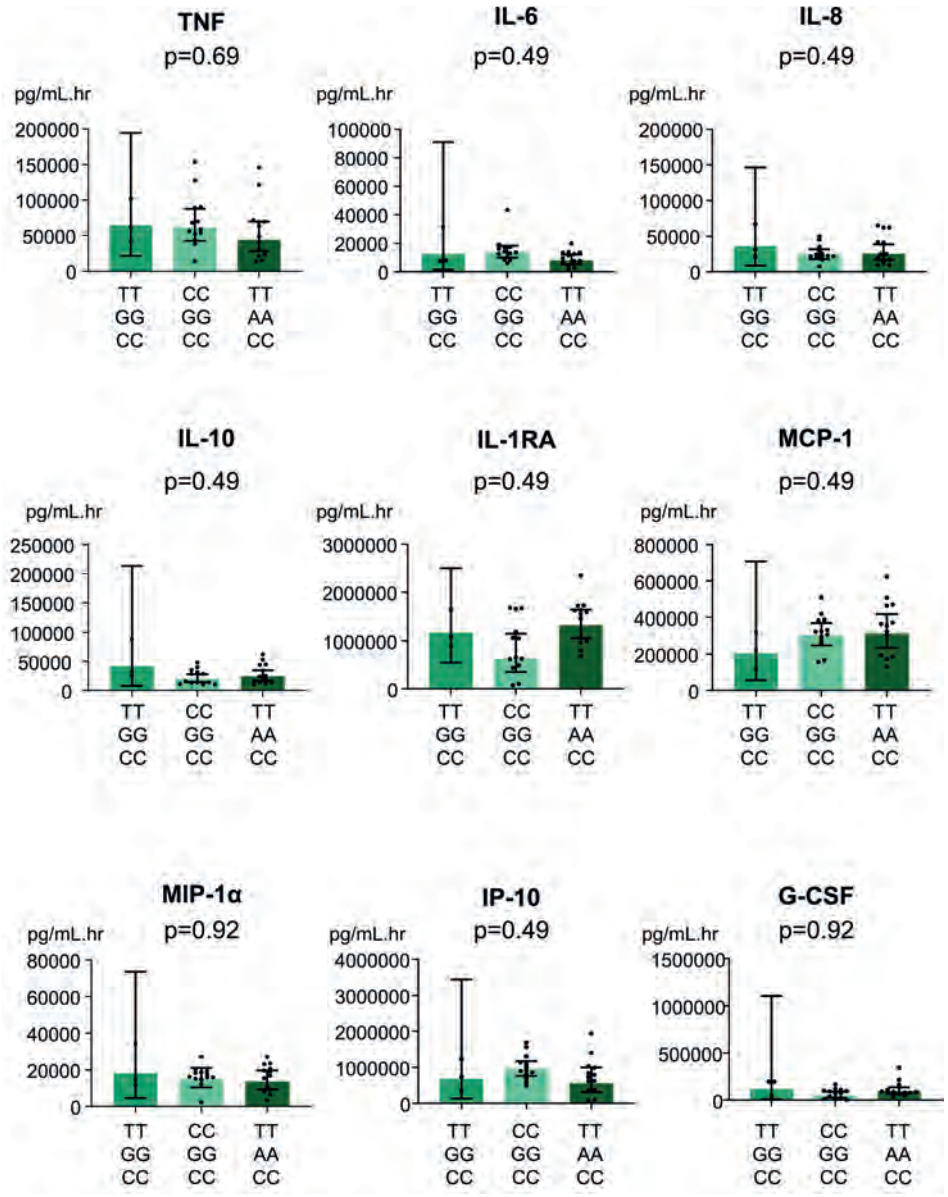
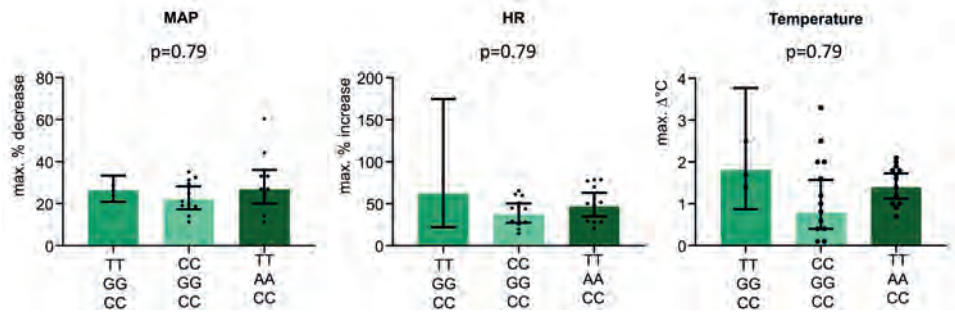


Supplemental Figure 1. In vivo cytokine production during experimental human endotoxemia is unaffected by ADRB2 genotype. Plasma concentrations of IL-1RA, MCP-1, MIP-1α and G-CSF during experimental human endotoxemia in 106 subjects (A-E) and area under curve cytokine responses (E-H) according to SNP genotype (rs1042711/rs1042714 CC/GG: n=13, TC/CG: n=58, TT/CC: n=35; rs1042713 GG: n=39, GA: n=54, AA: n=13). Data are expressed as geometric mean and 95% CI (A-E) or individual datapoints with geometric mean and 95% CI (E-H). P-values were calculated using linear regression analysis on log transformed data with SNP and sex as covariates and FDR correction was applied.



Supplemental Figure 2. In vivo cytokine production during experimental human endotoxemia is unaffected by ADRB2 haplotypes. Area under curve cytokine responses for TNF-α, IL-6, IL-8, IL-10, IL-1RA, MCP-1, MIP-1α, IP-10 and G-CSF according to haplotype (CysGlyGln [TTGGCC]: n=3, ArgGlyGlu [CCGGGG]: n=13, CysArgGln [TTAACC]: n=13). Data are expressed as individual datapoints with geometric mean and 95% CI. P-values were calculated using linear regression analysis on log transformed data with SNP and sex as covariates and FDR correction was applied.



Supplemental Figure 3. ADRB2 haplotype does not affect hemodynamic parameters or temperature during human endotoxemia. Percentage changes in mean arterial pressure (A), heart rate (B), and body temperature (C) during experimental human endotoxemia according to haplotype CysGlyGln [TTGGCC]: n=3, ArgGlyGlu [CCGGGG]: n=13, CysArgGln [TTAACC]: n=13). Data are expressed as individual datapoints with geometric mean and 95% CI. P-values were calculated using linear regression analysis on log transformed data with SNP and sex as covariates and FDR correction was applied.

CHAPTER 6

PHENYLEPHRINE IMPAIRS HOST DEFENCE MECHANISMS TO INFECTION: A COMBINED LABORATORY STUDY IN MICE AND TRANSLATIONAL HUMAN STUDY

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ABSTRACT

BACKGROUND

Immunosuppression after surgery is associated with postoperative complications, mediated in part by catecholamines that exert anti-inflammatory effects via the β-adrenergic receptor. Phenylephrine, generally regarded as a selective α-adrenergic agonist, is frequently used to treat perioperative hypotension. However, phenylephrine may impair host defence through β-adrenergic affinity.

METHODS

Human leukocytes were stimulated with lipopolysaccharide (LPS) in the presence or absence of phenylephrine and α- and β-adrenergic antagonists. C57BL/6J male mice received continuous infusion of phenylephrine (30–50 μg kg^{-1} min^{-1} i.v.) or saline via micro-osmotic pumps, before LPS administration (5 mg kg^{-1} i.v.) or caecal ligation and puncture (CLP). Twenty healthy males were randomised to a 5 h infusion of phenylephrine (0.5 μg kg^{-1} min^{-1}) or saline before receiving LPS (2 ng kg^{-1} i.v.).

RESULTS

In vitro, phenylephrine enhanced LPS-induced production of the anti-inflammatory cytokine interleukin (IL)-10 (maximum augmentation of 93%) while attenuating the release of pro-inflammatory mediators. These effects were reversed by pre-incubation with β-antagonists, but not α-antagonists. Plasma IL-10 levels were higher in LPS-challenged mice infused with phenylephrine, whereas pro-inflammatory mediators were reduced. Phenylephrine infusion increased bacterial counts after CLP in peritoneal fluid (+42%, P=0.0069), spleen (+59%, P=0.04), and liver (+35%, P=0.09). In healthy volunteers, phenylephrine enhanced the LPS-induced IL-10 response (+76%, P=0.0008) while attenuating plasma concentrations of pro-inflammatory mediators including IL-8 (–15%, P=0.03).

CONCLUSIONS

Phenylephrine exerts potent anti-inflammatory effects, possibly involving the β-adrenoreceptor. Phenylephrine promotes bacterial outgrowth after surgical peritonitis. Phenylephrine may therefore compromise host defence in surgical patients and increase susceptibility towards infection.

INTRODUCTION

Immunosuppression following major surgery is a well-recognized phenomenon with detrimental clinical consequences (1), for which there is currently no targeted treatment strategy. Surgery is accompanied by the release of both danger-associated molecular patterns (DAMPs) as well as pathogen-associated molecular patterns (PAMPs), the latter through translocation of bacterial (components) from the gut. In the perioperative setting, both DAMPs and PAMPs can bind to pattern recognition receptors and thereby elicit an inflammatory response, which is followed by suppression of host immunity (2, 3). In addition to increasing the risk for postoperative infections, this immune suppression can have protracted effects, such as increased risk of metastases in cancer patients (4, 5). There are several indications that catecholamines may play a role in these detrimental sequelae (6, 7). In accordance, studies in other setting have shown that catecholamines exert anti-inflammatory effects, such as decreased production of pro-inflammatory cytokines and increased release of the archetypical anti-inflammatory cytokine interleukin (IL)-10, via stimulation of the β-adrenergic receptor (8-11).

Phenylephrine, a synthetic sympathomimetic, is frequently used to treat hypotension in the perioperative setting. Phenylephrine is widely regarded as a selective α-adrenergic agonist (12), however, there are indications that it may exert some β-adrenergic affinity as well (13). For instance, β-adrenergic effects were unmasked in experiments where phenylephrine induced vasodilatation in the human forearm under selective α-adrenergic blockade with phentolamine (14). Furthermore, this effect was counteracted by pre-treatment with the β-blocker propranolol (14). Through these putative β-adrenergic effects, perioperative use of phenylephrine could contribute to surgery-induced immunosuppression. However, immunologic effects of phenylephrine have only sparsely been studied. *In vitro*, phenylephrine modestly enhanced IL-10 production upon stimulation with bacterial endotoxin (lipopolysaccharide [LPS]) (9), but exerted no effects on LPS-induced production of tumour necrosis factor (TNF)-α or IL-6 in human whole blood stimulation experiments (8). *In vivo* immunologic evidence is limited to studies in rats focused on cardiac inflammation, suggesting anti-inflammatory effects (15, 16).

In the present study, we evaluated immunologic effects of phenylephrine *in vitro*, in animal models of inflammation and surgical peritonitis, and finally *in vivo* in humans.

METHODS

Detailed information on study procedures, assays used, and analysis methods are described in the Supplementary data.

SUBJECTS

In vivo human endotoxemia experiments and blood withdrawal for in vitro studies were carried out in accordance with the declaration of Helsinki after approval of the local ethics committee of the Radboud University Medical Centre (CMO identifiers 2015–2079 and 2010–10, respectively). The human endotoxemia experiments were registered at Clinicaltrials.gov (identifier NCT02675868). All subjects provided written informed consent. All animal procedures were approved by the local animal ethics committee in Nijmegen and by the Dutch Council for Animal Care (identifier AVD103002016447). The Animal Research: Reporting of In Vivo Experiments (ARRIVE) checklist for animal experimentation is included in the online data supplement. The murine surgical peritonitis and human endotoxemia experiments were part of a broader project investigating (immunologic) effects of vasopressors (11, 17, 18). Therefore, data of control groups have been published previously as detailed below.

WHOLE BLOOD AND MONOCYTE STIMULATION EXPERIMENTS

Lithium heparin-anticoagulated venous blood was diluted five times in culture medium (RPMI 1640 Dutch modification [Invitrogen] supplemented with 10 µg ml^{-1} gentamicin, 10 mM Glutamax, and 10 mM pyruvate). This culture medium was used for all in vitro experiments (referred to as RPMI). Monocytes were isolated from the peripheral blood mononuclear cell (PBMC) fraction after Ficoll density gradient centrifugation using negative selection magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany). All whole blood and monocyte stimulation experiments were performed in duplicate.

Concentrations of the different compounds are provided in the figure legends. Diluted blood or monocytes (1×10^5) were pre-incubated in 96-well round bottom plates with phenylephrine (Sigma-Aldrich P6126; Sigma-Aldrich, St. Louis, MO, USA) or RPMI (control) for 60 min, after which 10 ng ml^{-1} Escherichia coli LPS (serotype 055:B5) or RPMI was added. After 24 h of incubation at 37°C and 5% CO2, plates were centrifuged and supernatants were collected and stored at –80°C until analysis. Additional monocyte and whole blood experiments were performed using adrenergic receptor (AR) antagonists (prazosin hydrochloride [α1 antagonist, Sigma-Aldrich P791]; yohimbine hydrochloride [α2 antagonist, Sigma-Aldrich Y3125], propranolol hydrochloride [non-selective β-antagonist, Sigma-Aldrich P0884], and ICI-118,551 hydrochloride [β2 antagonist, Sigma-Aldrich I127]), and using the protein kinase A (PKA) inhibitor H89 (Tocris 2910; Tocris Bioscience, Bristol, UK). Blood or monocytes were pre-incubated with AR antagonists for 30 min before the addition of phenylephrine or RPMI.

REACTIVE OXYGEN SPECIES PRODUCTION

A luminol-based luminescence assay was used to determine the production of reactive oxygen species (ROS) by monocytes, with each sample was measured in quadruplicate

(intra-assay CV%, 5.2). Monocytes (1×10^5 well^{-1}) were incubated (37°C, 5% CO2) with phenylephrine or RPMI for 60 min in flat-bottom 96-well plates (Eppendorf; Sigma-Aldrich). After addition of 100 μM luminol and 50 ng ml^{-1} phorbol 12-myristate 13-acetate (PMA), chemiluminescence was determined every 142 s at 37°C during 1 h. Cell death was quantified by was used to measure levels of lactate dehydrogenase (LDH) in stimulated whole blood culture supernatants (Cytotox96 colorimetric assay; Promega, Madison, WI, USA).

MURINE IN VIVO STUDIES

Male C57Bl/6J mice (Charles River, Germany) aged 6–9 weeks, used for all experiments, were housed in a light- and temperature-controlled room under specific pathogen-free conditions.

MICRO-OSMOTIC PUMPS

Isoflurane 5% for 2 min was used to induce anaesthesia, after which mice were anaesthetised with isoflurane 2–2.5% to maintain spontaneous respiration. Depth of anaesthesia was ascertained using the pedal withdrawal reflex (19). Micro-osmotic pumps (model 2001; Alzet Corporation, Cupertino, CA, USA) with connected jugular vein catheters (0007701; Alzet Corporation) were used in all murine experiments for continuous intravenous phenylephrine or phosphate-buffered saline (PBS) delivery.

LIPOPOLYSACCHARIDE CHALLENGE MODEL

Micro-osmotic pumps filled with phenylephrine (50 μg kg^{-1} min^{-1}) or PBS were implanted 3 h before i.v. LPS/saline challenge in 24 mice. Mice were killed by exsanguination under deep isoflurane anaesthesia 90 min after LPS or saline.

CAECAL LIGATION AND PUNCTURE

Caecal ligation and puncture (CLP; 60% ligation, 21G needle) or a sham operation was performed in 23 mice followed by placement of micro-osmotic pumps, filled with phenylephrine (30 μg kg^{-1} min^{-1}) or PBS. Mice were killed 48 h later. Plasma cytokine and bacterial count data of the sham and PBS + saline groups were published previously (11).

HUMAN IN VIVO STUDIES OF SYSTEMIC INFLAMMATION

Twenty healthy male volunteers participated in a randomised double-blind placebo-controlled experimental endotoxemia study. Subjects were randomised to receive either phenylephrine (0.5 μg kg^{-1} min^{-1}; Beacon Pharmaceuticals Ltd) for 5 h or sterile saline. To elicit a systemic inflammatory response, 2 ng kg^{-1} U.S. Reference E. coli LPS (serotype O:113; Clinical Center Reference Endotoxin, National Institute of Health, Bethesda, MD, USA) was administered i.v. 1 h after the start of phenylephrine/placebo infusion. Data from placebo infusions were published previously (11).

RANDOMISATION AND MASKING

An online random number generator (https://www.sealedenvelope.com) was used to randomise mice. Human participants were randomised by research nurses not otherwise involved in the study using the sealed envelope method. All biochemical assays were performed in a blinded fashion.

STATISTICAL ANALYSIS

Data are presented as mean (standard deviation, sd) or median and inter-quartile range (IQR) or (min–max), depending on distribution (determined using Shapiro–Wilk tests). Data were analyzed using one-way analysis of variance (anova) followed by Dunnet's post-hoc tests, t-tests, Mann–Whitney U tests, or Wilcoxon matched pairs tests. For the experimental human endotoxemia data, differences between phenylephrine and placebo groups over time were analyzed using repeated measures two-way anova (interaction term: time × treatment). A P value <0.05 was considered statistically significant. Statistical tests used are also specified in the figure legends. Note that for the caecal ligation and puncture experiments, it was not always possible to obtain sufficient blood to perform all measurements because of severity of illness, resulting in inadequate blood flow, leading to lower numbers of replicates for some of the presented data. Analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA).

SAMPLE SIZE ESTIMATION

For in vitro monocyte and whole blood stimulation experiments, sample size was based on previous in vitro experiments with vasopressors (11). For the human and murine endotoxemia experiments, sample size was calculated a priori based on earlier TNF-α results from previous experiments(20). For the murine CLP experiments, spleen CFU data from pilot experiments were used to calculate the appropriate sample size. Sample size was increased by 20% accommodate for loss of animals during the experiment.

RESULTS

EFFECT OF PHENYLEPHRINE ON RESPONSES OF PRIMARY HUMAN LEUKOCYTES AND MONOCYTES TO LPS AND ON ROS PRODUCTION

In LPS-stimulated whole blood, phenylephrine dose-dependently attenuated production of pro-inflammatory mediators TNF-α, IL-1β, and interferon gamma-induced protein (IP)-10 (maximum inhibition of 75%, 15%, and 77%, respectively; **Figure 1A**). In contrast, release of the anti-inflammatory cytokine IL-10 was enhanced by phenylephrine in a dose-dependent fashion (maximum augmentation of 93%; **Figure 1A**). Phenylephrine did not affect cell survival (**Supplementary Figure S1**). In keeping with the fact that monocytes are the main cytokine producers in short-term LPS stimulation experiments (21) we confirmed

that phenylephrine dose-dependently attenuated the production of TNF-α and enhanced the release of IL-10 in isolated primary human monocytes (**Figure 1B**). In addition to cytokine production, we assessed phenylephrine's effect on radical oxygen species (ROS) production, another important innate anti-bacterial defence mechanism. Phenylephrine did not affect ROS production by monocytes stimulated with the nuclear factor kappa B (NF-κB) activator phorbol myristate acetate (**Figure 1C and D**).

ADRENORECEPTOR MODULATION OF ANTI-INFLAMMATORY EFFECTS OF PHENYLEPHRINE

The non-selective β-AR antagonist propranolol nullified the effects of phenylephrine on TNF-α, IP-10, and IL-10 production in LPS-stimulated whole blood, whereas the phenylephrine-induced attenuation of TNF-α and IP-10 was not affected by the selective α1-AR antagonist prazosin or the selective α2-AR antagonist yohimbine (**Figure 2A**). Nevertheless, yohimbine reversed phenylephrine's IL-10-enhancing effects (**Figure 2A**). As previous work demonstrated that selective β2-agonists attenuate pro-inflammatory cytokine production(22) we investigated the effects of the selective and β2-AR antagonist ICI-118,551 in isolated primary human monocytes. ICI-118,551 reversed phenylephrine's effects on LPS-induced TNF-α and IL-10 production, indicating a role for the β2-AR in phenylephrine's anti-inflammatory effects (**Figure 2B**).

β-ARs are G-protein-coupled receptors that signal via the Gs protein, in turn increasing intracellular cyclic adenosine monophosphate (cAMP) levels. This results in activation of PKA, which prevents NF-κB translocation to the nucleus, ultimately reducing pro-inflammatory cytokine transcription and production while increasing anti-inflammatory IL-10 (23, 24). The PKA inhibitor H89 reversed the anti-inflammatory effects of phenylephrine, confirming the involvement of β2-AR signaling in the anti-inflammatory effects of phenylephrine (**Supplementary Figure S2**).

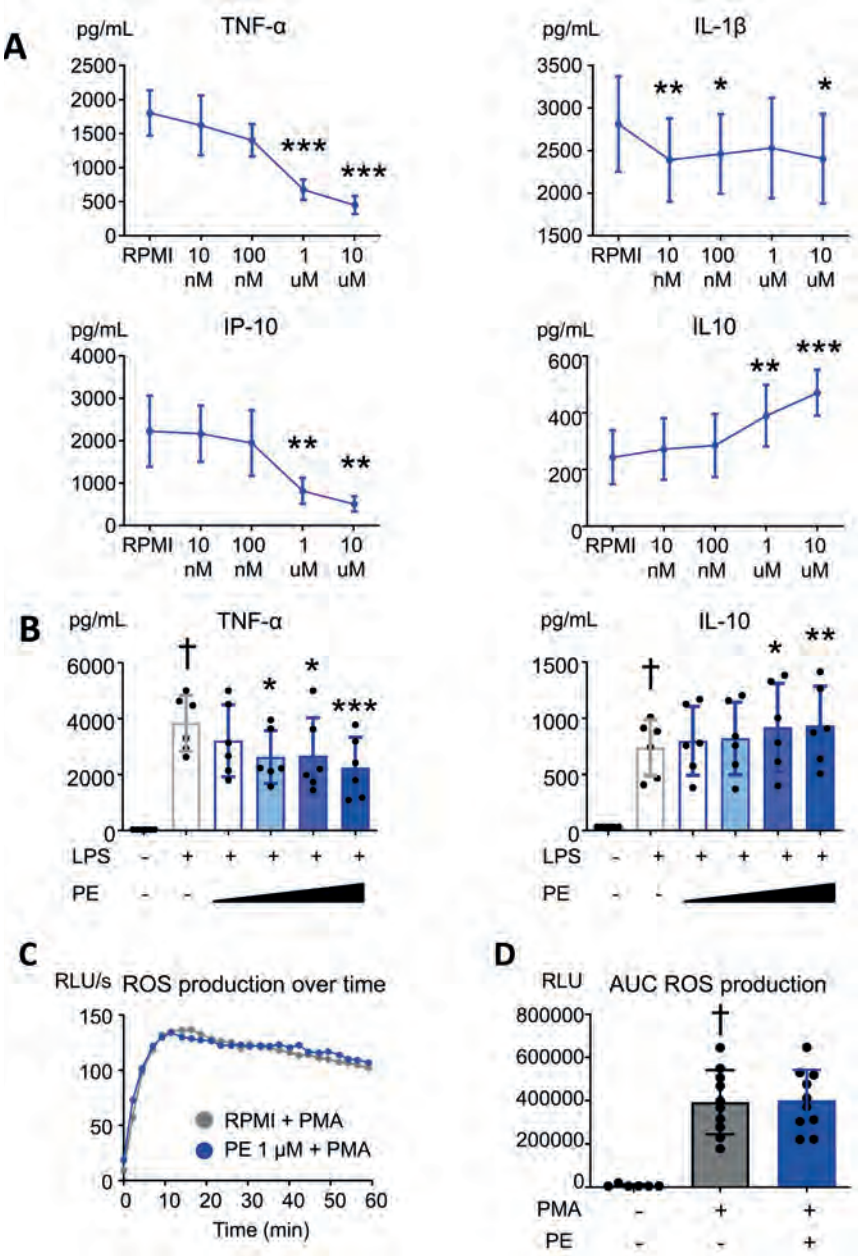

Figure 1. Phenylephrine modulates lipopolysaccharide (LPS)-induced cytokine production by human whole blood and primary human monocytes, whereas phorbol 12-myristate 13-acetate (PMA)-induced reactive oxygen species production is unaffected. (A) Concentrations of tumour necrosis factor (TNF)-α, induced protein (IP)-10, interleukin (IL)-1β, and IL-10 in supernatant of human whole blood pre-incubated with either RPMI (medium control), phenylephrine (PE) in escalating concentrations for 1 h and subsequently stimulated with LPS (10 ng ml−1) for 24 h. (B) Concentrations of TNF-α and IL-10 in supernatant of primary human monocytes pre-incubated with either RPMI or phenylephrine in escalating concentrations (10 nM, 100 nM, 1 µM, 10 µM) for 1 h and subsequently stimulated with LPS (10 ng ml−1) or RPMI for 24 h. (C and D) Primary human monocytes were incubated with RPMI

(medium control) or PE (1 µM) for 1 h and subsequently stimulated with PMA (50 ng ml−1) or RPMI in the presence of luminol, and luminescence, reflecting reactive oxygen species (ROS) production, was measured for 1 h. (C) Relative light units (RLU) per second; (D) area under the curve (AUC) of the data presented in panel (C). Data are expressed as mean (standard deviation, sd) (A), individual data points and mean (sd) (B and D), or means (C) of 6–10 individual donors. †P<0.05 compared with RPMI; ∗P<0.05, ∗∗P<0.01, ∗∗∗P<0.001 compared with LPS calculated using one-way analysis of variance with Dunnett's post hoc tests (a and b) or a t-test (D).

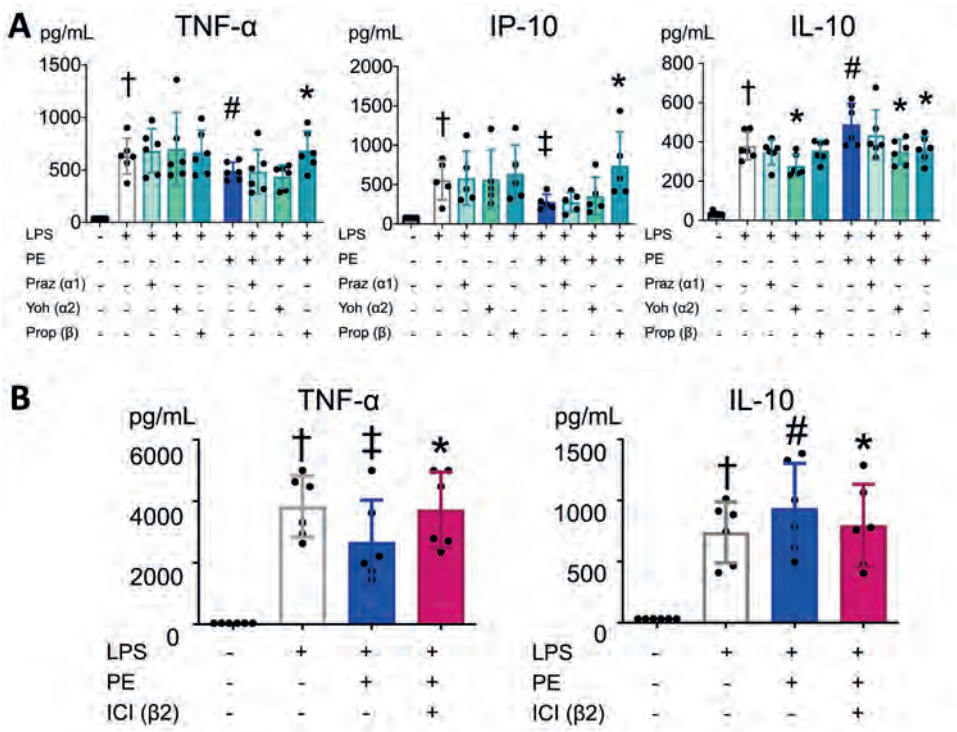

Figure 2. Phenylephrine modulates LPS-induced cytokine production via stimulation of the β2 adrenoreceptor. (A) Concentrations of TNF-α, IP-10 and IL-10 in supernatant of human whole blood preincubated with either RPMI (medium control) or adrenoceptor antagonists prazosin (Praz, α1, 1 µM), yohimbine (Yoh, α2, 1 µM), propranolol (Prop, β, 1 µM) for 30 minutes. This was followed by incubation with phenylephrine (PE, 1 µM) or RPMI for 1h and subsequent stimulation with LPS (10 ng/mL) or RPMI for 24h. (B) Concentrations of TNF-α, and IL-10 in supernatant of primary human monocytes preincubated with either RPMI or the β2-antagonist ICI-118,551 (ICI, 0.1 µM) for 30 min, followed by incubation with phenylephrine (PE, 1 µM) or RPMI for 1h and subsequent stimulation with LPS (10 ng/mL) or RPMI for 24h. Data are expressed as individual data points and mean±SD of 6 individual donors. †p<0.05 compared to RPMI; ‡p<0.05, #p=0.05–0.10 compared to LPS. *p<0.05, ***p<0.001 compared to PE + LPS calculated using t-tests.

MURINE LPS CHALLENGE

Under non-inflammatory conditions (saline-injected mice), phenylephrine infusion increased plasma concentrations of IL-6 (+117%) and IP-10 (+91%), whereas other cytokines were not affected (**Supplementary Figure S2**). In LPS-challenged mice (**Figure 3A**), phenylephrine infusion reduced plasma levels of TNF-α (–34%), IL-1β (–31%), IP-10 (–72%), macrophage inflammatory protein (MIP)-1α (–57%), macrophage chemoattractant protein (MCP)-1 (–64%), and keratinocyte-derived chemokine (KC, –32%), whereas concentrations of IL-10 were enhanced (+158%). Similar effects were observed in spleen and lung tissue of LPS-challenged mice (**Supplementary Figure S3**). LPS-induced increase in both basal neutrophilic ROS production and the PMA-induced maximal respiratory burst was reduced after phenylephrine infusion (–54% and –57%, respectively), whereas no such effects were observed under non-inflammatory conditions (**Figure 3B**). Phenylephrine infusion in saline-injected mice also attenuated IFN-γ production by splenocytes ex vivo stimulated with PMA and ionomycin (–57%), whereas statistical significance was not reached for TNF-α (–43%, P=0.09) (**Supplementary Figure S4**).

MURINE CAECAL LIGATION AND PUNCTURE

Compared with sham-operated mice, bacterial counts in peritoneal fluid, blood, liver, and spleen were profoundly increased in animals that underwent CLP (**Figure 4**). Compared with PBS-infused mice, phenylephrine infusion increased bacterial counts in the peritoneal fluid (+42%) and spleen (+59%), whereas a trend towards increased counts was observed in the liver (+35%, P=0.09). No robust augmentation in blood bacterial counts were observed (+51%, P=0.47; **Figure 4**). Phenylephrine treatment did not influence plasma cytokine levels determined 4 h after induction of CLP induction (**Supplementary Figure S5**).

HAEMODYNAMIC CHANGES AFTER LPS INFUSION IN PRESENCE OR ABSENCE OF PHENYLEPHRINE

Subjects randomised to receive a 5 h infusion of either low-dose phenylephrine (0.5 μg kg^{-1} min^{-1}; n=10) or saline (n=10) had similar characteristics (Supplementary data). After 1 h of infusion, phenylephrine increased MAP by 13 (3) mm Hg; **Figure 5A**) and reduced heart rate by 10 (1) beats min^{-1} (**Figure 5B**). One hour after start of phenylephrine/placebo infusion, 2 ng kg^{-1} LPS administration resulted in flu-like symptoms, lower MAP, and higher heart rate in both groups (**Figure 5A and B**). LPS-induced increase in body temperature was also similar between the two groups (**Figure 5C**). Endogenous circulating noradrenaline levels were lower after phenylephrine infusion (reduction of 24% in area under the time–concentration curve [AUC], P=0.053; **Figure 5D**); no effect on the LPS-induced increase in plasma adrenaline concentrations occurred (**Figure 5E**).

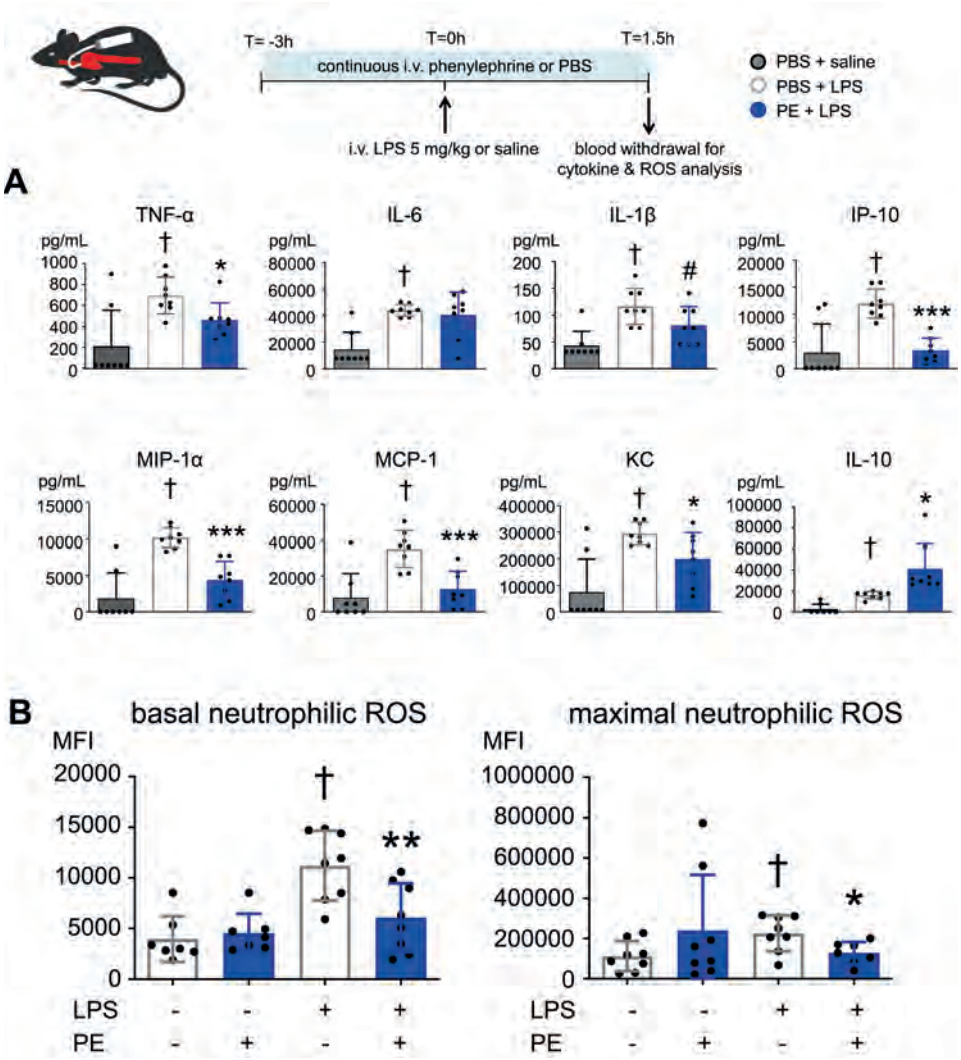

Figure 3 Phenylephrine modulates in vivo cytokine responses and ex vivo neutrophilic reactive oxygen species production in LPS-challenged mice. (A) Plasma concentrations of TNF-α, IL-6, IL-β, IP-10, MIP-1α, MCP-1, KC, and IL-10 in C57B/6J mice intravenously infused with phenylephrine (PE, 50 µg/kg/min) or PBS via a micro-osmotic pump connected to a jugular vein catheter for 4.5h and challenged intravenously with LPS (5 mg/kg) or saline 3h after start of infusion. (B) Reactive oxygen species (ROS) content and PMA-induced maximal respiratory burst of neutrophils isolated at the end of the experiment depicted in panel A. Data are expressed as individual data points and mean±SD of 8 animals per group. †p <0.05 compared to PBS + saline; #p=0.05-0.10, *p<0.05, **p<0.01, ***p<0.001 compared to PBS + LPS calculated using t-tests.

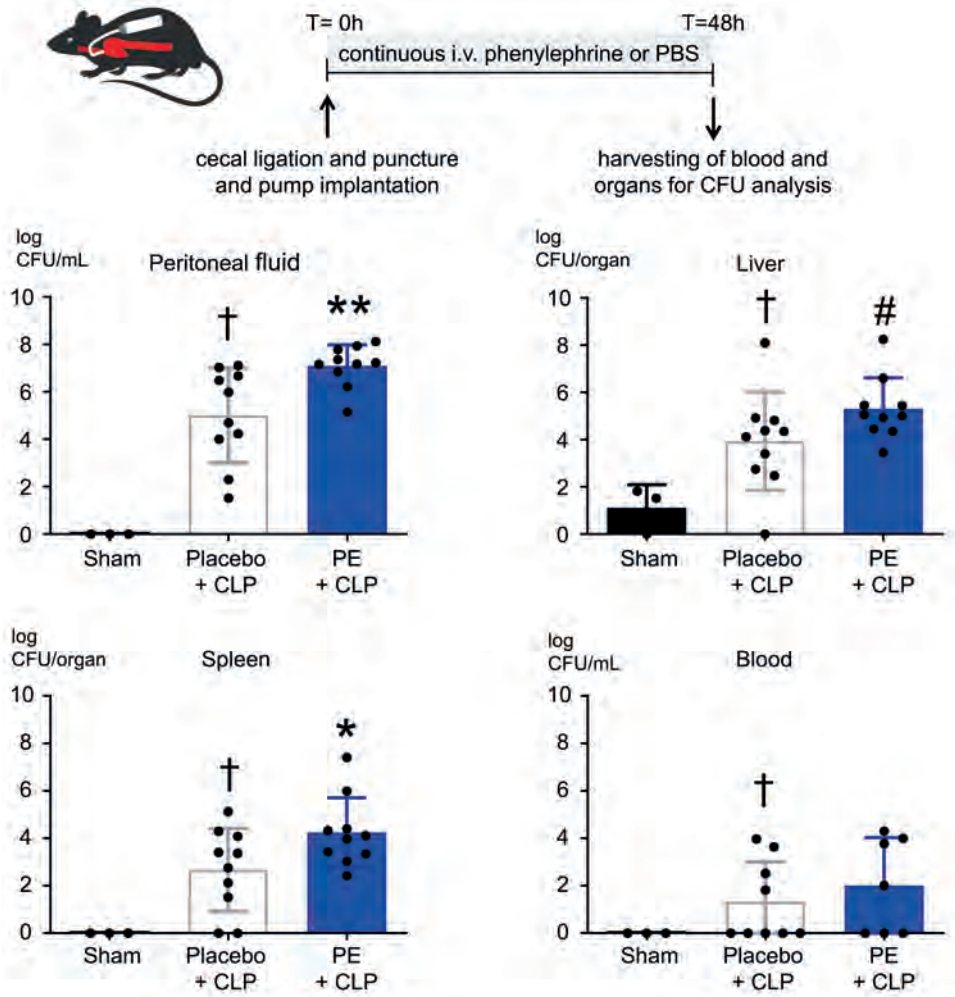

Figure 4. Phenylephrine treatment increases bacterial dissemination during surgical peritonitis. Bacterial load (expressed as colony forming units [CFU]) in peritoneal fluid, blood, liver, and spleen of C57B/6J mice intravenously infused with phenylephrine (PE, 30 µg/kg/min) or PBS for 2 days via a micro-osmotic pump connected to a jugular vein catheter and subjected to cecal ligation and puncture (CLP) to induce peritonitis or a sham operation. Data are expressed as individual data points and mean±SD of 10 animals per group.; †p<0.05 compared to sham; #p=0.05–0.10, *p<0.05, **p<0.01 compared to PBS + CLP, calculated using t-tests.

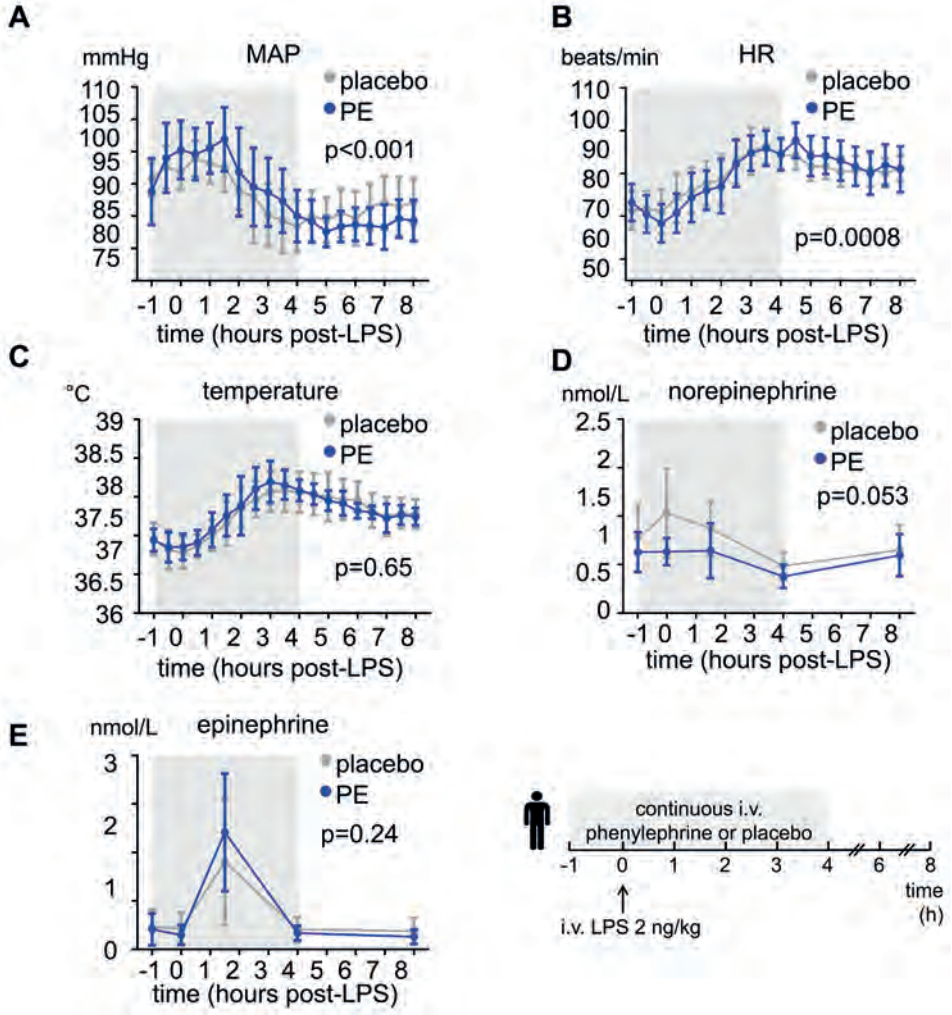

Figure 5. Hemodynamics, body temperature, and plasma (nor)adrenaline levels during experimental human endotoxemia. (A) Mean arterial pressure, (B) heart rate, (C) body temperature, (D) plasma noradrenaline levels, and (E) plasma adrenaline levels in healthy volunteers randomized to a 5h intravenous infusion with either saline (placebo) or low-dose phenylephrine (PE, 0.5 µg/kg/min) and challenged intravenously with 2 ng/kg LPS 1h after start of infusion to elicit a systemic inflammatory response. Data are expressed as mean±SD of 10 subjects per group. The grey area represents the period during which phenylephrine, or saline was infused. p-values were calculated using repeated measures two-way analysis of variance (interaction term: time*treatment) vs. the placebo group.

CIRCULATING LEUKOCYTES AND CYTOKINE LEVELS AFTER LPS INFUSION IN PRESENCE OR ABSENCE OF PHENYLEPHRINE

LPS-induced changes in circulating numbers of monocytes, neutrophils, or lymphocytes were not affected by phenylephrine infusion (**Figure 6A**). LPS administration caused a

transient increase in plasma concentrations of TNF-α, IL-6, IL-8, IP-10, MCP-1, granulocyte colony-stimulating factor (G-CSF), and IL-10 in all subjects (**Figure 6B**). Phenylephrine treatment significantly attenuated plasma levels of IL-8, IP-10, and MCP-1 (reduction in AUC of 15%, 41%, and 27%, respectively) while enhancing the IL-10 response (+76%; **Figure 6B**).

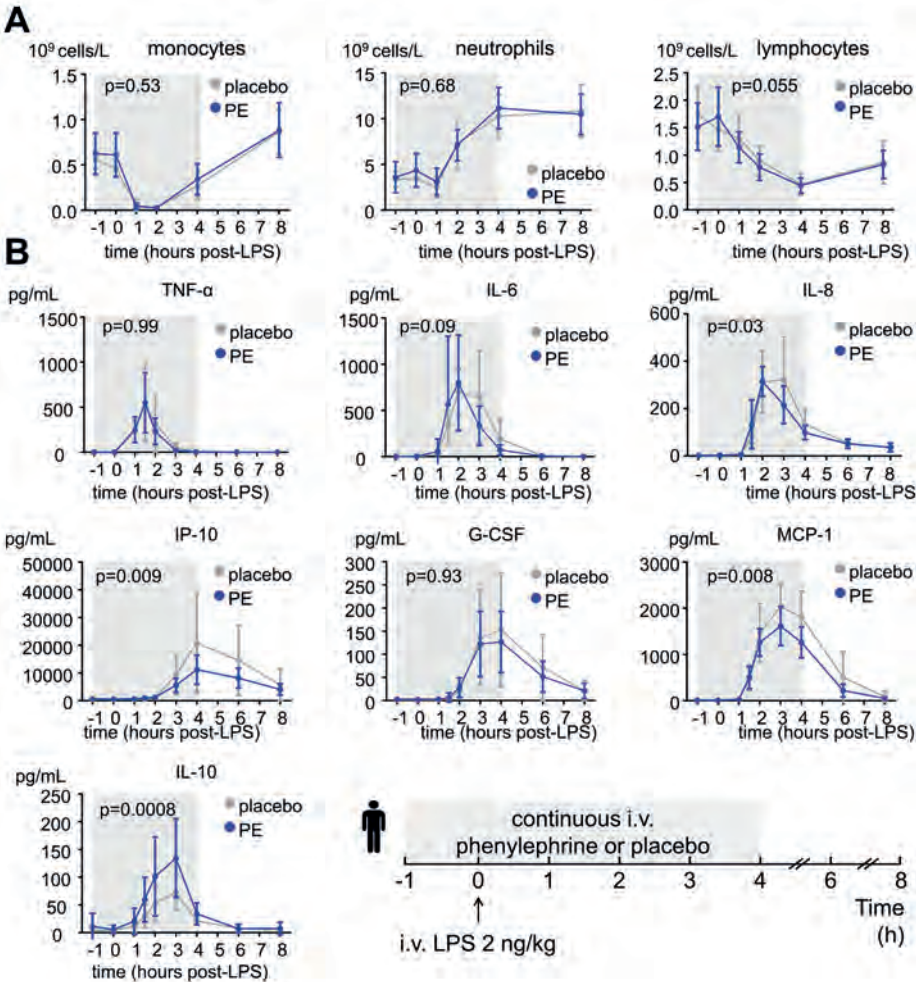

Figure 6. Low-dose phenylephrine infusion attenuates pro-inflammatory cytokine levels, while enhancing the anti-inflammatory IL-10 response during experimental human endotoxemia. (A) Circulating monocyte, neutrophil and lymphocyte numbers, (B) plasma concentrations of TNF-α, IL-6, IL-8, IP-10, G-CSF, MCP-1, and IL-10, in healthy volunteers randomized to a 5h intravenous infusion with saline (placebo) or low-dose phenylephrine (PE, 0.5 μg/kg/min) and challenged intravenously with 2 ng/kg LPS 1h after start of infusion to elicit a systemic inflammatory response. Data are expressed as mean±SD of 10 subjects per group. The grey area represents the period during which phenylephrine or saline was infused. p-values were calculated using repeated measures two-way analysis of variance (interaction term: time*treatment) vs. the placebo group.

DISCUSSION

We provide the first comprehensive translational study into the immunologic effects of phenylephrine. We demonstrate that phenylephrine attenuates LPS-induced pro-inflammatory cytokine production *in vitro,* whereas it enhances the release of the anti-inflammatory cytokine IL-10. These effects are not mediated through the α-adrenergic receptor, but instead appear to involve β-AR receptor stimulation, specifically the β2 receptor. Furthermore, we show that, in both mice and humans, phenylephrine infusion shifts the LPS-induced cytokine profile towards a distinct anti-inflammatory phenotype. The functional relevance of these immunosuppressive effects of phenylephrine is illustrated by increased bacterial dissemination during surgical peritonitis in mice, indicative of impaired host defence.

Phenylephrine is widely regarded as a selective α-adrenergic agonist, although some cardiovascular β-adrenergic effects have been reported (13, 14). Remarkably, we reveal that its immunomodulatory effects in terms of cytokine production are highly similar to those of specific β-AR agonists (22, 25) and to the β-AR-dependent effects of (nor)adrenaline (8-11). In accordance, our data show that the non-specific β-AR antagonist propranolol and the specific β2-AR antagonist ICI-118,551 block the effects of phenylephrine on LPS-induced cytokine production. Therefore, phenylephrine cannot be considered as a specific α-agonist, as it exerts functional immunological β-adrenergic effects as well. This concurs with previous data showing that vascular effects of phenylephrine were counteracted by co-administration of a β-AR antagonist (14). β-AR affinity was also implied by a study demonstrating uterine contraction by phenylephrine mediated by transient cAMP increases (26), which are also observed following β-AR stimulation with noradrenaline (23). Our data also show that phenylephrine-induced immunomodulation is mediated by the cAMP/PKA pathway, which concurs with our earlier results on noradrenaline (11) and those on other β-adrenergic agonists (27). Interestingly, investigations into the inotropic properties of phenylephrine revealed that its overriding effects are dependent on the relative distribution of α- and β-adrenergic receptors in the target tissue(28). As immune cells mainly express β-ARs(29), this may provide an explanation for the anti-inflammatory effects of phenylephrine observed in the present study. Our data are, to some extent, in contrast with previous reports showing no effects of phenylephrine on LPS-induced production of TNF-α by human whole blood (8), although the enhanced IL-10 production we observed was also demonstrated before (9). This discrepancy might be explained by the differences in the duration of stimulation. IL-10 is a potent negative regulator of pro-inflammatory cytokine production, but its production is delayed relative to that of pro-inflammatory cytokines such as TNF-α, with maximal expression observed only after 8 h (30). Therefore, in the previous study using a 4 h stimulation period (8), IL-10-mediated negative feedback on pro-inflammatory

cytokine production was likely limited compared to the 24 h stimulation period we employed, which may more accurately capture the complex interplay between the release of different inflammatory mediators.

In our murine studies, phenylephrine was administered via micro-osmotic pumps connected to a jugular vein catheter. This allowed for continuous i.v. administration in conscious animals, thereby excluding well-known confounding effects of sedatives (31). The phenylephrine dosages used were based on previous studies using similar dosages, which were shown to induce an increase in blood pressure but were nevertheless tolerated for more than 14 days (32). Previous animal studies investigating the immunomodulatory properties of phenylephrine are scarce. In a murine endotoxemia study, either one or two subcutaneous bolus injections of phenylephrine did not reduce plasma TNF-α levels (33); this might be related to the mode of administration used (bolus vs. continuous infusion) in conjunction with phenylephrine's short half-life and/or the timing relative to LPS injection (0-45 min after LPS administration instead of 1 hour before LPS challenge in our experiments), or both. Another study demonstrated protective effects of a single subcutaneous bolus administration of phenylephrine on myocardial dysfunction in rats subjected to CLP, exemplified by preservation of cardiac function as well as reduced myocardial TNF-α and IL-6 content, although plasma levels of TNF-α and IL-6 were not significantly reduced (16), which may again be related to timing and mode of administration. Furthermore, in our CLP experiments, we also did not observe clear effects of phenylephrine infusion on cytokine levels. Like in other studies, this is plausibly related to timing, as we measured cytokines only on a single, relatively early timepoint, on which concentrations were still relatively low. No other timepoints were assessed due to the limited amount of blood which can be obtained from mice. This represents a limitation of our work.

Although the anti-inflammatory effects of phenylephrine might be beneficial locally in the heart, our results indicate that these come at a cost of impaired host defence, and a subsequent increased susceptibility for infections. The most plausible underlying mechanism behind the increased bacterial dissemination observed in our CLP experiments is enhanced IL-10 release and an impaired pro-inflammatory cytokine response. This is illustrated by murine experiments showing that a more pronounced IL-10 response is associated with impaired bacterial clearance (34). The impaired IFN-γ production by splenocytes of phenylephrine-treated mice observed in the present work is also of interest, as this cytokine was also shown to be pivotal in the defence against bacterial infections in mice (34, 35) and its production is significantly attenuated in patients suffering from sepsis-induced immunoparalysis (36). Furthermore, exogenous IFN-γ administration has successfully been used to restore immunocompetence in human models of immunosuppression (37) and in several small studies in immunosuppressed

patients (38, 39). Although neutrophilic ROS generation was impaired in phenylephrine-infused mice, this may not represent a direct effect of phenylephrine for several reasons. First, phenylephrine did not decrease ROS production in our *in vitro* experiments. Second, the effect was only apparent in LPS-challenged mice and not in saline-challenged mice. As the inflammatory response elicited by LPS potentiated both basal and maximal neutrophilic ROS production, the attenuated ROS generation in phenylephrine-infused mice that were challenged with LPS is likely a consequence of the overall decreased inflammatory response in these animals.

To the best of our knowledge, this is the first study investigating immunomodulatory effects of phenylephrine in humans. Recently, we published a comprehensive investigation of immunomodulatory properties of noradrenaline, a catecholamine with both α- and β-adrenergic affinity (11). Noradrenaline is the cornerstone treatment for patients with septic shock, but is also frequently used in the operating theatre to counteract hypotension (40). We demonstrated that, similar to phenylephrine, noradrenaline enhances the IL-10 response and attenuates pro-inflammatory mediators *in vitro* and *in vivo* through β-adrenergic signalling (11). Unlike phenylephrine, noradrenaline also impaired monocytic ROS production (11). Possibly as a result of these effects on both cytokines and ROS, noradrenaline increased bacterial dissemination in CLP mice more profoundly than phenylephrine did in the present study (11). These dissimilarities might be caused by the different affinities of noradrenaline and phenylephrine for β-adrenergic receptors.

Clinically, phenylephrine is predominantly used in the surgical setting to treat hypotension and is either administered as a bolus or by continuous infusion. In this setting, higher dosages than those we employed in our human endotoxemia study (0.5 $\mu g\ kg^{-1}\ min^{-1}$) are commonly used. For instance, a median infusion rate of 0.95 $\mu g\ kg^{-1}\ min^{-1}$ was used in obstetric surgery patients to treat anaesthesia-associated hypotension (40). Nevertheless, we demonstrate that continuous infusion of this relatively low dose of phenylephrine enhances the IL-10 response and attenuates concentrations of several pro-inflammatory cytokines during experimental human endotoxemia (i.e. IL-8, IP-10 and MCP-1). Given the dose-dependent effects of phenylephrine in our *in vitro* experiments, stronger anti-inflammatory effects are anticipated in the clinical setting, which are likely of relevance. For example, augmented IL-10 levels have been associated with an increased incidence of sepsis in patients undergoing surgery as well as in trauma patients (41, 42). Furthermore, a 74% increase in the IL-10/TNF-α ratio (reflecting the anti/pro-inflammatory balance) has been linked to increased disease severity and mortality in sepsis patients (43). For comparison, the relatively low phenylephrine dose used in our human endotoxemia experiments increased the IL-10/TNF-α ratio by 93%. The effects of phenylephrine are neither mediated via enhanced

release of noradrenaline, nor by increased circulating concentrations of adrenaline, another catecholamine which was previously shown to increase IL-10 and impair pro-inflammatory responses during experimental human endotoxemia (9). As IL-8 plays a pivotal role in neutrophil chemotaxis(44), it is of interest to note that impaired neutrophil chemotaxis has been linked to increased susceptibility towards post-operative sepsis in patients (3). In addition to influencing such short-term post-operative outcomes, it has been suggested that perioperative immune suppression affects long-term outcomes as well, and there are several indications of β-adrenergic involvement. For example, use and release of catecholamines has been linked to suppressed anti-metastatic immunity and thereby increased risk of metastatic recurrence following cancer surgery (5). Furthermore, in a breast cancer model in rats, metastases dose-dependently increased following treatment with the β-agonist metaproterenol, an effect which could be mitigated by the β-antagonist nadolol (45). Likewise, in a rat model of surgical stress, it was demonstrated that pretreatment with nadolol prevented the surgery-induced suppression of T-and B-cell function (46).

Finally, in a clinical study, the use of β-blockers was associated with a significantly reduced incidence of sepsis and mortality after emergency colonic surgery, although this was a retrospective study with risk for selection bias and confounding (47). In a recent systematic review, this effect was not confirmed for non-cardiac surgery patients (48). Based on our experimental results, we argue that a clinical study aimed to investigate possible detrimental effects of phenylephrine-induced immunomodulation is warranted, and that use of alternative vasopressors should be further explored.

A limitation of our study is that we only included male subjects. There are considerable differences in the cytokine response to LPS between males and females (49). This is likely influenced by menstrual cycle-related hormonal variations that can affect the immune response. As human endotoxemia experiments are very labour-intensive and costly studies, and for ethical reasons (we want to expose as few volunteers as possible to endotoxemia), we therefore only include male subjects in virtually all of our endotoxemia studies. However, as no sex differences were demonstrated for anti-inflammatory effects of other adrenergic agonists (50), it is unlikely that phenylephrine will have different effects in females.

In conclusion, phenylephrine, a sympathomimetic frequently used during surgery, exerts anti-inflammatory effects *in vitro* and *in vivo.* These effects are not mediated through α-adrenergic receptor agonism, but via the β2-adrenoceptor, and functionally result in impaired bacterial clearance. Phenylephrine could therefore compromise host defence in surgical patients, with potential detrimental effects on both the short and long term outcome.

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SUPPLEMENTARY MATERIALS

IN VITRO STUDIES

Whole blood and monocyte stimulation experiments

For whole blood stimulation experiments, lithium heparin-anticoagulated venous blood was diluted five times in culture medium (RPMI 1640 Dutch modification [Invitrogen] supplemented with 10 µg/mL gentamicin, 10 mM Glutamax and 10 mM pyruvate). This culture medium was used for all *in vitro* experiments and is henceforth referred to as RPMI. For monocyte stimulation experiments, peripheral blood mononuclear cells (PBMCs) were isolated from ethylenediaminetetraacetic acid (EDTA)-anticoagulated venous blood using Ficoll (Ficoll-Paque Plus, GE healthcare) density gradient centrifugation in SepMate™ tubes (STEMCELL Technologies, 10 min, 1200 g, RT. Subsequently the PBMCs were washed thrice in cold phosphate-buffered saline (PBS, 10 min, 1700 rpm, 4°C) and resuspended in RPMI. Monocytes were isolated from the PBMC fraction using magnetic separation (negative selection using the Pan Monocyte Isolation Kit from Miltenyi Biotec) following the manufacturer's instructions and resuspended in RPMI. The average purity of the monocyte fraction was 91% (SD 8%), whereas the mean yield from the PBMC fraction was 76% (SD 17%). All whole blood and monocyte stimulation experiments were performed in duplicate. Concentrations of the different compounds are provided in the figure legends.

Diluted blood or monocytes (1×10^5) were pre-incubated in 96-wells round bottom plates with phenylephrine (Sigma-Aldrich P6126) or RPMI (control) for 60 min, after which 10 ng/mL *Escherichia coli* (*E.coli*) LPS (serotype 055:B5) or RPMI was added. After 24 h of incubation at 37°C and 5% CO_2, plates were centrifuged and supernatants were collected and stored at -80°C until analysis. Additional monocyte and whole blood experiments were performed using adrenergic receptor (AR) antagonists (*prazosin hydrochloride* (α1 antagonist, Sigma-Aldrich P791); *yohimbine hydrochloride* (α2 antagonist, Sigma-Aldrich Y3125), *propranolol hydrochloride* (non-selective β-antagonist, Sigma-Aldrich P0884), and *ICI-118,551 hydrochloride* (β2 antagonist, Sigma-Aldrich I127), as well as using the protein kinase A (PKA) inhibitor H89 (Tocris 2910). Blood or monocytes were pre-incubated with AR antagonists for 30 mins prior to the addition of phenylephrine or RPMI. Enzyme-linked immunosorbent assays (ELISA, Duosets, R&D Systems) were optimized in house and used to measure concentrations of TNF-α, interferon gamma-induced protein (IP)-10, IL-1β, and IL-10 in cell culture supernatants. Intra-assay coefficients of variation (CV%) for these assays are 2.3, 3.2, 1.7, and 5.4 for TNF-α, IP-10, IL-1β, and IL-10, respectively, whereas inter-assay CV% are 3.9, 1.2, 7.2, and 5.9 for TNF-α, IP-10, IL-1β, and IL-10, respectively.

ROS production by monocytes

A in-house developed luminol-based luminescence assay was used to determine the

production of reactive oxygen species (ROS) by monocytes. Each sample was measured in quadruplicate, and the intra-assay CV% was 5.2. Monocytes ($1x10^5$/well) were incubated (37°C, 5% CO_2) with phenylephrine or RPMI for 60 min in flat-bottom 96-well plates (Eppendorf). After addition of 100 µM luminol and 50 ng/mL phorbol 12-myristate 13-acetate (PMA), chemiluminescence was determined every 142 s at 37°C during 1 h. All measurements were carried out in quadruplicate.

Cytotoxicity assay

The Cytotox96 colorimetric assay (Promega, Madison, USA) was used to measure levels of lactate dehydrogenase (LDH) in stimulated whole blood culture supernatants (see section above for experimental details) according to the manufacturer's instructions. Background controls consisted of untreated cells (RPMI only) while otherwise untreated samples treated with lysis solution served as positive controls. LDH levels in the samples were stratified according to the amount of LDH measured in the positive controls, which was set at 100%.

MURINE *IN VIVO* STUDIES

Priming of micro-osmotic pumps

Pumps were filled 3-5 days prior to implantation and placed in sterile NaCl 0.9% at 37°C in the dark to ensure complete priming of the connected jugular vein catheter.

Endotoxemia experiments

Under isoflurane inhalation anesthesia, the right jugular vein was catheterized and connected to a micro-osmotic pump pre-filled with phenylephrine (Sigma-Aldrich P6126, for delivery of 50 µg/kg/min) or PBS for continuous intravenous delivery. The phenylephrine dose was based on literature(1, 2), where comparable dosages demonstrated vasopressor activity in mice. A subcutaneous pocket was created on the back of the mouse to place the pump and remaining part of the catheter. Thereafter, mice recovered in a 37°C incubator for 30 min before being put back in their cages. LPS (5 mg/kg, serotype 0111:B4, L2630, Sigma-Aldrich) or saline was injected in the tail vein 3 hours after osmotic pump implantation. Ninety minutes after LPS/saline administration, animals were culled by exsanguination under deep isoflurane anesthesia. For *ex vivo* neutrophilic ROS production, lithium heparin-anticoagulated blood was collected and kept on ice until analysis. EDTA-anticoagulated blood was centrifuged (10 min, 2000 g, RT) and plasma was stored at -80°C until analysis of cytokine & catecholamine concentrations. The right lung was harvested, snap frozen in liquid nitrogen, and stored at -80°C until cytokine analysis. The spleen was harvested and divided in two parts. One part was snap frozen in liquid nitrogen and stored at -80°C until cytokine analysis, whereas. the remaining part was kept on ice in RPMI containing 20% Fetal Bovine Serum (FBS) for *ex vivo* stimulation assays. In total 24 mice were included in these experiments,

of which 6 mice served as PBS-saline controls and 8 mice were allocated to both the PBS-LPS and phenylephrine-LPS groups.

Surgical peritonitis experiments

Surgical peritonitis was induced by cecal ligation and puncture (CLP) surgery. Under isoflurane anesthesia, a 1 cm incision was made in the shaved and disinfected lower abdomen. This was followed by incision of the muscular and peritoneal wall. The cecum was located and exteriorized, after which it was ligated at 60% and punctured once ("through and through") with a 21 gauge needle. After puncture, a small amount of feces was extruded and the cecum was placed back in the abdominal cavity. The peritoneal wall was closed with running sutures and the skin was closed with clips. Sham mice underwent the exact same procedures with the exception of cecal ligation and puncture. Micro-osmotic pumps containing either phenylephrine (30 µg/kg/min, dose was slightly lowered compared to the endotoxemia experiments because of the longer infusion period) or PBS were implanted directly following CLP, as described in the section above. Mice were resuscitated with 1 mL of saline post-surgery. Analgesia was provided by twice daily subcutaneous injections of 0.05 mg/kg buprenorphine on the day of surgery (of which one dose was administered 1 h before the procedure) and on the day afterwards. The health status of the animals was scored twice daily based on weight loss, posture and fur, activity, dehydration, and seclusion of the group. Based on this scoring system, presence of humane endpoints was determined. In case these were reached, animals were euthanized. For cytokine determination, 30 µL of lithium-heparin-anticoagulated blood was obtained via a small tail incision 4 hours after surgery and centrifuged (2000 g, RT), after which plasma was stored at -80°C until analysis. Forty-eight hours post-CLP, mice were culled by exsanguination under deep isoflurane anesthesia. Lithium heparin-anticoagulated blood was kept on ice for determination of bacterial counts. Peritoneal fluid was obtained by injecting 5 mL of sterile PBS into the peritoneal cavity followed by gentle agitation of the animal for 1 min. Subsequently, peritoneal fluid was harvested aseptically using a syringe and kept on ice for determination of bacterial counts. Spleen and liver were harvested aseptically and kept on ice for determination of bacterial counts. In total 23 mice were included in these experiments, of which 3 mice served as sham-operated controls and 10 mice were allocated to both the placebo-CLP and phenylephrine-CLP groups.

Homogenization of spleen and lung tissue and cytokine measurements

Frozen tissue samples were placed in a pre-cooled 2 mL eppendorf tube containing a 5 mm stainless steel bead (Qiagen) and incubated for 15 min on a dry ice/ethanol slurry. Tubes were then kept at RT for 2 min, after which 1 mL T-PER™ Tissue Protein Extraction Reagent (Thermo Scientific) containing cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche) was added. Samples were homogenized using a TissueLyser LT instrument

(Qiagen) for 5 min (spleen) or 7 min (lung) at 50 Hz. Tubes were centrifuged (10 min, 15000 rpm, 4°C), and supernatants were collected and stored at -80°C until cytokine analysis. Plasma, spleen, and lung concentrations of TNF-α, IL-1β, IL-6, IL-10, IP-10, keratinocyte chemoattractant (KC), monocyte chemoattractant protein 1 (MCP-1), and macrophage inflammatory protein 1 alpha (MIP-1α) were determined using a Luminex assay (Milliplex MAP Mouse Cytokine/Chemokine Panel) according to the manufacturer's instructions (Millipore). This assay has an intra-assay CV% of 2.6, 2.3, 1.5, 1.9, 2.5, 2.2, 3.3, and 1.4 for TNF-α, IL-6, MIP-1α, MCP-1, IL-1β, IP-10, KC and IL-10, respectively. The inter-assay CV% is 9.3, 5.4, 5.5, 4.3, 5.8, 3.4 and 5.1 TNF-α, IL-6, MIP-1α, MCP-1, IL-1β, IP-10, KC, and IL-10, respectively. Cytokine levels in spleen and lung homogenates were normalized to total protein content measured using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific) according to the manufacturer's instructions.

Neutrophilic ex vivo ROS production

One-hundred µL of lithium heparin-anticoagulated blood was added to tubes with preheated (10 min, 37°C) dihydrorhodamine 123 (DHR; 0.5 µg/mL, Sigma-Aldrich) or DHR supplemented with PMA (50 ng/mL, Sigma-Aldrich), and incubated for 15 min at 37°C with gentle vortexing every 5 min. After lysis of red blood cells (RBCs) using ammonium chloride solution for 5 min and subsequent washing, cells were resuspended in 100 µL PBA (PBS containing 1% bovine serum albumin [BSA, Sigma-Aldrich]) and incubated with an APC/Cy7-conjugated Ly-6G antibody (1:25; Biolegend, clone 1A8) for 5 min at RT for identification of neutrophils. Cells were washed (5 min, 500 g at 4°C) and resuspended in PBA, and measured on CytoFLEX flow cytometer (Beckman Coulter). Data were analyzed using Kaluza Analysis 1.5a (Beckman Coulter).

Ex vivo stimulation of splenocytes

Single cells suspensions were obtained by passing spleen tissue through a 70 uM cell strainer (BD), after which splenocytes were centrifuged (5 min, 460 g, 4°C). After lysing of RBCs using ammonium chloride solution for 10 min and washing, splenocytes were resuspended in cold RPMI containing 20% FBS. Subsequently, 250,000 cells per well were seeded in 96-well flat-bottom plates and PMA (20 ng/mL) and ionomycin (1 µg/mL) were added to the wells. Cells were incubated for 20 h at 37°C, 5% CO_2, after which plates were centrifuged and supernatants were stored at -80°C until determination of TNF-α and interferon (IFN)-γ concentrations using ELISA (Duosets, R&D systems). The intra-assay CV% was 2.4 and 3.1 for TNF-α and IFN-γ, respectively, whereas the inter-assay CV% was 3.2 and 1.2 for TNF-α and IFN-γ, respectively.

Plasma adrenaline and noradrenaline measurements

Plasma adrenaline and noradrenaline levels were determined using ELISA Fast Track kits (LDN, Nordhorn, Germany) according to the manufacturer's instructions with an intra-

assay CV% of 11.2 for adrenaline and 11.7 for noradrenaline.

Bacterial counts

Bacterial colony forming units (CFUs) were determined in blood and peritoneal fluid as well as in liver and spleen tissue. Liver tissue was homogenized in M-tubes using the "RNA" program on a gentleMACS instrument (both from Miltenyi). The homogenized samples were then filtered through a 70 µM cell strainer (BD). Spleen samples were mechanically homogenized through a 70 µM cell strainer. Thereafter, serial ten-fold dilutions from all samples were prepared, plated in triplicate on blood-agar plates (5% sheep blood, BD), and incubated overnight at 37°C, 5% CO_2 under aerobic conditions. Bacterial colonies were counted and results are specified as CFU per mL (blood, peritoneal fluid) or per organ (spleen and liver).

HUMAN *IN VIVO* STUDIES

Subjects and study design

Screening was performed before entry into the study; all subjects had normal physical examination, electrocardiography and routine laboratory results. Exclusion criteria were febrile illness during the 2 preceding weeks before trial, use of prescription drugs, history of hypertension or spontaneous vagal collapse and participation in earlier endotoxemia trials. Subjects were similar between phenylephrine and saline groups, respectively: median [min-max] age of 22 [19-28] vs. 23 [18-30] years, p=0.35; BMI of 22.7 [18.5-27.7] vs. 20.0 [18.0-27.8] kg/m², p=0.21; heart rate of 68 [48-85] vs. 62 [51-87] bpm, p=0.19; and mean arterial pressure of 90 [69-101] vs. 92 [80-99] mmHg, p=0.35.

Experimental human endotoxemia procedures and monitoring of vital signs

Endotoxemia experiments were conducted at the research unit of the intensive care department of the Radboud university medical center according to our standard protocol(3). Subjects refrained from caffeine and alcohol in the 24 hours before the experiment and from food and drinks for 10 hours before the experiment. In the antecubital vein two venous canulae were placed, one for fluid infusion and endotoxin administration and the other for study drug administration. In the hour before LPS administration, subjects were prehydrated with 1.5L 2.5% glucose/0.45% saline, followed by infusion of 150 mL/h saline for 6 hours and 75 mL/h saline for the rest of the experiment. Blood pressure monitoring and blood withdrawals were performed via an arterial cannula placed in the radial artery. There was continuous ECG and SaO_2 monitoring. Vital signs were recorded on a Phillips MP50 patient monitor using an in-house developed data capturing system. Body temperature was measured every 30 mins with infrared tympanic measurements (FirstTemp Genius 2, Covidien). The dose of phenylephrine was based upon dosing of noradrenaline (0.05 µg/kg/min) in a previous endotoxemia study by our group(4). As the vasopressor effect of phenylephrine is

approximately tenfold less potent than that of noradrenaline(5), a tenfold higher dose of phenylephrine was used.

Determination of plasma cytokines, adrenaline and noradrenaline levels, and hemocytometry

Blood was collected in EDTA tubes (Vacutainer, BD) and immediately centrifuged at 2000g at 4 °C for 10 min for cytokine analysis. Plasma was subsequently stored at -80°C until levels of TNF-α, IL-6, IL-8, IP-10, granulocyte colony-stimulating factor (G-CSF), MCP-1, and IL-10 were measured using a luminex assay (Milliplex, Millipore), with an intra-assay CV% of 1.8, 1.6, 2.0, 1.9, 2.6, 1.5, and 2.6 for G-CSF, IL-10, IL-6, IL-8, IP-10, MCP-1, and TNF-α, respectively. The inter-assay CV% is 15.5, 16.8, 18.3, 3.5, 15.3, 7.9, and 13.0 for G-CSF, IL-10, IL-6, IL-8, IP-10, MCP-1, and TNF-α, respectively. For determination of adrenaline and noradrenaline levels, blood was collected in into lithium heparin tubes (Vacutainer, BD) that were immediately placed on ice and centrifuged at 2000 g at 4 °C for 10 min, after which plasma was stored at −80 °C until analysis using HPLC with fluorometric detection, as described previously(6). The total imprecision of the adrenaline and noradrenaline assays are 6.8% at 0.133 nmol/L and 4.4% at 1.52 nmol/L, respectively. Accuracy, as assessed by recovery experiments, is between 98.6 – 104.6% and 94.9 – 99.9% for adrenaline and noradrenaline, respectively. Hemocytometry was performed in EDTA-anticoagulated blood using routine analysis methods also used for patient samples (flow cytometric analysis on a Sysmex XE-5000).

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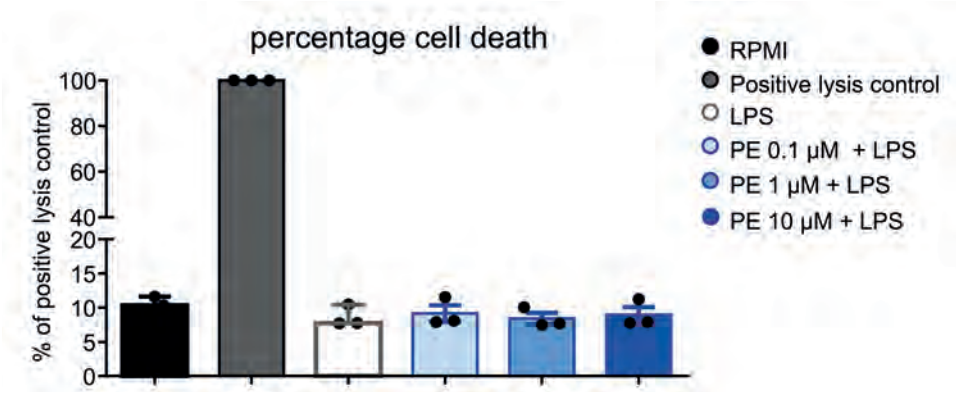
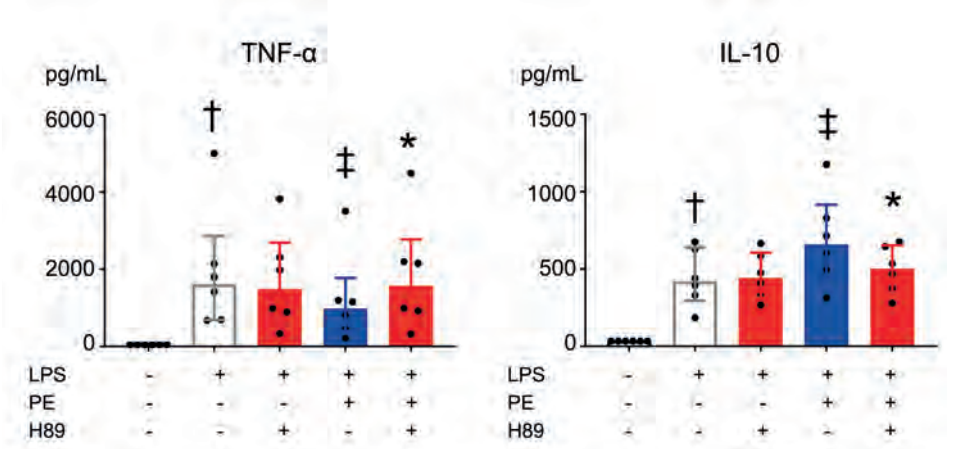
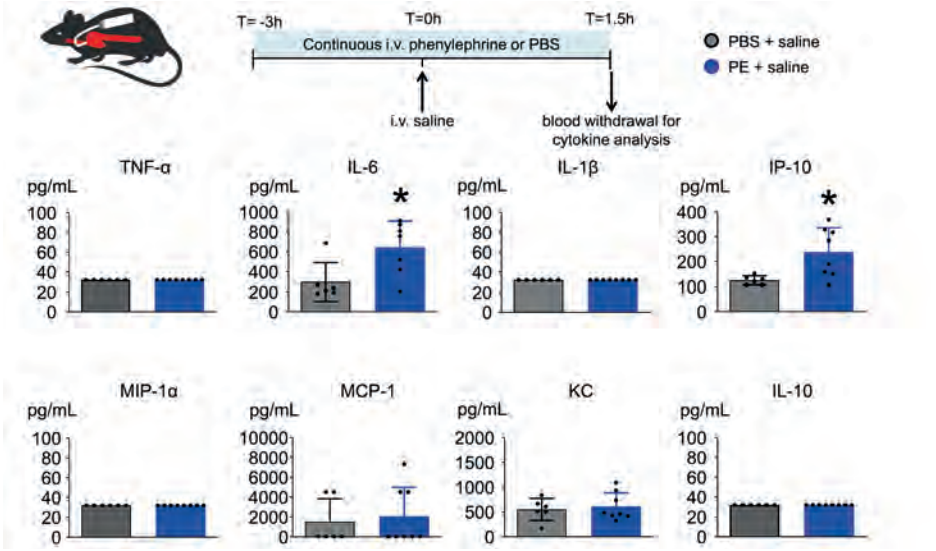


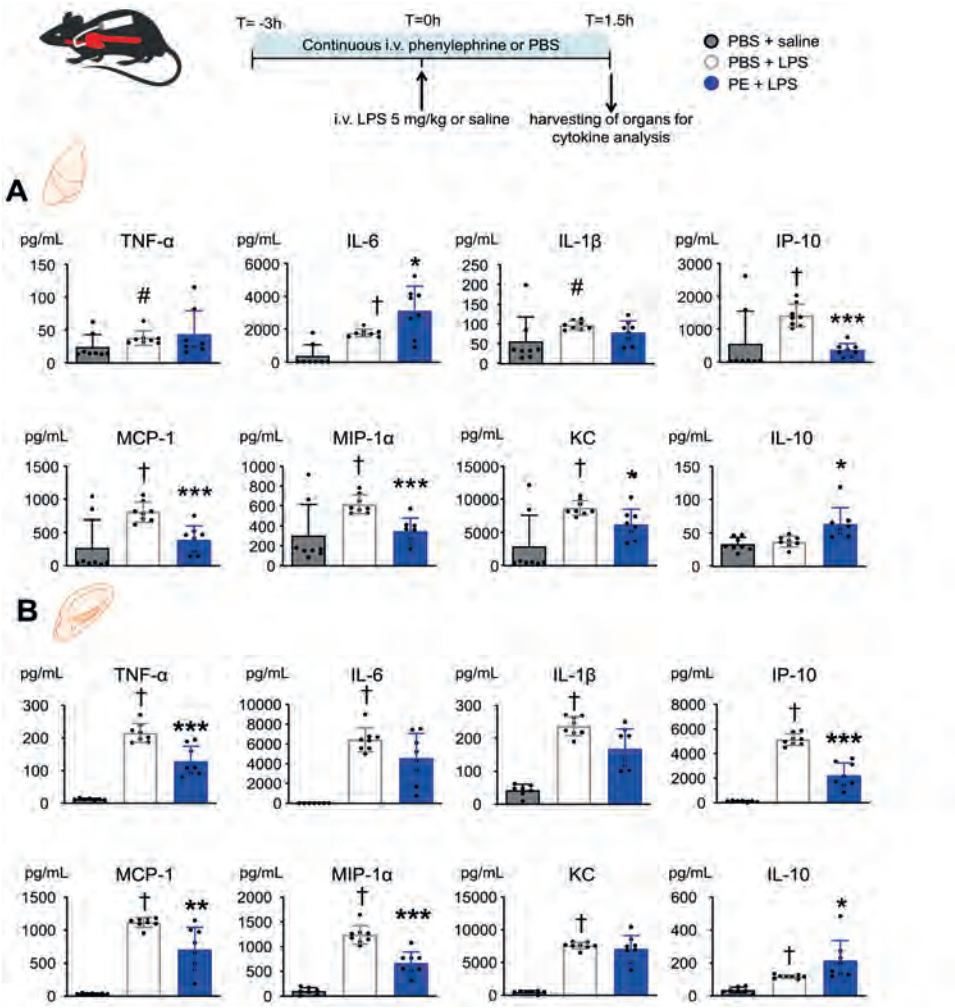
Supplementary Figure 1. Phenylephrine does not affect cell survival. Percentage cell death measured by lactate dehydrogenase (LDH) release in human whole blood cultures pre-incubated with either RPMI (medium control) or phenylephrine (PE, 0.1 µM, 1 µM, 10 µM) for 1h and subsequently stimulated with LPS (10 ng/mL) or RPMI for 24h. Positive controls are otherwise untreated samples incubated with lysis solution. The amount of LDH measured in the positive controls was set at 100% and LDH levels in the experimental samples were calculated accordingly. Data are expressed as individual data points and median and IQR of 3 individual donors.



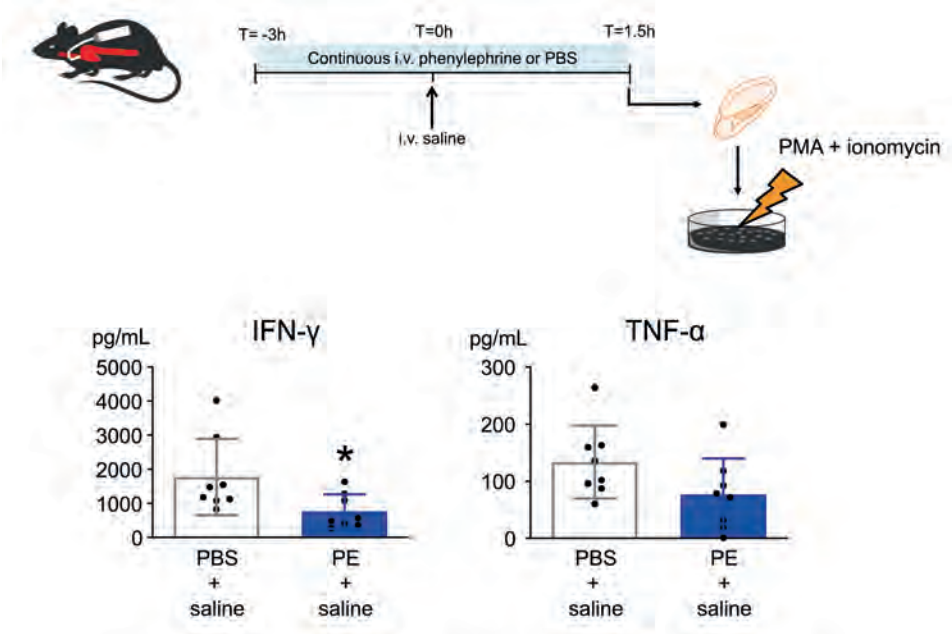
Supplementary Figure 2. Protein kinase A inhibition reverses the phenylephrine-induced modulation of cytokine production. Concentrations of TNF-α and IL-10 in supernatants of primary human monocyte cultures preincubated with either RPMI (medium control) or the protein kinase A (PKA) inhibitor H89 (3 µM) for 30 min, followed by incubation with PE (1 µM) for 1h and subsequent stimulation with LPS (10 ng/mL) or RPMI for 24h. Data are expressed as median and IQR of 6 individual donors. †$p<0.05$ compared to RPMI; ‡$p<0.05$ compared to LPS; *$p<0.05$, compared to PE + LPS calculated using Wilcoxon matched pairs tests.



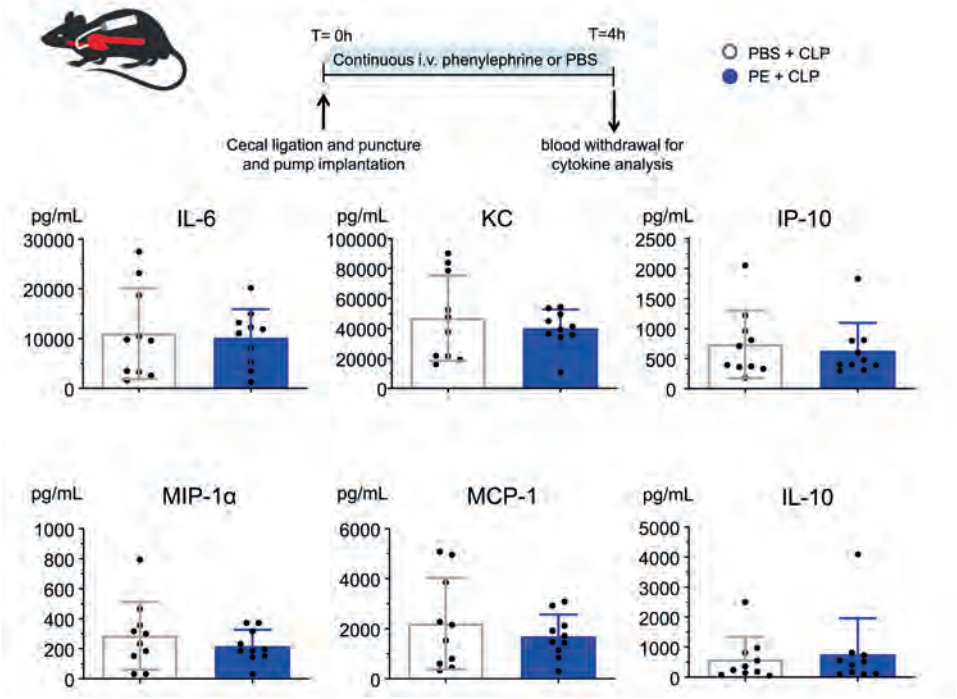
Supplementary Figure 3. Phenylephrine infusion enhances circulating IL-6 and IP-10 concentrations in non LPS-challenged mice. Plasma concentrations of TNF-α, IL-6, IL-β, IP-10, MIP-1α, MCP-1, KC, and IL-10 in C57B/6J mice intravenously infused with phenylephrine (PE, 50 µg/kg/min) or PBS via a micro-osmotic pump connected to a jugular vein catheter for 4.5h and injected intravenously with saline 3h after start of infusion. Data are expressed as individual data points and mean±SD of 6-8 animals per group. *p<0.05 compared to PBS + saline calculated using t-tests.



Supplemental Figure 4. Phenylephrine infusion modulates cytokine levels in lungs and spleens of LPS-challenged mice. (A) Lung and (B) spleen concentrations of TNF-α, IL-6, IL-β, IP-10, MIP-1α, MCP-1, KC, and IL-10 in C57B/6J mice intravenously infused with phenylephrine (PE, 50 µg/kg/min) or PBS via a micro-osmotic pump connected to a jugular vein catheter for 4.5h and challenged intravenously with LPS (5 mg/kg) or saline 3h after start of infusion. Data are expressed as individual data points and mean±SD of 7-8 animals per group. #p=0.05-0.10, †p<0.05 compared to PBS + saline; *p<0.05, **p<0.01, ***p<0.001 compared to PBS + LPS calculated using t-tests.



Supplementary Figure 5. Phenylephrine infusion modulates IFN-γ production by *ex vivo* stimulated splenocytes. Concentrations of IFN-γ and TNF-α in supernatant of splenocytes ex vivo stimulated with PMA (20 ng/mL) and ionomycin (1 µg/mL) for 20h. Splenocytes were obtained from C57B/6J mice that were intravenously infused with phenylephrine (PE, 50 µg/kg/min) or PBS via a micro-osmotic pump connected to a jugular vein catheter for 4.5h and injected intravenously with saline 3h after start of infusion. Data are expressed as individual data points and mean±SD of 8 animals per group. *p<0.05 compared to PBS + saline calculated using a t-test.



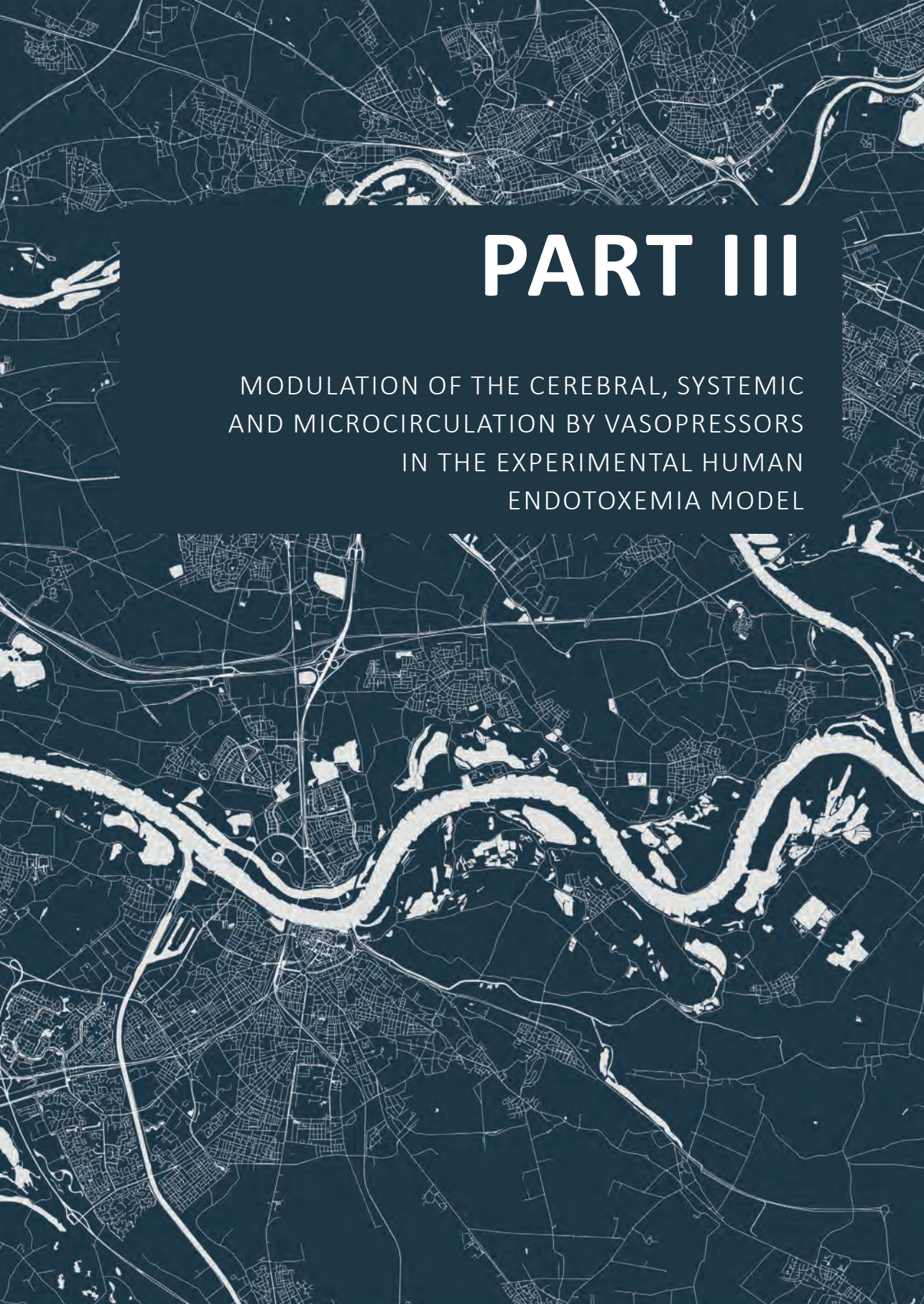
Supplementary Figure 6. Phenylephrine infusion does not modulate cytokine production during surgical peritonitis. Plasma concentrations of IL-6, KC IP-10, MIP-1α, MCP-1, and IL-10 in C57B/6J mice intravenously infused with phenylephrine (PE, 30 μg/kg/min) or PBS for 2 days via a micro-osmotic pump connected to a jugular vein catheter and subjected to cecal ligation and puncture (CLP) to induce sepsis. Blood was withdrawn 4h after induction of CLP. TNF-α concentrations were below the detection limit (3.2 pg/mL) in all animals. Data are expressed as individual data points and mean±SD of 10 animals per group.

THE ARRIVE ESSENTIAL 10: AUTHOR CHECKLIST

Item	Recommendation	Section/line number
Study design	a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. b. The experimental unit (e.g. a single animal, litter, or cage of animals).	a & b. Supplementary materials & methods, section "Murine *in vivo* studies"
Sample size	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used.	a. Supplementary materials & methods, section "Murine *in vivo* studies"
	b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	b. Materials & methods, section "Murine *in vivo* studies"
Inclusion and exclusion criteria	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori*. If no criteria were set, state this explicitly.	a. Supplementary materials & methods, section "Murine *in vivo* studies"
	b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so.	b. No exclusions, see materials & methods, section "Murine *in vivo* studies"
	c. For each analysis, report the exact value of *n* in each experimental group.	c. Materials & methods, section "Murine *in vivo* studies" and figure legends
Randomisation	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence.	a. Materials & methods, section "Murine *in vivo* studies"
	b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	b. Materials & methods section, Murine *in vivo* studies
Blinding	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	Materials & methods, section "Murine in vivo studies"

THE ARRIVE ESSENTIAL 10. CONTINUED

Item	Recommendation	Section/line number
Outcome Markers measures	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes). b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	a. Supplementary materials & methods, section "Murine *in vivo* studies" b. Materials & methods, section "Murine *in vivo* studies"
Statistical methods	a. Provide details of the statistical methods used for each analysis, including software used. b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	a & b. Materials & methods, section "Statistical analysis"
Experimental Strain Animals	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight. b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	a & b. Materials & methods, section "Murine *in vivo* studies"
Experimental procedures	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. What was done, how it was done and what was used. b. When and how often. c. Where (including detail of any acclimatisation periods). d. Why (provide rationale for procedures).	Supplementary materials & methods, section "Murine *in vivo* studies"
Results	For each experiment conducted, including independent replications, report: a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range). b. If applicable, the effect size with a confidence interval.	a & b. Results, sections "Continuous infusion of phenylephrine exerts anti-inflammatory effects in mice" and "Continuous infusion of phenylephrine increases bacterial dissemination during surgical peritonitis"



PART III

MODULATION OF THE CEREBRAL, SYSTEMIC AND MICROCIRCULATION BY VASOPRESSORS IN THE EXPERIMENTAL HUMAN ENDOTOXEMIA MODEL

CHAPTER 7

VASOPRESSORS DO NOT INFLUENCE CEREBRAL CRITICAL CLOSING PRESSURE DURING SYSTEMIC INFLAMMATION EVOKED BY EXPERIMENTAL ENDOTOXEMIA AND SEPSIS IN HUMANS

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ABSTRACT

AIM

The aim of this study was to investigate the effects of different vasopressors on the cerebral vasculature during experimental human endotoxemia and sepsis. We used the critical closing pressure (CrCP) as a measure of cerebral vascular tone.

METHODS

We performed a prospective pilot study, at the intensive care department (ICU) of a tertiary care university hospital in the Netherlands, in 40 healthy male subjects during experimental human endotoxemia (administration of bacterial lipopolysaccharide [LPS]) and in 10 patients with severe sepsis or septic shock. Subjects in the endotoxemia study were randomized to receive a 5 hour infusion of either 0.05µg/kg/min noradrenaline (n=10, "LPS-nor"), 0.5 µg/kg/min phenylephrine (n=10, "LPS-phenyl"), 0.04 IU/min vasopressin (n=10, "LPS-AVP") or saline (n=10, "LPS-placebo") starting 1 hour before intravenous administration of 2 ng/kg LPS. In patients with sepsis, fluid resuscitation and vasopressor use was at the discretion of the medical team, aiming at normovolemia and a mean arterial pressure (MAP) >65mmHg using noradrenaline. The mean flow velocity in the middle cerebral artery (MFV_{MCA}) was measured by transcranial doppler (TCD) with simultaneously recording of heart rate, arterial blood pressure, respiratory rate and Oxygen saturation. CrCP was estimated using the cerebrovascular impedenance model.

RESULTS

The CrCP decreased in the LPS-placebo group from 52.6 [46.6-55.5] mmHg at baseline to 44.1 [41.2-51.3] mmHg at 270 min post-LPS (P=0.03). Infusion of phenylephrine increased the CrCP in the period before LPS administration from 46.9 [38.8-53.4] to 53.8 [52.9-60.2] mmHg (P=0.02), but after LPS administration, a similar decrease was observed compared with the LPS-placebo group. Noradrenaline or vasopressin prior to LPS did not affect the CrCP. The decrease in CrCP after LPS bolus was similar in all treatment groups. The CrCP in the sepsis patients equaled 35.7 [34.4-42.0] mmHg, and was lower compared to that in the LPS-placebo subjects from baseline until 90 min after LPS (P<0.01).

CONCLUSIONS

Experimental human endotoxemia results in a decreased CrCP due to a loss of vascular resistance of the arterial bed. Vasopressors did not prevent this decrease in CrCP. Findings in patients with sepsis are comparable to those found in subjects after LPS administration. Patients with sepsis, despite treatment with vasopressors, have a risk for low cerebral blood flow and ischemia.

INTRODUCTION

Sepsis-associated encephalopathy (SAE) is a common complication of sepsis and septic shock and associated with increased mortality and long-term cognitive impairment (1). Reduced cerebral blood flow (CBF) is one of the major features in the pathophysiology of SAE. In addition, autoregulation is frequently disturbed in patients with severe sepsis and SAE, and may further increase the risk of ischemia if blood pressure decreases below the lower limit of autoregulation (2).

The critical closing pressure (CrCP) is a method to describe and quantify characteristics of the cerebrovascular bed and is defined as the lower limit of arterial blood pressure below which vessels collapse and flow ceases (3, 4). CrCP is a valuable and clinically relevant tool in cerebrovascular research, as it allows for estimation of the minimal cerebral perfusion pressure required to prevent collapse of vessels and ischemia (5-7). The CrCP is mainly a feature of the arteriolar bed and the difference between the arterial (inflow) pressure and the CrCP is regarded as the driving pressure for cerebral perfusion (8). Because CrCP cannot be measured directly, several models have been developed to estimate CrCP indirectly from other physiological parameters or their derivatives. Varsos et al. proposed a model using cerebrovascular impedance to determine the CrCP. This model can accurately detect changes in vascular properties induced by changes in intracranial pressure (ICP), $PaCO_2$ and blood pressure (9).

Vasopressors are frequently applied to counteract hypotension and subsequent decreased cerebral perfusion in sepsis and septic shock (10). The effects of different vasopressors on cerebral hemodynamics in sepsis are however unknown. Directly studying the effects of different vasopressors on cerebral perfusion in sepsis patients is hampered by the heterogeneity of the patient population, encompassing large variability in hemodynamic compromise and vasopressor requirements. In addition, current sepsis resuscitation protocols dictate a central role for noradrenaline, with vasopressin only used as an additional drug in 'catecholamine-resistant' shock. The experimental human endotoxemia model is a safe and reproducible model of systemic inflammation in humans *in vivo*, capturing many (hemodynamic) hallmarks of early sepsis, including a reduced MAP and increased heart rate. As such, this model allows for a head-to-head comparison of the effects of different vasopressors on cerebral hemodynamics in sepsis-like conditions (11).

Taken together, microvascular and macrovascular alterations play a key role in the pathogenesis of SAE. The effects of vasopressors on the cerebrovascular tone in SAE are unknown, despite the widespread use these agents in daily clinical practice. The effects of vasopression on CBF may be mediated by a change in systemic MAP. Alternatively,

different vasopressors may induce changes in cerebrovascular resistance through direct effects on the cerebral vasculature.

The aim of this study was to investigate the effects of different vasopressors on the cerebral vasculature during experimental human endotoxemia and to compare these to data obtained in sepsis patients. We focused on the parameter CrCP as a measure of cerebral vascular tone.

METHODS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The human endotoxemia study was approved by the local Institutional Review Board (document number 2015-2079, NCT02675868) and written informed consent was obtained from all participants prior to the study.
The local Institutional Review Board waived the need for informed consent in the sepsis patients.

STUDY DESIGN, SETTING, AND SUBJECTS

We performed a randomized controlled experimental endotoxemia pilot study in healthy subjects. Furthermore, we performed a prospective observational pilot study in patients with severe sepsis or septic shock. Both studies were performed at the intensive care department (ICU) of a tertiary care university hospital in the Netherlands.

Forty healthy, non-smoking, male volunteers, aged 18 to 35 years, were included in the human endotoxemia study that was registered at Clinicaltrials.gov under NCT02675868. All subjects provided written informed consent and experiments were in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines, and approved by the local ethics committee (document number 2015-2079). Subjects were screened before start of the experiment and had a normal physical examination, electrocardiography and routine laboratory values. Subjects were excluded from participation in the endotoxemia trial in case of febrile illness in the two weeks preceding the experiment, use of any medication or nicotine, signs and symptoms of cardiovascular disease, renal or liver impairment, or in case they previously participated in an endotoxemia experiment. Subjects refrained from alcohol or caffeine intake 24 hours before the experiment and refrained from food 12 hours before the start of the endotoxemia experiment.

Ten patients with severe sepsis or septic shock were included. The local Institutional Review Board waived the need for informed consent. Severe sepsis or septic shock was defined by the international sepsis definition conference (12), requiring the use

of vasopressors. Sepsis patients were treated according to international guidelines for management of severe sepsis and septic shock (13). All sepsis patients were sedated and mechanically ventilated. Sedation was performed using propofol and/or midazolam and sufentanil to achieve a target Richmond Agitation and Sedation Scale (RASS) score of-3 to 0.

General exclusion criteria for all participants were an irregular heart rhythm, insufficient transtemporal bone window, age <18 years, pregnancy, thrombolytic therapy, refractory cardiogenic shock or life expectancy less than 24 hours.

EXPERIMENTAL HUMAN ENDOTOXEMIA

Purified lipopolysaccharide (LPS, US Standard Reference Endotoxin *Escherichia coli* O:113) obtained from the Pharmaceutical Development Section of the National Institutes of Health, supplied as a lyophilized powder, was reconstituted in 5 mL saline 0.9% for injection and vortex mixed for at least 20 minutes. The LPS solution was administered as an intravenous bolus injection at a dose of 2 ng/kg body weight in 1 min at *T*= 0 min as described previously (14). All subjects received 1.5 L of 2.5% glucose/0.45% saline solution in the hour before the administration of LPS, followed by 150 mL/h 2.5% glucose/0.45% saline solution during the first 6 hours after the LPS administration and 75 mL/h until the end of the experiment. LPS-induced symptoms, including headache, nausea, shivering, muscle and back pain, were scored every 30 min on a six-point Likert scale (0 = no symptoms, 5 = worst ever experienced), resulting in a total score of 0–25.

VASOPRESSORS

Subjects in the LPS trial were randomized using the sealed envelope method to receive either a 5 hour infusion of 0.05µg/kg/min noradrenaline (n=10, "LPS-nor"), 0.5 µg/kg/min phenylephrine (n=10, "LPS-phenyl"), 0.04 IU/min vasopressin (n=10, "LPS-AVP") or saline (NaCl 0,9%) (n=10, "LPS-placebo"). Continuous infusion of the vasopressors started at one hour before LPS administration until 4 hours afterwards. A visual outline of the study is depicted in **Figure 1**.

In sepsis patients, fluid resuscitation and vasopressor use was at the discretion of the medical team, aiming at normovolemia and a mean arterial pressure (MAP) >65mmHg, using noradrenaline.

DATA COLLECTION

In subjects participating in the endotoxemia study, the radial artery was cannulated using a 20 gauge arterial catheter (Angiocath; Becton Dickinson) which was connected to an arterial pressure monitoring set (Edwards Lifesciences). Heart rate, blood pressure, respiratory rate and oxygen saturation were recorded continuously, starting

2 hours before administration of LPS until discharge from the ICU 8 hours after LPS administration. In sepsis patients the radial artery was cannulated using a 20 gauge arterial catheter (Angiocath; Becton Dickinson) which was connected to an arterial pressure monitoring set (Edwards Lifesciences). Heart rate, blood pressure, respiratory rate and oxygen saturation were recorded continuously during 30 minutes.

The mean flow velocity in the middle cerebral artery (MFV_{MCA}) in both healthy volunteers and patients was measured using transcranial Doppler (TCD) through the temporal window with a 2-Mhz probe (Multi-Dop T Digital, Compumedics DWL, Singen, Germany) according to the method developed by Aaslid et al (15). The probe was positioned over the temporal bone window above the zygomatic arch and fixed. This procedure ensured that the angle and the individual depth of insonation remained constant during the investigation. The TCD was measured on both sides. For the recordings, the bone window with the most optimal signal was chosen. All recordings were made with subjects/patients in the supine position with the head elevated to 30°.

A minimum of 10-12 minute windows of MFV, heart rate (ECG) and arterial blood pressure (ABP) were simultaneously recorded on a laptop computer and stored on a hard disk with a sample rate of 200 Hz by an A/D converter (NI USB-6211, National Instrument, Austin, TX, USA). In subjects participating in the endotoxemia study, recordings were performed at 90 and 30 minutes before, and at 90, 210 and 270 minutes after LPS administration. Sepsis patients were measured once, under stable hemodynamic (normotensive) and respiratory conditions (normocapnic).

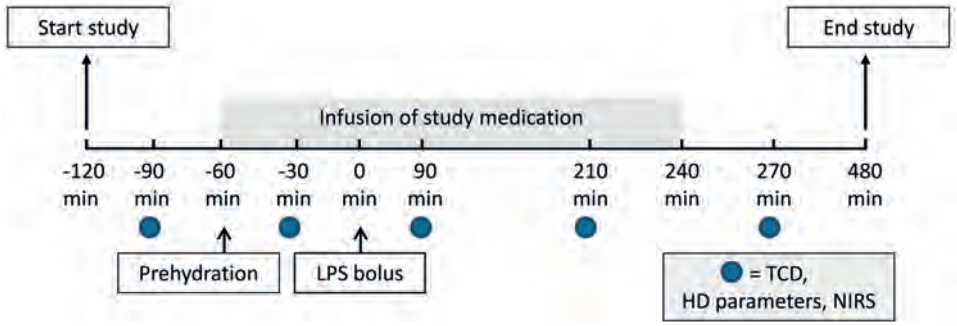

Figure 1. Outline of the human endotoxemia trial, HD Hemodynamic, LPS Lipopolysaccharide, TCD Transcranial Doppler, NIRS Near Infrared Spectroscopy

DATA ANALYSIS

ABP and MFV data were analysed using custom-written MATLAB scripts (Matlab R2014b, The MathWorks Inc. Massachusetts, USA). First, the time series were filtered with an 5th-order low-pass Butterworth filter (25 Hz), to ascertain signal stationarity. Second, periods of 5 minutes of artefact- and calibration-free data were selected by visual inspection for subsequent analysis. Finally,mean blood pressure and cerebral blood flow velocity were obtained simultaneously using a 4th order low-pass Butterworth filter.

CRITICAL CLOSING PRESSURE (CRcP)

CrCP was determined according to the method suggested by Varsos et al. (9, 16).

$$CrCP = ABP - \frac{CPP}{\sqrt{(CVR \cdot Ca \cdot HR \cdot 2\pi)^2 + 1}}$$

With CVR the cerebrovascular resistance, C_a the compliance of the vascular bed of the brain and HR the heart rate. The multiplication of CVR and C_a is also called the timeconstant Tau (τ). Cerebral perfusion pressure (CPP) is defined as ABP – ICP, however in this study the ICP was not measured. Therefore the mean ABP was used as an approach of CPP, as described by Varsos et al. (9). CVR was calculated by dividing ABPmean by MFVmean. To determine the C_a, the cerebral arterial blood volume (CABV) was calculated by integrating the MFV signal over time. Then C_a was calculated by dividing the amplitude of the first harmonic of the CABV by the amplitude of the first harmonic of the ABP. Heart rate (HR) was defined as the first harmonic frequency of ABP.

Calculations and statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). All baseline characteristics data are presented as means +/- SD, as they were normally distributed (determined by Shapiro-Wilk tests). All other results are presented as median with 25th and 75th percentile (interquartile range [IQR]), as they were not normally distributed (determined by Shapiro-Wilk tests). Changes over time were analyzed with Friedman tests. A P-value of <0.05 was considered to indicate significance.

RESULTS

POPULATION

Baseline characteristics of the healthy volunteers participating in the experimental human endotoxemia study are presented in **Table 1**. There were no differences in baseline characteristics between the 4 treatment groups. All subjects developed the typical flulike symptoms, starting approximately 75 minutes after LPS administration. As

expected, symptoms completely subsided after 5-6 hours.

TABLE 1. Demographic and clinical data at baseline of LPS subjects

	All subjects (n=40)	LPS-NE (n=10)	LPS-PE (n=10)	LPS-VP (n=10)	LPS- placebo (n=10)	
Age (years)	22.4 (±2.6)	20.9 (±1.6)	22.4 (±2.8)	22.9 (±2.2)	23.3 (±3.2)	p = 0.09
BMI (kg/m²)	22.6 (±2.7)	23.70 (±2.2)	22.61 (±2.7)	23.5 (±2.3)	20.0 (±2.9)	p = 0.34
HR (BPM)	66.6 (±10.5)	64.5 (±8.5)	67.8 (±10.8)	71.6 (±11.8)	62.50 (±10.0)	p = 0.41
MAP (mmHg)	89.0 (±7.4)	86.44 (±6.2)	87.6 (±10.3)	92.9 (±6.0)	91.5 (±5.2)	p = 0.27
Temperature (°C)	36.9 (±0.3)	36.9 (±0.4)	36.9 (±0.3)	36.9 (±0.4)	36.8 (±0.3)	p = 0.98
Hb (mmol/L)	8.9 [8.5 – 9.1]	8.9 [8.5 – 9.4]	8.8 [8.5 – 9.1]	8.8 [8.5 – 9.2]	8.9 [8.5 – 9.1]	p = 0.93

NE Noradrenaline, PE phenylephrine, VP Vasopressin, BMI Body mass index, HR Heart rate, MAP Mean arterial pressure, LPS Lipolypsaccharide

The 10 sepsis patients had a SAPS 2 score of 55.0 [48.5-76.8] and an APACHE 2 score of 24.5 [18.3-28.8]; 6 sepsis patients died in the ICU due to refractory shock and multi-organ failure. All patients in the sepsis population received noradrenaline (0.30 [0.24-0.45] µg/kg/min). Baseline characteristics of sepsis patients are presented in **Table 2**.

Table 2. Demographic and clinical data at baseline of sepsis patients

	Sepsis (n=10)
Age (years)	63.1 (±12.8)
BMI (kg/m²)	26.2 (±3.3)
HR (BPM)	105.2 (±13.3)
MAP (mmHg)	68.1 (±10.2)
Temperature (°C)	37.4 (±1.3)
Hb (mmol/L)	6.0 (±1.1)

BMI Body mass index, HR Heart rate, MAP Mean arterial pressure

HEMODYNAMIC AND CLINICAL DATA

The MAP in the LPS-placebo group was 91.0 [78.8-92.4] mmHg at the start of the experiment and decreased to 78.9 [71.3-88.5] mmHg after LPS administration (P<0.01) (**Figure 2**). Infusion of noradrenaline and phenylephrine significantly increased MAP by 12.6 [11.5-15.2] and 22.2 [9.4-37.1] % in the period before LPS administration (P=0.01), whereas vasopressin had no effects on MAP. LPS administration decreased MAP in all treatment groups, with no significant differences between groups. MAP in sepsis patients was lower than in healthy volunteers at all time-points (69.7 [66.4-73.5] mmHg, P<0.01). The temperature increased from 36.8 [36.6-37.2] °C at baseline to 38.4 [37.5-38.6] °C after LPS administration in the LPS-placebo group (P<0.01) and a similar temperature response was observed in endotoxemic subjects receiving vasopressors (data not shown).

The respiratory rate in the LPS-placebo group increased from 16.8 [14.0-20.0] resp/min to 19.4 [18.0-21.9] resp/min at 210 min post-LPS and 20.4 [19.7-21.5] resp/min at 270 min post-LPS (P<0.01). Administration of vasopressors neither affected respiratory rates before LPS administration, nor the LPS-induced increase in respiratory rate. Septic patients were ventilated aiming at normocarbia.

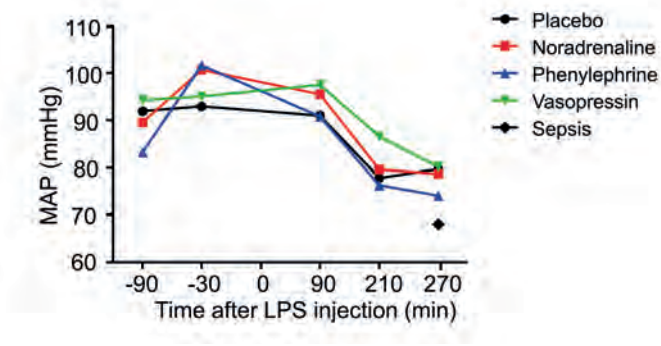

Figure 2. MAP after LPS injection. Time 0 = time of LPS bolus, LPS Lipopolysaccharide, MAP Mean arterial pressure

MFV AND CrCP

The MFV_{MCA} in the LPS-placebo group decreased significantly during human endotoxemia from 69.9 [64.2-85.3] cm/sec at baseline to 59.0 [54.3-63.1] cm/sec at 270 min post-LPS (P=0.01) (**Figure 3**). None of the vasopressors increased the MFV_{MCA} in the period before LPS administration. Despite infusion of the vasopressors, the MFV_{MCA} decreased upon LPS injection to values similar to that observed in the LPS-placebo group. The MFV_{MCA} in sepsis patients was 69.8 [54.6-83.3 cm/sec].

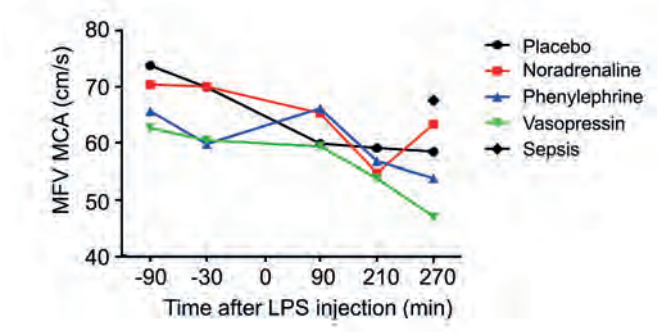

Figure 3. MFV_{MCA} in sepsis patients and after LPS injection. Time 0 = time of LPS bolus, LPS Lipopolysaccharide, MFVMCA Mean flow velocity in the middle cerebral artery.

The CrCP decreased significantly in the LPS-placebo group from 52.6 [46.6-55.5] mmHg at baseline to 44.1 [41.2-51.3] mmHg at 270 min after LPS administration (P=0.03) (**Figure 4**). Infusion of phenylephrine before LPS injection increased the CrCP significantly from 46.9 [38.8-53.4] to 53.8 [52.9-60.2] mmHg (P=0.02). Noradrenaline or vasopressin had no effect on the CrCP prior to LPS administration. The decrease in CrCP after LPS bolus was similar in all treatment groups. The CrCP in the sepsis patients was 35.7 [34.4-42.0] mmHg, which was significantly lower compared to values in the LPS-placebo subjects from baseline until 90 min post-LPS (P<0.01).

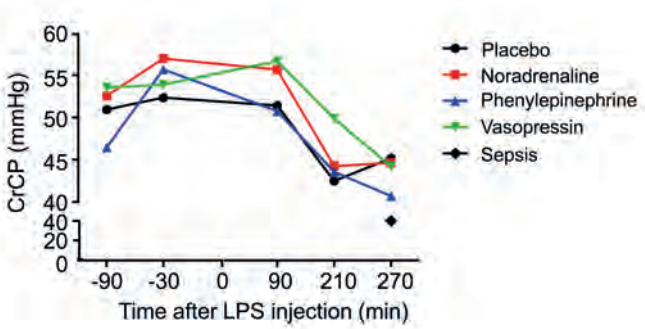

Figure 4. CrCP in sepsis patients and after LPS injection. Time 0 = time of LPS bolus CrCP Critical closing pressure, LPS Lipopolysacharride

The cerebrovascular arterial compliance (Ca) represents the change of arterial blood volume in response to change in arterial pressure and is estimated as a ratio of pulse amplitude of cerebral arterial blood volume and pulse amplitude of the arterial blood pressure. In the LPS-placebo group, Ca decreased from 0.16 [0.12-0.26] mmHg/cm^3 before LPS admission to 0.11 [0.10-0.14] mmHg/cm^3 at 270 min post-LPS (P=0.03) (**Figure 5a**). None of the vasopressors changed the Ca significantly before LPS administration. Furthermore, no significant changes compared to the placebo group were observed in subjects treated with vasopressors after LPS administration. Ca values in sepsis patients were lower compared to values in healthy volunteers before LPS administration (0.10 [0.07-0.13] mmHg/cm^3, P<0.01).

The CVR represents the resistance of small cerebral arteries and arterioles, which was estimated from the MAP and MFV. The initial CVR in endotoxemia subjects was 1.37 [1.01-1.45] mmHg*sec/cm and did not change significantly after LPS administration (P=0.63) (**Figure 5b**). Phenylephrine increased the CVR from 1.32[1.07-1.54] to 1.71[1.52-1.87] mmHg*sec/cm (P=0.02). No changes in CVR occurred in any of the other treatment groups. The CVR in sepsis patients was 1.11 [0.87-1.56] mmHg*sec/cm, and did not differ from the LPS subjects (P=0.50).

Tau (τ) is the time constant of the cerebral arterial bed and is the product of brain arterial compliance Ca and resistance CVR. It provides an estimation as to how quickly cerebral blood arrives in the cerebral arterial bed during each cardiac cycle. Tau decreased from 0.21 [0.17-0.25] sec to 0.15 [0.15-0.18] sec in the LPS-placebo group (P<0.01) (**Figure 5c**). None of the vasopressors changed Tau significantly in the period before LPS injection. Furthermore, no significant changes compared to the placebo group were found in the subjects treated with vasopressors after LPS administration. Tau in the sepsis group was 0.12 [0.08-0.15] sec, and significantly lower compared to values before LPS administration (P<0.01).

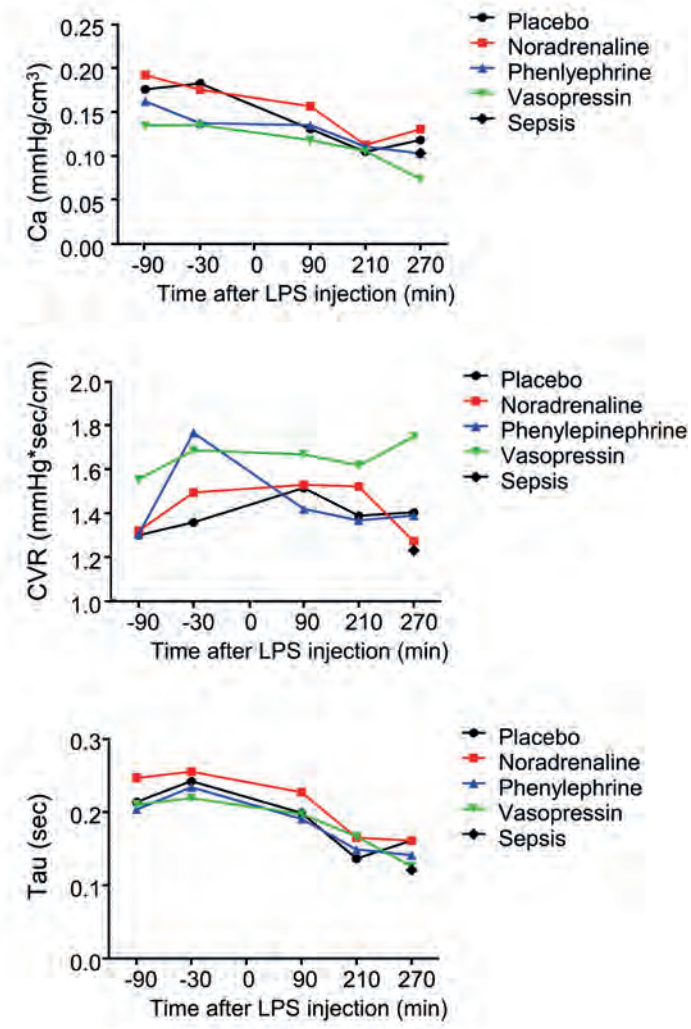

Figure 5. Ca, CVR and Tau in sepsis patients after LPS injection. (A) C_a Compliance of the vascular bed of the brain, (B) CVR Cerebrovascular resistance, (C) Tau Timeconstant, LPS Lipopolysaccharide, Time 0 = time of LPS bolus.

DISCUSSION

Endothelial dysfunction and disturbed NO production play a key role in the pathogenesis of SAE (17, 18). After LPS administration, cerebral blood flow velocity decreases (19) most likely as a result of decreased outflow from the circle of Willis to the middle and anterior cerebral arteries (20). The decrease in cerebral blood flow velocities was accompanied by a decrease in CrCP. This decrease in CrCP probably maintains adequate cerebral driving pressure and thereby serves as a mechanism to protect the brain against ischemia. The decrease in cerebrovascular compliance in our study is consistent with previous work indicating that systemic vasoparalysis does not affect the brain circulation in sepsis (21). In contrast to the systemic vasodilation in sepsis, decreased blood flow velocities together with higher pulsatility indices in patients with SAE strongly suggest vasoconstriction of the resistance arterioles in the brain (22).

Remarkably, CVR was unmodified during endotoxemia . Ideally, CVR is calculated by dividing pressure in the resistance arterioles by MFVmean. In this study, the CVR was estimated from the MAP and MFV in the MCA and thus not measured in the cerebral resistance arterioles. This may explain the fact that CVR did not change in endotoxemic subjects. Ca decreased in our study, reflecting a reduced ability of arteries to expand and contract with changes in blood pressure. Generally, Ca and CVR are inversely correlated, but the correlation between these parameters may change in pathological states, such as SAE (23).

Infusion of noradrenaline and phenylephrine before LPS bolus injection increased MAP, whereas vasopressin had no effect on blood pressure. The latter finding was not unexpected, because the hemodynamic effect of vasopressin in septic shock is based on the relative vasopressin deficiency in these patients (24, 25). Therefore, exogenous vasopressin infusion will not change the vascular tone in healthy subjects with adequate endogenous vasopressin levels. The increased MAP after initiation of noradrenaline and phenylephrine infusion did not result in a change of the MFV_{MCA}. Probably, the simultaneous increase in CrCP ensured a constant cerebral driving pressure despite the increase in MAP, suggestive of cerebrovascular adaptation in these subjects.

None of the vasopressors used in this study prevented the decrease in MFV_{MCA} after LPS administration. In addition, the decrease in CrCP in the subjects receiving vasopressors was similar to that in the placebo group. So far, few studies have compared the effectiveness of commonly used agents to augment cerebral perfusion, and the available literature is restricted patients suffering from traumatic brain injury (26-30). None of these studies found significant differences in cerebral hemodynamic responses or oxygenation between different vasopressors. However, several animal studies have

examined the effects of different vasopressors on cerebral perfusion. For instance, augmentation of cerebral perfusion with noradrenaline resulted in a more pronounced increase in brain tissue oxygenation compared to phenylephrine in a large animal model of pediatric closed head injury (31). The lack of effect of the α-agonist phenylephrine and the combined α-β-agonist noradrenaline in our study may reflect the low density of α and β receptors in the human cerebral vasculature. Alternatively, the dose of phenylephrine (0.5 μg/kg/min) and noradrenaline (0.05μg/kg/min) may have been too low to induce an effect on CBF, either directly by influencing cerebrovascular tone or indirectly by augmentation of MAP. The fact that CrCP decreased simultaneously with decreasing MAP, indicates that cerebrovascular adaptation was not affected by the use of phenylephrine and noradrenaline.

The reported effects of vasopressin on the cerebral vasculature are heterogeneous, with cerebral vasoconstriction or dilation depending on the species, artery size and vasopressin dose (32-35). The relevance of these studies for the treatment of patients with septic shock remains to be established. In our population of healthy subjects during endotoxemia, no effects of vasopressin (administered in a clinically relevant dose) on CBF were observed.

The cerebrovascular effects in the human endotoxemia model strongly resembled the findings in septic patients. In both endotoxemia and sepsis the MFV_{MCA} decreased, together with a lowered CrCP. Previously, dynamic cerebral autoregulation was believed to be enhanced in human endotoxemia and impaired in sepsis (19, 36). More recently it was suggested that dynamic cerebral autoregulation is also enhanced in the earlier stages of sepsis, indicating that in early sepsis cerebrovascular adaptation to changes in cerebral perfusion are similar to those observed in human endotoxemia (37). This suggests that the human endotoxemia model is a relevant model to study the efficiency of therapeutic approaches in SAE.

This study has a number of limitations. First, CrCP cannot be measured directly but is estimated using a mathematical model, with its inherent risks of bias. Most importantly, ICP is required for the most accurate calculation of CrCP, but this parameter was not obtained in the current study. Nevertheless, since ICP is low under septic conditions, it is unlikely that the absence of ICP data significantly influenced the estimation of CrCP (38). Second, we measured MAP through a catheter in the radial artery, at heart level instead of brain level. Measurement of the MAP in the MCA would have resulted in a more accurate estimation of cerebral perfusion pressure but is not feasible. Thirdly, cerebral perfusion changes after a LPS bolus or in sepsis may be heterogeneously distributed through the brain, with some areas more affected than others. As CrCP is derived from the MFV_{MCA}, this heterogeneity in flow cannot be assessed using this technique.

Fourth, as mentioned before, because healthy subjects were studied in the endotoxemia study, only low-dose vasopressors were administered. We cannot exclude a possible effect on cerebrovascular adaptation with higher dosing.

A fifth limitation of this study is the small number of subjects and that the population in the endotoxemia group differed from the sepsis group in terms of sex and age. We included only men in the endotoxemia study to limit subject variability, because we know from earlier experimental human endotoxemia studies that females show a more pronounced proinflammatory innate immune response associated with less attenuation of noradrenaline sensitivity (39). We included only young volunteers in the endotoxemia study to limit subject variability and for reasons of safety (comorbidity). Injection of E. coli endotoxin in elderly patients (median age 66yrs) is associated with a more pronounced reduction in blood pressure compared to younger control subjects (40). Aging is associated with decreasing cerebral blood flow velocities. However this occurs mainly in the posterior cerebral circulation and at significantly older ages than those of our sepsis population. A study measuring cerebral blood flow velocity and pulsatility index by transcranial Doppler in 303 healthy elderly subjects indicated a slight but non-significant decrease in cerebral blood flow velocities in the middle cerebral artery (33.8±0.9 to 32.2±1.1 and 34.8±0.9 to 32.8±1.1 cm/s) between patient groups 70-74 yrs to > 85 yrs. The pulsatility index (reflecting cerebrovascular resistance) increased from 1.55±0.04 to 1.66±0.04 and from 1.59±0.04 to 1.64±0.04 in these age groups (41). In our study, the CrCP in the (older) septic population was significantly lower compared to the (younger) endotoxemia group. In addition, the CVR was similar in both populations. This strongly suggests that in our population, changes in CrCP and CVR were mainly influenced by sepsis, rather than the age per se.

A sixth limitation of the study is that data obtained in healthy volunteers were not controlled for carbon dioxide tension: as the respiratory rate and body temperature increased after LPS administration, we cannot exclude an influence of altered levels of CO_2 on reduced MFV and cerebrovascular adaptation.

CONCLUSIONS

Cerebral blood flow velocity decreases during human endotoxemia and vasopressors do not prevent this decrease. In addition, vasopressors do not influence the change in CrCP during human endotoxemia. These results have important clinical implications as they indicate that titration of the systemic circulation to a specific minimal MAP with vasopressors will not automatically restore cerebral perfusion in patients with septic shock. Monitoring of cerebral perfusion parameters such as CrCP may assist in titration

of the optimal systemic MAP to prevent cerebral hypoperfusion and brain ischemia.

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CHAPTER 8

EFFECT OF VASOPRESSORS ON THE MACRO- AND MICROCIRCULATION DURING SYSTEMIC INFLAMMATION IN HUMANS *IN VIVO*

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ABSTRACT

AIM

Comparing the effects of different vasopressors in septic shock patients is hampered by high heterogeneity and the fact that current guidelines dictate the use of noradrenaline. Herein, we studied the effects of three vasopressor agents, noradrenaline, phenylephrine and vasopressin, on the macro- and microcirculation during experimental human endotoxemia, a standardized, controlled model of systemic inflammation in humans in vivo.

METHODS

We performed a randomized controlled study in which 40 healthy male volunteers were assigned to a five-hour infusion of either 0.05 µg/kg/min noradrenaline (n=10), 0.5 µg/kg/min phenylephrine (n=10), 0.04 IU/min vasopressin (n=10), or saline (n=10), starting one hour before intravenous administration of two ng/kg lipopolysaccharide (LPS). The macrocirculation was monitored using arterial catheter-derived parameters with additional blood pressure waveform contour analysis (PCA) until 4.5 hours following LPS administration. Sublingual microcirculatory density and flow were assessed using a handheld video microscope until 6 hours post-LPS.

RESULTS

LPS administration affected all macrocirculatory and microcirculatory parameters. The LPS-induced decrease in blood pressure and systemic vascular resistance (SVR) was refractory to low-dose noradrenaline and phenylephrine, and to a lesser extent, to vasopressin. Only vasopressin exerted effects on PCA parameters compared to placebo, by mitigating the LPS-induced decrease in diastolic blood pressure by stabilizing SVR and cardiac output. The endotoxemia-induced decreased indices of microvascular flow and density were not influenced by vasopressor therapy.

CONCLUSIONS

In a highly controlled model of systemic inflammation in humans in vivo, a five-hour infusion of various vasopressors revealed distinctive effects on macrohemodynamic variables without affecting the sublingual microcirculation.

INTRODUCTION

During septic shock, both systemic hemodynamics and the microcirculation are severely affected, and these alterations are associated with organ failure and impaired outcome (1). Noradrenaline is by far the most widely used vasopressor for septic shock. However, other vasopressors such as vasopressin or phenylephrine might hold an advantage when considering effects on both the microcirculation and systemic hemodynamics (2). Comparing the effects of different vasopressors in septic shock patients is hampered by the high heterogeneity of the disease and the fact that current guidelines dictate the use of noradrenaline as the first line vasopressor, and only advise the use of other compounds as 'add-on' treatment in catecholamine-resistant shock (3). Experimental human endotoxemia is a controlled, safe and reproducible model of systemic inflammation that mimics several of the microcirculatory and macrocirculatory changes observed in sepsis (4-6). In the present study, we aimed to study the effects of three vasopressor agents, noradrenaline, phenylephrine and vasopressin, on both the microcirculation and systemic hemodynamics during experimental human endotoxemia.

MATERIALS AND METHODS

SUBJECTS, STUDY DESIGN, AND ETHICS

We performed a randomized controlled experimental endotoxemia study in forty healthy male volunteers (18-35 years) at the intensive care department of a tertiary care university hospital in the Netherlands (Clinicaltrials.gov NCT02675868). All subjects provided written informed consent and the study was approved by the local ethics committee (registration no. 2015-2079). Experiments were carried out in accordance with the Declaration of Helsinki, including recent revisions, and Good Clinical Practice guidelines.

EXPERIMENTAL HUMAN ENDOTOXEMIA PROCEDURES

All subjects received an intravenous bolus injection with 2 ng/kg lipopolysaccharide (*E. coli*-derived LPS), and were randomized to receive either a five-hour infusion of 0.05 µg/kg/min noradrenaline (n=10), 0.5 µg/kg/min phenylephrine(n=10), 0.04 IU/min vasopressin (n=10) or placebo (NaCl 0,9%, n=10). Experimental procedures are detailed in our previous work (7). Infusion was started one hour before LPS administration. Furthermore, the study subjects received 1500 mL 2.5% glucose/0.45% saline during the hour prior to LPS administration, followed by 150 mL/h until six hours after LPS administration, and 75 mL/h for the remaining two hours. Both macro- and microcirculatory measurements were performed at baseline (T1), 30 minutes after initiation of vasopressor administration but before LPS administration (T2), 90

[macrocirculation] or 120 [microcirculation] minutes following LPS administration (T3, the height of the inflammatory response, characterized by peak levels of pro-inflammatory cytokines and flu-like symptoms(5)), 210 minutes post-LPS administration (T4, maximum hemodynamic effects (5), only macrocirculatory parameters were obtained at this timepoint), and 270 [macrocirculation] or 360 [microcirculation] minutes following LPS administration (T5, after cessation of vasopressor infusion).

MACROCIRCULATION MEASUREMENTS

All macrocirculation parameters were blood-pressured derived. The radial artery was cannulated using a 20-gauge arterial catheter (Angiocath, Becton Dickinson Pty Ltd, Franklin Lake, NJ, USA) which was connected to an arterial pressure monitoring set (Edwards. Lifesciences LLC, Irvine, California, USA). The arterial blood pressure (ABP) signal was recorded on a laptop computer and stored on a hard disk with a sample rate of 200Hz by an A/D converter (NI USB-6211, National Instrument, Austin, TX, USA) for off-line analysis. The ABP signal was analysed using custom-made MATLAB scripts (Matlab R2017b, The MathWorks Inc. Massachusetts, USA). Mean arterial blood pressure (MAP) was acquired by taking a fourth order Butterworth low-pass filter with a cut-off frequency of 0.02 Hz from the raw ABP signal. Heart rate (HR) was acquired by automatic detection of R-peaks from the ECG-signal. The used pulse contour analysis (PCA) accounts for the dependence of arterial compliance on arterial pressure by scaling its cardiac output (CO) estimate to pulse pressure, with stroke volume (SV) equalling pulse pressure divided by the sum of systolic (SBP)- and diastolic blood pressure (DBP) as proposed by Liljestrand and Zander (8, 9). SV was subsequently multiplied by HR to calculate cardiac output (CO). Systemic vascular resistance (SVR) was approximated by dividing MAP by CO.

MICROCIRCULATION MEASUREMENTS

A minimum of five steady video clips of at least 10 seconds were obtained from the sublingual region using a video microscope (CytoCam-IDF, Braedius Medical, Huizen, The Netherlands). Video microscopy was performed by a trained investigator (LvL) after removal of saliva while avoiding pressure artefacts. Video scoring was performed according to Massey *et al* (10). Vessel density was calculated as the number of vessels crossing arbitrary lines divided by the total length of these lines (i.e. Number of crossings). Quantification of flow (i.e. microvascular flow index (MFI) was categorized as 0:no flow, 1: intermittent flow, 2: sluggish flow, and 3: continuous flow, as described previously(4).

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). Normality was assessed using Shapiro-Wilk tests. Effects of vasopressor agents before LPS administration were analysed using paired Student's T-tests on T1 and

T2. LPS-induced changes over time were analysed using repeated measures one-way ANOVA on T2, T3, T4, and T5 in the placebo group only. Differences between vasopressor and placebo-treated subjects over time during endotoxemia were tested using repeated measures two-way ANOVA (interaction term) on T2, T3 and, for macrocirculatory parameters only, T4. A two-sided p-value of <0.05 was considered statistically significant.

RESULTS

SUBJECTS AND SYMPTOMS

There were no differences in baseline characteristics between the treatment groups, which are reported elsewhere (7). All subjects developed typical flu-like symptoms, peaking at 90 minutes following LPS administration, which were completely subsided 7-8 hours after the LPS challenge.

EFFECTS OF VASOPRESSORS PRIOR TO LPS ADMINISTRATION

Administration of noradrenaline and phenylephrine caused an immediate increase in blood pressure, but did not affect other macrocirculatory parameters prior to LPS administration (**Figure 1**). Vasopressin did not affect any of the macrocirculatory parameters, and none of the vasopressors affected microcirculatory parameters before LPS administration (**Figure 2**).

EFFECTS OF VASOPRESSORS DURING ENDOTOXEMIA

Except for SV, LPS administration resulted in significant changes of all macrocirculatory parameters (**Figure 1**). All blood pressure variables decreased, accompanied by a compensatory increase in HR, increased CO (at constant SV) and decreased SVR. MAP kinetics in the noradrenaline and phenylephrine groups were not significantly different from placebo. Vasopressin mitigated the LPS-induced decrease in DBP by stabilizing SVR and CO. The static blood pressures did not correlate to their corresponding PCA parameters (SVR, CO and SV) in any of the groups (Pearson correlation p-values >0.10). LPS administration resulted in decreased microvascular density and flow, which were not changed by any of the vasopressors (**Figure 2**).

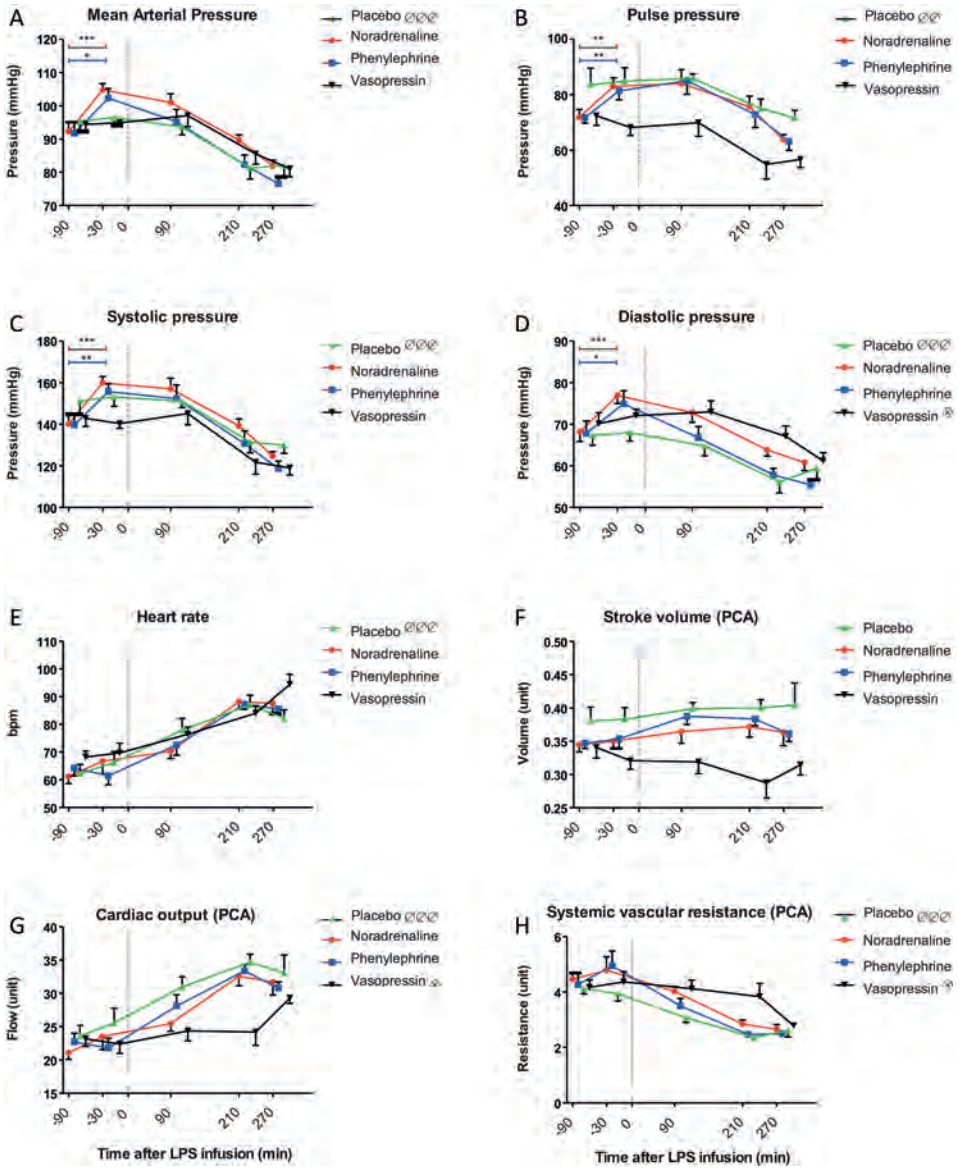

Figure 1. Macrocirculatory parameters before and after LPS administration. (A) Mean arterial pressure, (B) Pulse Pressure, (C) Cardiac output (pulse contour analysis), (D) Systemic vascular resistance (pulse contour analysis), (E) Heart rate (pulse contour analysis), (F) Stroke volume (pulse contour analysis), (G) Cardiac output (pulse contour analysis), and (H) Systemic vascular resistance (pulse contour analysis). LPS was administered at timepoint 0, indicated by the vertical dashed line. Data are expressed as mean and SEM. *: p<0.05, **: p<0.01 and ***: p<0.001 (paired Student's t-tests on-90 [T1] and-30 [T2] within treatment groups). ØØ: p<0.01 and ØØØ: p<0.001 over time (-90 – 270 [T1-T2]) within placebo group (repeated measures one-way ANOVA). ⊗: p<0.05 over time (-30 - 210 [T1-T4]) vs. placebo (repeated measures two-way ANOVA, time*treatment interaction term).

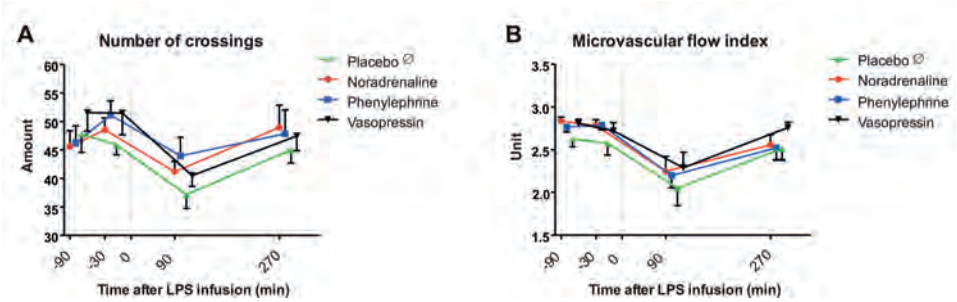

Figure 2. Sublingual microcirculatory parameters before and after LPS administration. (A) Number of crossings and (B) Microvascular Flow index. LPS was administered at timepoint 0, indicated by the vertical dashed line. Data are expressed as mean and SEM. ∅: $p<0.05$ over time (-90- 270 [T1-T5]) within placebo group (repeated measures one-way ANOVA).

DISCUSSION

Our study demonstrates that the decrease in blood pressure and SVR during experimental endotoxemia is refractory to low-dose noradrenaline and phenylephrine therapy, and to a lesser extent, to vasopressin administered at a dosage used in clinical practice for the treatment of septic shock. Vasopressin prevented the endotoxin-induced increase in CO and decrease in SVR. Furthermore, endotoxemia resulted in decreased indices of sublingual microvascular flow, which were not affected by any of the vasopressors.
Expectedly, both noradrenaline and phenylephrine caused an increase in blood pressure prior to LPS administration. While these elevated levels were maintained during the peak of the inflammatory response, the LPS-induced decrease in blood pressure was not prevented. Vasopressin did not increase blood pressure prior to LPS administration. Unlike patients with sepsis, this can be explained by the fact that vasoconstrictive effects of vasopressin infusion are antagonized by intrinsic activation of the baroreflex in healthy volunteers under non-inflammatory conditions (11). PCA allowed us to break down blood pressure into flow and resistance. Complementary to our previous findings, we anew showed that experimental human endotoxemia results in a loss of vascular resistance of the arterial bed (7). Interestingly, vasopressin mitigated the LPS-induced decrease in SVR, a hallmark of sepsis-induced hypotension (12).

Our study underscores that limiting hemodynamic monitoring in critically ill patients to solely blood pressure is insufficient, as it neglects the causative physiological processes (CO and SVR) and its ultimate goal (improving microvascular perfusion). The lack of coherence between blood pressure and these other parameters is a well-known phenomenon in sepsis (13, 14) . Accordingly, in our model, there were no correlations between blood pressure and PCA parameters. Furthermore, despite clear effects on the macrocirculation both prior to (noradrenaline and phenylephrine) and

after LPS administration (vasopressin), the different vasopressors did not influence sublingual microcirculatory parameters. In accordance with earlier work (4), the sublingual microcirculation was profoundly altered during endotoxemia but remained intact (indicated by high >2 MFI-values). Previous work in a model of septic shock in pigs revealed that noradrenaline and phenylephrine improved macrocirculatory parameters (e.g. MAP and cardiac index) (2). However, both pressors only marginally affected microcirculatory flow measured in seven organs: noradrenaline decreased microcirculatory blood flow in the jejunal mucosa, whereas phenylephrine increased microcirculatory jejunal muscularis flow (2). As such, the sole measurement of blood pressure can be misleading, as it may suggest that vasopressor therapies or resuscitation manoeuvres are adequate, while perfusion at the tissue level is or remains markedly compromised (14).

Several study limitations deserve attention. First, knowing that the ideal model of sepsis does not exist, our model has proven to be highly controlled, reproducible and representative for several hallmarks of sepsis (5). Nevertheless, since healthy subjects were studied, only low dosages of noradrenaline and phenylephrine could be safely administered. Higher dosages of these agents may affect the microcirculation. Second, microcirculatory parameters were determined in the sublingual vascular bed. Although the sublingual area is the preferred site for non-invasive microcirculation measurements and this approach is widely accepted as a measure of the systemic microcirculation, we cannot exclude the possibility of heterogeneity between different tissues. Third, because PCA converts pressure measurements into volume parameters using assumptions of the dynamic characteristics of the arterial vasculature, uncalibrated PCA may not yield accurate results upon changes in SVR. Furthermore, PCA remains arduous for implementation in everyday clinical practice, partly because of the use of inscrutable algorithms. We advocate for the use of well-documented, open source, and straightforward formulas, as employed in the present work.

In conclusion, various vasopressors exert distinctive effects on macrohemodynamic variables without affecting the sublingual microcirculation in a highly standardized controlled model of systemic inflammation in humans *in vivo*. Furthermore, our data indicate that blood pressure measurements do not adequately reflect physiological parameters that are of vital importance in the critical care setting, such as CO, SVR, and microvascular perfusion. Uncalibrated PCA could be a helpful, less-invasive tool in monitoring hemodynamic responses to interventions and in disease.

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CHAPTER 9

SUMMARY

SUMMARY

In this thesis, our primary aim was to study the effects of vasopressors on host defense mechanisms. Secondly, we investigated vascular effects of vasopressors during systemic inflammatory conditions. In **Part I,** the concept of sepsis-induced immunoparalysis and its clinical consequences is explained, immunological effects of vasopressors are reviewed, noradrenaline's potential contribution to immune dysregulation in sepsis is hypothesized, and alternative vasopressors are proposed. In **Part II,** we investigated the effects of several vasopressors, including noradrenaline, on the immune response and host defense using a translational approach, and we studied the contribution of genetic variation in the β2 adrenergic receptor to noradrenaline-induced immune dysregulation and the systemic inflammatory response *per se*. **Part III** focuses on the effects of several vasopressors on cerebral, microcirculatory and macrocirculatory parameters during systemic inflammation in humans.

Chapter 1 contains the general introduction and outline of this thesis. Sepsis is the leading cause of death worldwide, and the WHO has designated it a global health priority. It is defined as a dysregulated host response to infection, comprising both pro-and anti-inflammatory responses. A too pronounced pro-inflammatory or 'hyperinflammatory' response, for example observed in the so-called 'macrophage activation syndrome' clearly has detrimental consequences. However, it has become increasingly evident that rather a profound or protracted anti-inflammatory response, known as 'sepsis-induced immunoparalysis' is associated with the majority of sepsis deaths. Immunoparalysis entails several features, such as reduced production of pro-inflammatory cytokines and enhanced production of anti-inflammatory cytokines, reduced HLA-DR expression on antigen presenting cells and impaired immunometabolism. This may render patients unable to clear their primary infection and increases vulnerability towards secondary infections, often with opportunistic pathogens. Outcome is particularly poor for patients with septic shock, the most severe form of sepsis, as they suffer from hypotension and extended organ dysfunction due to vascular leakage and loss of peripheral vascular resistance. This necessitates the administration of vasopressors, aimed at restoration of organ perfusion, of which noradrenaline is the primary agent of choice worldwide. However, *in vitro* studies indicate that noradrenaline can exert anti-inflammatory effects and therefore may compromise host defense. However, *in vivo* data on immunomodulatory effects of noradrenaline and alternative vasopressors were largely lacking before the conduct of the studies described in this thesis.

PART I IMMUNOMODULATION BY VASOPRESSORS; AN OVERVIEW OF THE LITERATURE AND POSSIBLE CLINICAL CONSEQUENCES

In **Chapter 2,** we provide a comprehensive review of the available evidence of noradrenaline's inadvertent immunomodulatory effects in light of sepsis-induced immunoparalysis. *In vitro* studies indicate that noradrenaline exerts anti-inflammatory effects via stimulation of the β-adrenergic receptor. These include attenuation of pro-inflammatory cytokine production, enhanced production of anti-inflammatory cytokines, and diminished cytotoxicity of natural-killer cells. In addition, limited animal studies extended the *in vitro* findings to the *in vivo* situation by demonstrating anti-inflammatory effects after noradrenaline administration. Finally, there exists circumstantial clinical evidence for noradrenaline's role in the development and perpetuation of sepsis-induced immunoparalysis, such as the fact that circulating noradrenaline levels are a risk factor for sepsis mortality, independently from disease severity and hemodynamic parameters, and that administration of β-blockers dramatically improved outcome in a clinical trial in septic shock patients. The available evidence for immunologic effects of potential alternative vasopressors is reviewed as well, although, due to the sparsity of data, this section is brief. We conclude that translational work on the immunologic effects of noradrenaline and alternative vasopressors are highly warranted. **Chapter 3** focuses on the intermediary roles that noradrenaline and immunoparalysis play in adverse outcomes in sepsis patients. This chapter starts with a brief overview of the features and importance of immunoparalysis in sepsis patients. We highlight that patients admitted with high illness severity scores are prone to contract ICU-acquired infections, have a prolonged ICU length of stay and a higher mortality. Therefore, immunoparalysis represents an intermediary factor which links disease severity in sepsis patients to detrimental consequences. This illustrates the need for strategies aimed at reversing sepsis-induced immunoparalysis and necessitates reevaluation of standard of care practices. More severely ill patients are more likely to suffer from hemodynamic instability, and therefore to receive noradrenaline, which exerts anti-inflammatory effects. Hence, we note that noradrenaline acts as an intermediary factor as well, linking disease severity and immunoparalysis. This is of clinical relevance, as noradrenaline is a modifiable factor which can be exploited to prevent immunoparalysis. We describe and discuss several knowledge gaps that need to be bridged to further explore this concept.

PART II IMMUNOMODULATION BY VASOPRESSORS, TRANSLATIONAL STUDIES

Chapter 4 comprises translational studies into the immunomodulatory effects of noradrenaline and the alternative non-catecholaminergic vasopressor vasopressin, based on the knowledge gaps identified in part I of this thesis. We demonstrate potent anti-inflammatory effects of noradrenaline *in vitro*, exemplified by diminished production of pro-inflammatory cytokines such as TNF-α, IP-10 and IL-1β and enhanced anti-inflammatory IL-10 production in response to stimulation with a plethora of

specific TLR- and pathogen-derived stimuli. Monocytes are identified as the blood cell type responsible for these effects. In addition, we show that noradrenaline inhibits metabolic pathways in monocytes and reduces generation of reactive oxygen species, an important anti-bacterial defense mechanism. Using specific antagonists and blockers, we demonstrate that these effects are mediated via β-adrenergic receptors and increased levels of intracellular downstream molecules cAMP and PKA. Importantly, vasopressin did not exert any of these immunomodulatory effects across all of these *in vitro* setups. Subsequently, these findings are translated to the *in vivo* situation using murine endotoxemia and sepsis models. In these experiments, noradrenaline and vasopressin were continuously infused intravenously via implanted micro-osmotic pumps to mimic the clinical situation. A 3- or 24-hour infusion with noradrenaline dose-dependently attenuated circulating pro-inflammatory cytokine levels while enhancing IL-10 concentrations in endotoxin-challenged mice. Furthermore, noradrenaline impaired *ex vivo* production of reactive oxygen species. The functional consequences of noradrenaline's pleiotropic attenuating effects on host defense mechanisms were explored in a murine sepsis model, where noradrenaline administration led to increased systemic bacterial dissemination. In addition, similar to the observations in endotoxin-challenged mice, noradrenaline skewed the pro-anti-inflammatory balance towards an anti-inflammatory phenotype. Again, vasopressin did not modulate any of the investigated host defense parameters across our murine experiments. Thereafter, we translated the findings to the human situation using the experimental human endotoxemia model (intravenous endotoxin administration in healthy volunteers). In this model, low-dose noradrenaline infusion significantly reduced circulating pro-inflammatory IP-10 levels, while enhancing IL-10 concentrations. Once again, vasopressin did not modulate the immune response. Finally, in an observational study in sepsis patients, we show that higher noradrenaline infusion rates are associated with a more anti-inflammatory cytokine balance, whereas concurrent β-blocker use was associated with a more pro-inflammatory cytokine balance. Collectively, our results clearly show that noradrenaline dysregulates the immune response in mice and humans, and compromises host defense, thereby facilitating infections. Therefore, it may significantly contribute to sepsis-induced immunoparalysis, whereas vasopressin does not have these untoward immunologic effects.

Genetic variation is an important determinant of the host response. As we showed in chapter 4 that noradrenaline compromises host defense via stimulation of the β2-adrenergic receptor, we hypothesized that common non-synonymous variants (either individual single nucleotide polymorphisms [SNPs] or SNP haplotypes in the β2-adrenergic receptor gene could render subjects more vulnerable or resistant towards these untoward effects of noradrenaline. This hypothesis is investigated in **chapter 5**. Peripheral blood mononuclear cells of 109 healthy participants were stimulated with

noradrenaline and endotoxin *ex vivo*. Furthermore, all subjects of this cohort were genotyped to investigate associations between genetic variation in the β2 receptor and noradrenaline-induced attenuation of pro-inflammatory cytokine production and enhanced anti-inflammatory cytokine release. Additionally, in order to assess associations between SNPs and *in vivo* immunologic and hemodynamic parameters, all 109 subjects underwent experimental endotoxemia. Examined SNPs were rs1042711, rs1042713 and rs1042714, and haplotypes of the these SNPs. We found no associations between any of the individual SNPs or SNP haplotypes in the *ADRB2* gene and noradrenaline-mediated immunosuppression *ex vivo* or the systemic *in vivo* immune and hemodynamic response. Consequently, we conclude that these genetic variants do not influence the susceptibility towards catecholamine-induced dysregulation of the host response.

The effects of phenylephrine, a synthetic sympathomimetic vasopressor commonly used in the peri-operative setting, on host defense mechanisms are investigated in **chapter 6.** Immunosuppression after major surgery is a well-recognized phenomenon that can increase the risk for post-operative infections. Furthermore, it can have protracted detrimental effects as well, such as increased risk for metastases after cancer surgery. Phenylephrine is regarded as a pure α-adrenergic agonist, however, several studies have indicated it can exert β-adrenergic effects as well. Therefore, based on our work presented in chapters 4 and 5, it is not inconceivable that phenylephrine has potential immunosuppressive properties. We demonstrate that, similar to noradrenaline, phenylephrine dose-dependently attenuates the production of TNF-α, IP-10 and IL-1β, and enhances the production of anti-inflammatory IL-10 in human leukocytes *in vitro*. Moreover, we reveal that these effects are indeed mediated via the β-adrenergic receptor. *In vivo,* continuous intravenous administration of phenylephrine using micro-osmotic pumps strongly reduced circulating pro-inflammatory cytokine levels in endotoxin-challenged mice. Other indications of phenylephrine-induced impairment of host defense mechanisms in mice included attenuated *ex vivo* production of IFN-γ by splenic T-cells and reduced reactive oxygen species production by neutrophils. In terms of functional effects, phenylephrine treatment resulted in increased systemic bacterial dissemination to spleen and liver after surgical induction of peritonitis in mice. Finally, we translate these findings to the human *in vivo* setting, again making use of the experimental endotoxemia model, in which phenylephrine infusion attenuated plasma levels of pro-inflammatory cytokines IL-8, IP-10, and MCP-1, while enhancing IL-10 concentrations. We conclude that phenylephrine may compromise host defense and increase susceptibility towards infection in surgical patients.

Collectively, the studies described in Part II indicate that commonly used vasopressors exert extensive immunomodulatory effects which may comprise host defense in a variety of patients suffering from dysregulated immune responses, such as those with

sepsis or following major surgery.

PART III MODULATION OF THE CEREBRAL, SYSTEMIC AND MICROCIRCULATION BY VASOPRESSORS IN THE EXPERIMENTAL HUMAN ENDOTOXEMIA MODEL

In addition to inflammatory consequences, we investigated whether vasopressors exerted differential effects on the cerebral, systemic, and microcirculation during systemic inflammation. As sepsis patients represent a heterogenous group, exemplified by differences in age, co-morbidities, co-medication, causative pathogen, duration of the inflammatory hit and disease severity, we used the human endotoxemia model to address this research question. In **chapter 7** the effects of noradrenaline, phenylephrine and vasopressin on the cerebral vasculature before and during experimental human endotoxemia are described. The critical closing pressure (CrCP) was used as measure of cerebral vascular tone. CrCP is defined as the lower limit of arterial blood pressure below which vessels collapse and flow to the brain ceases, it is a reliable parameter to quantify characteristics of the cerebral vascular bed. Administration of noradrenaline and phenylephrine significantly increased mean arterial pressure before administration of endotoxin, whereas vasopressin had no effect. Furthermore, before endotoxin administration, phenylephrine significantly increased CrCP, in contrast to noradrenaline and vasopressin, which had no effect on this parameter. Induction of endotoxemia resulted in a decreased CrCP consequential to loss of vascular resistance in the arterioles. None of the vasopressors prevented the decrease in CrCP after endotoxin administration. In **chapter 8** we evaluated the effects of the same three vasopressors on both micro-and macrocirulation parameters before and after induction of systemic inflammation. Macrocirculation parameters were derived from pulse-contour analysis of the arterial blood pressure signal. The microcirculation was evaluated using sublingual video microscopy. Before endotoxin administration, infusion of noradrenaline and phenylephrine increased mean arterial pressure, but other macrocirculatory parameters were unaffected. None of the vasopressors affected the microcirculation under non-inflammatory conditions. Endotoxemia induced a decrease in systemic vascular resistance and thereby blood pressure. This decrease was refractory to infusion to noradrenaline or phenylephrine, whereas vasopressin infusion mitigated the endotoxin-induced decrease in diastolic blood pressure by stabilizing systemic vascular resistance and cardiac output. Endotoxin administration also induced a reduction in microvascular density and flow but none of the vasopressors influenced these parameters. In conclusion, we could not discern major differences on cerebral or microcirculatory effects of the different vasopressors investigated, while there were differences on the macrocirculatory level.

CHAPTER 10

GENERAL DISCUSSION AND
FUTURE PERSPECTIVES

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Despite advances in supportive care over the last decades mortality and morbidity in critically ill septic patients remains high. This emphasizes the need for novel approaches, especially regarding modulation of the immune response. In addition, it calls upon us to reevaluate our current standard of care. Previous *in vitro* studies indicated that the broadly applied vasoactive drug noradrenaline potentially exerts immunomodulatory effects. Furthermore, circumstantial clinical evidence suggested these effects might be detrimental to patients. This drug has been in use for approximately 70 years at relatively low costs, but it appears that its risk profile is hitherto not yet completely understood. This thesis comprises bench-to-bedside studies into the effects of noradrenaline, the related catecholaminergic vasopressor phenylephrine, and the non-catecholaminergic vasopressor vasopressin on host defense mechanisms and on the circulation. Our main finding is that we characterized previously unrecognized sources of inadvertent immunomodulation in daily clinical practice and identified alternatives to mitigate this problem.

In this chapter, the results of the work presented in this thesis are discussed in the perspective of our current understanding of the immune response in sepsis and in critically ill patients in general, adrenergic pathways, agonists and antagonists as well as alternative vasopressors and effects of these agents on the circulation. We discuss our findings in light of recent studies in the field that were published during the conduct of our own studies and present recommendations for future research in these areas.

THE CURRENT STATE OF IMMUNOLOGICAL SEPSIS RESEARCH

Sepsis is not a homogenous well-defined pathological condition, and this has hampered progress of research aimed at novel treatments. The immunological response in sepsis patients is very complex; pro-and anti-inflammatory responses are mounted simultaneously and their magnitude or dominance can vary over time within a patient (1, 2). Earlier clinical trials were solely aimed at attenuation of the immune response, but these have, without exception, failed to significantly improve outcome. The prevailing conviction has long been that septic patients were dying of an 'overactive' hyperinflammatory response to infection that causes collateral tissue damage and organ failure. However, more recently the pendulum has shifted towards increased recognition of the importance of a too pronounced anti-inflammatory response, a phenomenon known as sepsis-induced immunoparalysis (2). This dysregulated immune response rather than solely an overactive one is acknowledged in the most recent definition of sepsis from 2016: *a life-threatening organ dysfunction caused by a dysregulated host-response to infection* (3). This new definition is a substantial improvement over the old one from 1991, which has been vastly outpaced by advances in our understanding of sepsis pathobiology, or more accurately: the recognition of our lack of understanding,

and is based on three pillars: provocation by a pathogen, a dysregulated host response and organ dysfunction.

In general, current sepsis research is shifting towards a better characterization of the host response in sepsis patients in order to unlock targeted immunotherapy in a so-called 'precision medicine' approach (4, 5). The studies in this thesis have been performed to evaluate a critical component of our current supportive therapy, namely vasopressors, which we show to be a dysregulating factor for the host response in sepsis. Reappraisal of our current standards of vasopressor therapy in light of the immunologic effects of different vasopressors could therefore lead to improved outcome, and even pave the way towards precision vasopressor medicine, as outlined below.

IMMUNOLOGIC EFFECTS OF VASOPRESSORS IN SEPSIS

Until now, vasopressor therapy was not regarded as an immunomodulatory intervention. Noradrenaline is administered in sepsis patients because of hemodynamic failure despite increased levels of endogenous catecholamines (6). The need for exogenous catecholamine administration in sepsis is attributed to altered adrenergic receptor signaling, for instance due to downregulation of receptor density on cell surfaces and attenuated intracytosolic cAMP levels after adrenergic stimulation. Consequently, the endogenous adrenergic drive falls short to restore hemodynamic homeostasis and organ perfusion (7, 8). Noradrenaline stimulates both the α- and β-adrenergic receptors, but whereas there is conflicting data on the presence and function of the α-receptors on immune cells (9), β-receptors are abundantly present on most of these cells (10, 11). The findings described in this thesis demonstrate that noradrenaline dysregulates the immune response via β-receptor stimulation and compromises host defense. It may therefore significantly contribute to sepsis-induced immunoparalysis and impair outcome of sepsis patients. In support of this hypothesis, a growing body of clinical evidence illustrates the potential detrimental effects of noradrenaline administration. In two observational studies, high circulating noradrenaline levels were a risk factor for mortality, independent of disease severity score and hemodynamic parameters (12, 13). Nevertheless, these findings should be interpreted with caution as there is a high risk of confounding due to bias by indication. Recently, another observational study yielded similar findings, but this time it was also demonstrated that mortality risk by vasopressor load is impacted by the amount of fluids administered for resuscitation and timing of vasopressor infusion (14). Moreover, a catecholamine sparing 'permissive-hypotension' strategy was recently demonstrated to improve outcome in older critically ill patients (15), which is congruent with an earlier meta-analysis of high versus low mean arterial pressure (MAP) targets in septic shock patients (16). Finally, pooled data from a meta-analysis of various non-adrenergic vasopressors showed improved survival in patients with vasodilatory shock of various etiologies (17). Unfortunately, in none of these studies

secondary infections or other hallmarks of immunoparalysis were assessed, providing no mechanistic insight concerning these pathophysiological aspects.

Importantly, we compared the immunologic effects of noradrenaline to vasopressin, a synthetic analog of the endogenous antidiuretic hormone which is currently used as an adjunctive therapy to noradrenaline in patients with a severe septic shock (3). Vasopressin has no adrenergic affinity, instead it activates the V1 receptor, which is present on the smooth muscle cells of the vasculature and induces vasoconstriction, and the V2 receptor, which regulates water resorption in the kidney (18, 19). We found no evidence of immunomodulation by vasopressin in any of our experiments which makes vasopressin an interesting 'immunologically inert' alternative vasopressor for further exploration. In the VASST-trial, add-on use of vasopressin reduced mortality in a subset of sepsis patients compared to noradrenaline (20). However, no overall mortality benefit was demonstrated in this trial (20), nor in another large clinical study (21), although vasopressin did reduce the need for renal replacement therapy (21). In addition, selepressin, a V1-selective vasopressor, did not improve outcome compared to noradrenaline as well (22). A critical consideration of these trials is that vasopressin and selepressin have been used as adjunctive therapy; therefore, patients had already been treated with noradrenaline before these vasopressors were started alongside noradrenaline. In most cases, patients had already received much higher dosages of noradrenaline than those we used in our human endotoxemia study, which already significantly dysregulated the host response, so this precludes clear interpretation of the clinical trial results. Furthermore, no infectious complications or other signs of immunoparalysis were assessed in any of the trials, so again, no conclusions can be drawn on immunological effects. Because of the lack of overt clinical benefit in unselected septic shock patients, the surviving sepsis campaign still advocates the use of vasopressin as an add-on treatment and not as a monotherapy (3). Our results clearly warrant further clinical investigation, which should first be aimed at evaluation of immunologic effects. Critically, these would have to be designed as monotherapy studies to avoid the confounders mentioned above. Importantly, both vasopressin and selepressin have been shown to be suitable as first-line agents (i.e. initial monotherapy) for the treatment of septic shock (23-25). If our results are recapitulated in a clinical trial, a larger follow-up study is warranted comparing vasopressin/selepressin monotherapy with noradrenaline monotherapy. Such a trial could lead to reappraisal of the current guidelines for vasopressor use in shock patients. Alternative vasopressors other than vasopressin/selepressin have recently been gaining attention as well. For example, synthetic angiotensin II, which activates the angiotensin II receptor type 1 and causes peripheral vasoconstriction as well as sodium and water retention, has been demonstrated to reduce noradrenaline use in septic shock patients (26), although effects on mortality still have to be demonstrated. It would be of interest to investigate the immunological profile of this new vasopressor as well in future studies.

An interesting future perspective is the implementation of precision medicine regarding which vasopressor to use for (septic) shock patients. Precision medicine entails the targeted use of different types of medication, in addition to monitoring of the effects, for instance to facilitate dosage adjustments (4). For trials, selection of patients that are more likely to benefit from a certain therapy based on biomarkers related to the pathways/mechanism targeted by the drug is known as predictive enrichment (27). Efforts are underway to improve characterization of the host response on an individual levels, which is imperative for precision immunotherapy to succeed. An example of a biomarker which has shown promise in studies is ferritin, as a predictive marker for macrophage activation-like syndrome (28), a condition characterized by excessive activation of several immune cells leading to an overwhelming pro-inflammatory reaction, with high morbidity and mortality (29). In contrast, reduced monocyte HLA-DR expression is the most widely used marker to identify immunoparalysis (30, 31). The first major trial evaluating the contribution of a personalized medicine approach in sepsis patients is the Immunosep trial (clinicaltrials.gov NCT04990232). In this RCT, personalized adjunctive treatment targeted at either fulminant hyperinflammation or immunoparalysis is compared to placebo. Patients are characterized as hyperinflammatory or immunoparalyzed based on the aforementioned biomarkers and then randomized towards additional treatment with anakinra (to counter hyperinflammation), interferon (IFN)-γ or placebo. In a similar setup, a comparative trial between noradrenaline and a non-catecholaminergic vasopressor could be performed. The anti-inflammatory actions of noradrenaline might be advantageous in patients suffering from hyperinflammation, while being detrimental in patients demonstrating signs of immunoparalysis, whereas the opposite could be true for an alternative vasopressor. Ideally, the future treatment of sepsis patients should entail targeted immunotherapy in combination with personalized vasopressor treatment, based on a biomarker which can be evaluated rapidly and preferably at the bedside.

In addition to single biomarkers or combinations of several biomarkers, transcriptomic profiling of circulating leukocytes, providing a snapshot of genome-wide expression patterns, has shown promising results for immunological characterization of patients as well. In pneumonia patients, genomic profiling of leucocytes was able to identify patients who exhibited an immunosuppressed phenotype (i.e. endotoxin tolerance and T-cell exhaustion). These patients had a worse prognosis compared to patients who did not exhibit these features (32). In a comparable study, four transcriptomics-based endotypes in sepsis patients were associated with marked differences in outcome, with increased mortality observed for the endotype characterized by decreased expression of innate and adaptive immune response pathways (33). In both of these studies, patients exhibiting immunosuppressed endotypes were more likely to suffer from shock and therefore required higher dosages of noradrenaline. Unfortunately, noradrenaline therapy was not included as a potential confounder in these trials and so, based on our

results, it could be speculated that endotypes in sepsis are not only driven by pathogen and host factors, but also by treatment. Therefore, the relation between noradrenaline infusion and expression of genes underlying the different endotypes in the mentioned trials should be further assessed. Furthermore, although genomic profiling is an attractive methods to facilitate precision medicine in sepsis patients, it is still time-consuming, not available at the bedside, and difficult to interpret for clinicians.

The use of pharmacogenomics, i.e. how a person's genetic makeup affect their response to drugs, provides another approach to individualize drug therapy. In addition, pharmacogenomics could be regarded as a form of predictive enrichment, as mentioned earlier, to enhance patient selection for certain therapies, clinically and in trials. Pharmacogenomics already have shown promise for commonly used medications on the ICU in order to improve efficacy and reduce adverse drug reactions. For example, voriconazole is used for severe fungal infections such as invasive aspergillosis but has a wide effective variability and narrow therapeutic range, with potentially severe side effects including hepatotoxicity, visual disturbances and hallucinations(34). This drug is metabolized by CYP2C19, of which several genetic variants importantly influence its function, leading to ultra-rapid metabolizers, normal metabolizers and poor metabolizers (35). Currently, it is recommend by the Dutch pharmacogenetics working group to monitor serum plasma concentrations for voriconazole in poor metabolizers (36). Our findings demonstrate that variants in the β2-adrenoceptor gene *(ADRB2)* do not influence inflammation *in vivo* nor immunosuppression by noradrenaline in the *ex vivo* setting. Importantly, the CysGlyGln haplotype, earlier associated with increased mortality in septic patients (37), was only present in three subjects in our cohort, so we cannot draw conclusions pertaining to this specific haplotype. These results may implicate that there is no value of *ADRB2* genotyping (with the potential exception of the CysGlyGln haplotype) in identifying subjects susceptible towards noradrenaline-induced immune dysregulation, but affirmation in other, preferable larger (and therefore including more CysGlyGln carriers), studies is warranted.

Another important discussion sparked by our present work is the use of β-blockers in (septic) shock patients. We demonstrated that the immunological effects of noradrenaline and phenylephrine are mediated via the β-adrenergic receptor and can be reversed by the non-selective β-antagonist propranolol and the β2-selective antagonist ICI-118-551. Furthermore, we show that septic shock patients who concurrently used β-blockers and were treated with noradrenaline display a significantly higher TNF-α/IL-10 ratio than patients not on β-blockers, a marker that has been associated with improved survival in sepsis patients (38). These data indicate that the immune-dysregulating effects of β-adrenergic stimulation are mitigated by these commonly used drugs. In support of this, β-blockers have been associated with improved outcome in both sepsis and emergency surgery (39-

41). This has been further investigated in interventional trials. For example, esmolol, a short-acting selective β1-antagonist was demonstrated to dramatically improve mortality in septic shock patients with tachycardia and high noradrenaline requirements (42), although the mortality in the control group (~80%) was extremely high, warranting cautious interpretation. Nevertheless, beneficial effects of β-blockers have been demonstrated by others as well (43), and was shown to be safe in sepsis patients (44, 45). However, these reported studies are all single center studies of relatively limited sizes. Currently, two large multicenter RCTs (LANDI-SEP and STRESS-L studies) are being performed in sepsis patients, in which the effects of the highly selective ultrashort acting β1-antagonist landiolol are investigated, although these may also not be insightful regarding immunological effects, as we show that mainly the β2-receptor is involved in the anti-inflammatory effects of noradrenaline. Noteworthy in this context is that the use of β-blockers in sepsis patients is not associated with increased vasopressor requirements (40).

The exciting developments outlined in the previous sections will be further deployed and their place in clinical practice will be elucidated over the coming decades, but what can be done now to improve vasopressor treatment? The results put forward in this thesis provide arguments for detrimental immunological effects of catecholamines in sepsis patients. Next to this, treatment with high doses of catecholamines have been associated with a number of non-immunological adverse effects. For example, reversible myocardial depression has been described in hyperadrenergic states such as pheochromocytoma crises and severe emotional stress (46) and excess catecholamines induce insulin resistance and inhibit glycogen synthesis, which induces hyperglycaemia (47). Furthermore, our own group has shown that catecholamines can aggravate enterocyte damage (48), which may contribute to decreased barrier function and aid bacterial translocation. Finally, long term cardiovascular health might be compromised by increased and repeated catecholamine exposure, as has been demonstrated in pheochromocytoma patients, and this is related to an increased risk for cardiovascular events (49). Interestingly, recent evidence has been put forward which attributes this increased cardiovascular risk to hyperresponsiveness of monocytes in the period after exposure to catecholamines. Perhaps counterintuitively, this hyperresponsiveness includes enhanced production of pro-inflammatory mediators and increased glycolysis and oxidative phosphorylation due to epigenetic alterations, signatures of a phenomenon known as 'trained immunity'. This is essentially the opposite of innate immune tolerance; a key hallmark of immunoparalysis in patients. Therefore, catecholamines clearly exert direct short-term anti-inflammatory effects as demonstrated in this thesis, but appear to have more pro-inflammatory properties on the longer term (50). Collectively, the emerging evidence of detrimental effects of catecholamines are causing a shift towards so-called 'decatecholaminisation' of sepsis patients. Selecting appropriate blood pressure targets for individual patients, reducing use of sedatives, and optimizing

the fluid balance are general critical care principles which can contribute to such minimization of noradrenaline administration. Furthermore, earlier use of alternative vasopressors has been shown to reduce noradrenaline requirements (20, 26), and a meta-analysis suggests that use of non-catecholaminergic vasopressors as a group can reduce mortality (17), although this has not been demonstrated yet for one single agent.

IMMUNOLOGIC EFFECTS OF VASOPRESSORS BEYOND SEPSIS

Sepsis is the archetypal syndrome defined by immune dysregulation. However, after trauma, burns and major surgery, similar immunological sequelae are observed. In these patients the immune system is activated via release of danger associated molecular patterns (DAMPs) rather than pathogen-associated molecular patterns (PAMPs), which can activate and dysregulate the immune response (51, 52). Perhaps not surprising in this respect, these patients display a high incidence of infections following the inciting event (i.e. trauma, burns or major surgery), which is comparable to the incidence of secondary infections in sepsis patients (53). Immunosuppression by DAMP release has been further substantiated by an investigation in which changes in the leukocyte transcriptome in trauma and burn injury patients was compared to those in early experimental human endotoxemia. The authors found a large overlap: more than 80% of cellular functions were similarly affected among the different conditions (54). Also similar to sepsis, patients suffering from trauma, burns and after major surgery often require vasopressor support. Consequently, inadvertent immune dysregulation might be of importance in these patients as well. Phenylephrine, a synthetic sympathomimetic is frequently used to treat hypotension in the perioperative period and is regarded as a selective α-adrenergic agonist (55). However, our results demonstrate that it exerts potent anti-inflammatory actions *in vitro* and *in vivo*, which are mediated trough β-adrenergic stimulation. Similar to our recommendations for sepsis research regarding noradrenaline, this warrants further clinical examination of possible harmful effects of phenylephrine in patients and exploration of alternative non-catecholaminergic vasopressors in patients normally treated with phenylephrine. Another implication of these results could be the further investigation of peri-operative use of β-blockers, although clinical evidence of its effectiveness has been ambiguous up till now (39, 56).

EFFECTS OF VASOPRESSORS ON THE CIRCULATION UNDER INFLAMMATORY AND NON-INFLAMMATORY CONDITIONS

In the final part of this thesis, we first investigated the effects of three vasopressors (noradrenaline, phenylephrine and vasopressin) on cerebral hemodynamics using the human endotoxemia model. During endotoxemia, the mean flow velocity in the middle cerebral artery decreased and this was unaffected by any of the vasopressors. In addition, critical closing pressure (CrCP), defined as the lower limit of arterial blood pressure, below which vessels collapse and flow ceases, was unaffected by the

vasopressors as well during endotoxemia. For noradrenaline and phenylephrine, this might be explained by the low dose, and for vasopressin, the lack of an effect might be due to the normal endogenous vasopressin status of these healthy individuals, as opposed to the deficiency observed in sepsis patients (57). To date, no studies have been performed in sepsis patients, although a few studies have compared effects of different vasopressors in traumatic brain injury patients, but these have not identified clinically relevant differences in terms of cerebral blood flow (58-60). However, sepsis is a far more heterogeneous condition in which cerebral blood flow is disconnected from the systemic circulation (61). Therefore, further studies have to be performed to establish an optimal vasopressor regimen to maintain cerebral blood flow in this population.

Microcirculatory failure is closely related to the pathophysiology of sepsis as well as sepsis-related morbidity (62), although the different effects of vasopressors have scarcely been examined. In animal models, varying effects of vasopressors have been established, as noradrenaline caused a significant reduction in mesenteric artery flow and a reduced microcirculatory flow in the jejunal mucosa, whereas phenylephrine, while increasing MAP, had no effect on regional or microcirculatory flow (63). In our healthy volunteer study, systemic vascular resistance was reduced during endotoxemia, leading to decreased blood pressure. Although noradrenaline and phenylephrine increased MAP before endotoxin administration, the endotoxin-induced decrease was refractory to infusion of both agents. Vasopressin infusion stabilized systemic vascular resistance and cardiac output during endotoxemia, and thereby it mitigated the aforementioned endotoxin-induced effects. Endotoxin administration induced a reduction in microvascular density and flow but none of the vasopressors changed these parameters. So, while we did observe discernable differences of the investigated vasopressors on macrocirculation during human endotoxemia, we did not find differential vasopressor effect on microcirculatory parameters (64). Again, these distinct effects might be explained by the low dosages used for noradrenaline and phenylephrine, however, vasopressin was used in a clinical relevant infusion rate. Moreover, this distinction might point towards the uncoupling of the systemic circulation and microcirculation, a phenomenon which is also observed in sepsis patients. For example, increasing the MAP to 85 mmHg with noradrenaline did not improve microperfusion in septic shock patients (65). However, a recent small study did suggest that addition of vasopressin to noradrenaline might improve microcirculation in some patients (66), although the most important determinant of microcirculatory improvement was found to be the noradrenaline dose (66). In conclusion, our study provides no definite proof of superiority for any vasopressor to improve microcirculatory parameters. Clearly, our results do demonstrate the diverging effects of vasopressors on macro- and microcirculation parameters in humans *in vivo*. This finding implicates the need for monitoring of other (bedside) parameters to evaluate vasopressor effects as opposed to simply measuring blood pressure alone.

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CHAPTER 11

NEDERLANDSE SAMENVATTING

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Patiënten met een zeer ernstige infectie (bloedvergiftiging, ook wel sepsis genoemd) die op de intensive care zijn opgenomen en andere ernstig zieke patiënten hebben frequent een ontregeld afweersysteem (immuunsysteem). Tevens worden deze patiënten zeer vaak behandeld met medicijnen die de bloeddruk verhogen (vasopressors). Het primaire doel van dit proefschrift was het onderzoeken van de effecten van vasopressors op het immuunsysteem. Verder werden de effecten van vasopressors op de doorbloeding van de hersenen, kleine bloedvaten en grote bloedvaten tijdens een afweerreactie in kaart gebracht.

DEEL I EFFECTEN VAN VASOPRESSORS OP HET IMMUUNSYSTEEM: EEN OVERZICHT VAN DE LITERATUUR EN MOGELIJKE GEVOLGEN VOOR PATIËNTEN

Hoofdstuk 1 bevat de algemene inleiding en opzet van dit proefschrift. Sepsis is wereldwijd de belangrijkste doodsoorzaak en de Wereldgezondheidsorganisatie (WHO) heeft het bestrijden van sepsis dan ook als een mondiale gezondheidsprioriteit bestempeld. Bij sepsis spelen zowel ontstekingsbevorderende (pro-inflammatoire) en ontstekingsremmende (anti-inflammatoire) processen een rol, die idealiter in balans zijn. Bij sepsis is dit echter niet het geval. Zo kan er sprake zijn van een te uitgesproken pro-inflammatoire reactie, die naast het doden van de ziekteverwekker ook schade aan gezond weefsel veroorzaakt. Een te uitgesproken en langdurige anti-inflammatoire reactie kan uitmonden in zogenaamde “sepsis-geïnduceerde immuunparalyse”. Deze “verlamde” toestand van het immuunsysteem leidt ertoe dat de ziekteverwekker niet kan worden opgeruimd in de infectie blijft bestaan. Verder maakt het patiënten erg vatbaar voor een nieuwe infectie, vaak met moeilijk te behandelen ziekteverwekkers. Het is de laatste jaren duidelijk geworden dat sepsis-geïnduceerde immuunparalyse een belangrijke rol speelt in de meerderheid van de sterfgevallen die veroorzaakt worden door sepsis.

Immuunparalyse wordt op het niveau van de afweercel gekenmerkt door verminderde productie van pro-inflammatoire cytokines (ontstekingsbevorderende stoffen) en verhoogde productie van anti-inflammatoire cytokines (ontstekingsremmende stoffen), verder is ook de stofwisseling van afweercellen verstoord. Patiënten met een ernstige vorm van sepsis (septische shock) hebben vaak een erg lage bloeddruk. Dit vereist toediening van vasopressors op de intensive care (IC). Noradrenaline is wereldwijd de meest gebruikte vasopressor. Enkele eerder uitgevoerde maar beperkte laboratoriumonderzoeken met afweercellen hebben laten zien dat noradrenaline ontstekingsremmende effecten kan hebben en daarom de afweer zou kunnen onderdrukken. Echter, de effecten van noradrenaline en andere vasopressoren op het immuunsysteem bij proefdieren en mensen waren vrijwel geheel onbekend vóór de

uitvoering van de onderzoeken beschreven in dit proefschrift.

De afweerreactie is bij sepsispatiënten moeilijk te onderzoeken. Sepsispatiënten vormen namelijk een zeer diverse patiëntengroep, bijvoorbeeld door verschillen in leeftijd, onderliggende ziektes, medicijngebruik en ziekteverwekkers die de infectie hebben veroorzaakt. We kunnen sepsis echter nabootsen in gezonde vrijwilligers, door gebruik te maken van een zogenaamd onderzoeksmodel, in dit geval het experimentele endotoxinemiemodel. Zo kunnen we gemakkelijker en beter de effecten van verschillende behandelingen op de afweerreactie onderzoeken. In het experimentele endotoxinemiemodel wordt een bestanddeel van de celwand van een bacterie, endotoxine genoemd, geïnjecteerd in de bloedbaan van gezonde vrijwilligers. Endotoxine is het deel van de bacterie wat wordt herkend door het immuunsysteem. Het veroorzaakt daardoor een kortdurende afweerreactie met griepachtige symptomen zoals koorts en de productie van ontstekingseiwitten, cytokines, die meetbaar zijn in het bloed. Verder heeft endotoxinemie effecten op de bloedsomloop die vergelijkbaar zijn met de effecten die worden waargenomen in patiënten met sepsis. Omdat het een dood bestanddeel van een bacterie betreft is dit model veilig voor de gezonde vrijwilligers die deelnemen.

In **hoofdstuk 2** geven we een overzicht van de effecten van noradrenaline op het afweersysteem voor zover deze bekend waren voor aanvang van het onderzoek beschreven in dit proefschrift. Verder wordt beschreven of en hoe deze effecten zouden kunnen bijdragen aan het ontstaan van sepsis-geïnduceerde immuunparalyse. Laboratoriumonderzoeken met afweercellen laten zien dat noradrenaline anti-inflammatoire effecten heeft via stimulatie van de β-adrenerge receptor (één van de receptoren waaraan noradrenaline bindt op cellen). Deze effecten bestaan uit vermindering van productie van pro-inflammatoire cytokines, verhoogde productie van anti-inflammatoire cytokines en een verminderde doding van ziekteverwekkers. Deze effecten zijn tevens in beperkte mate *in vivo* (in een levend organisme, dus niet alleen op geïsoleerde afweercellen) aangetoond bij proefdieren. Ten slotte bestaat er indirect bewijs verkregen uit patiëntenonderzoek voor de rol die noradrenaline speelt in sepsis-geïnduceerde immuunparalyse. Zo blijken verhoogde noradrenalineconcentraties in het bloed van sepsispatiënten een risicofactor voor sterfte aan sepsis, onafhankelijk van de ernst van de ziekte en andere patiëntkarakteristieken. Daarnaast zorgde toediening van β-blokkers (medicijnen die de β-adrenerge receptor afschermen voor de werking van noradrenaline) voor drastisch minder sterfte in een onderzoek bij patiënten met septische shock. Het beschikbare bewijs voor immunologische effecten van andere vasopressors dan noradrenaline is zeer schaars. We concludeerden dat er behoefte is aan meer onderzoek om te beoordelen of de immunologische effecten van de vasopressors ook echt *in vivo* in mensen aanwezig zijn en een relevante rol spelen bij sepsis-geïnduceerde immuunparalyse.

Hoofdstuk 3 richt zich op de rol die immuunparalyse en noradrenaline spelen bij sterfte bij sepsispatiënten. Sepsispatiënten die worden opgenomen met hoge ziektescores (een maat voor de ernst van de ziekte) zijn vatbaar voor het krijgen van nieuwe infecties op de IC, hun afweersysteem functioneert dus niet goed. Daarbij hebben ze een langere opnameduur op de IC en een hogere sterfte. Immuunparalyse is dus een mogelijk verklarende factor tussen ernst van de ziekte bij sepsispatiënten en sterfte. Ernstig zieke patiënten hebben daarnaast vaak een lage bloeddruk en krijgen daarom veelal (hoge doseringen) noradrenaline, dat anti-inflammatoire effecten heeft. Daarom is noradrenaline een mogelijk verklarende factor tussen de ernst van de ziekte en immuunparalyse. Dit is van belang voor de patiënt, omdat, in tegenstelling tot ernst van ziekte, het type vasopressor wat gebruikt wordt een beïnvloedbare factor is waarmee immuunparalyse dus mogelijk voorkomen of verminderd kan worden.

DEEL II EFFECTEN VAN VASOPRESSORS OP HET IMMUUNSYSTEEM: VAN LABORATORIUMONDERZOEK NAAR BEVINDINGEN BIJ PATIËNTEN

Hoofdstuk 4 omvat ons eigen onderzoek naar de effecten van noradrenaline en een andere vasopressor, namelijk vasopressine, op het immuunsysteem. Onze bevindingen tonen sterke anti-inflammatoire effecten van noradrenaline aan op geïsoleerde afweercellen van gezonde vrijwilligers. Bij afweercellen die werden gestimuleerd met verschillende (bestanddelen van) ziekteverwekkers vonden we dat toevoeging van noradrenaline leidt tot een verminderde productie van verschillende pro-inflammatoire cytokines en verhoogde productie van het anti-inflammatoir cytokine interleukine (IL)-10, alsmede verschillende andere onderdrukkende effecten op afweercellen. We toonden verder aan dat deze effecten van noradrenaline tot stand komen via stimulatie van de β-adrenerge receptor. Een belangrijke bevinding is dat vasopressine geen effecten uitoefende op afweercellen. Vervolgens vertaalden we onze bevindingen naar de *in vivo* situatie met behulp van proefdieren, in dit geval muizen, die ofwel endotoxine kregen toegediend of waarbij sepsis werd veroorzaakt door een gaatje in de darm te maken waardoor bacteriën zich kunnen verspreiden. De muizen kregen in deze experimenten noradrenaline, vasopressine óf een nepmedicijn (placebo) toegediend. Wederom ontdekten we sterke onderdrukkende effecten op het immuunsysteem door noradrenaline. Deze effecten zorgden ervoor dat bacteriën slechter bestreden werden en zich dus makkelijker konden vermenigvuldigen en verspreiden. Vasopressine beïnvloedde de afweerreactie wederom niet bij deze experimenten in muizen. Daarna hebben we de bevindingen vertaald naar mensen met behulp van het experimentele endotoxinemiemodel. Ook in dit experiment verminderde toediening van een lage dosis noradrenaline de bloedspiegels van enkele pro-inflammatoire cytokines, terwijl de concentraties van het anti-inflammatoire IL-10 werden verhoogd. In deze proefpersonen had vasopressine opnieuw geen effect op het immuunsysteem. Tenslotte toonden we in een onderzoek bij sepsispatiënten aan dat hogere noradrenaline doseringen geassocieerd zijn met lagere bloedspiegels van de

pro-inflammatoire cytokine TNF en hogere spiegels van het anti-inflammatoire IL-10. Daarnaast lieten we zien dat gelijktijdig gebruik van β-blokkers dit effect deels teniet doet. Uit ons onderzoek concludeerden we dat noradrenaline het immuunsysteem onderdrukt en de afweer tegen ziekteverwekkers vermindert, waardoor de vatbaarheid voor infecties toeneemt en infecties tevens ernstiger kunnen verlopen. Daarom kan noradrenaline in belangrijke mate bijdragen aan sepsis-geïnduceerde immuunparalyse, terwijl vasopressine deze ongewenste immunologische effecten niet heeft.

Ons onderzoek toont aan dat noradrenaline de afweer onderdrukt via stimulatie van de β-adrenerge receptor. Tegelijkertijd bepaalt iemands genetische opmaak voor een belangrijk deel zijn afweerreactie op ziekteverwekkers. Daarom formuleerden we de hypothese dat personen vatbaarder of resistenter kunnen zijn voor de effecten van noradrenaline op het immuunsysteem op basis van genetische variatie in het *ADRB2* gen (het gen dat codeert voor de β-adrenerge receptor). Deze variatie wordt ook wel uitgedrukt in single nucleotide polymorphisms [SNPs], waarbij er tussen personen verschil bestaat in de bouwstenen van het DNA, de nucleotiden. Als er een combinatie van verschillende SNPs aanwezig is noemen we dat een haplotype. Onze hypothese hebben we onderzocht in **hoofdstuk 5**. Afweercellen van 109 gezonde vrijwilligers werden via bloedafname verkregen en in het laboratorium gestimuleerd met endotoxine in de aan- en afwezigheid van noradrenaline. Tevens werd de aanwezigheid van SNPs binnen het *ADRB2* gen bij deze personen vastgesteld. Zo konden we verbanden onderzoeken tussen SNPs en het effect van noradrenaline op het immuunsysteem. Daarnaast ondergingen alle 109 proefpersonen experimentele endotoxinemie om associaties tussen de SNP's en de *in vivo* afweerreactie te beoordelen. In ons onderzoek vonden we geen associaties tussen de individuele SNPs of SNP-haplotypes in het *ADRB2* gen en het effect van noradrenaline op afweercellen. Ook de *in vivo* afweerreactie tijdens experimentele endotoxinemie werd niet beïnvloed door de aanwezigheid van individuele SNPs of SNP-haplotypes in het *ADRB2* gen.

In **hoofdstuk 6** werden de effecten van fenylefrine, een andere vasopressor waarvan verondersteld werd dat het alleen bind aan de α-adrenerge receptor, op het immuunsysteem onderzocht. Fenylefrine wordt vaak gebruikt om de bloeddruk op peil te houden bij patiënten die een operatie ondergaan. Onderdrukking van het immuunsysteem na een grote operatie is een bekend fenomeen dat het risico op infecties verhoogt. Op basis van de veronderstelling dat fenylefrine alleen aan de α-adrenerge receptor bindt zou het weinig of geen effecten op het immuunsysteem moeten hebben. Echter, we toonden aan dat fenylefrine vrijwel vergelijkbare effecten heeft op het immuunsysteem als noradrenaline, waaronder remming van de productie van pro-inflammatoire cytokines en verhoging van de productie van het anti-inflammatoire IL-10, zowel in geïsoleerde afweercellen als bij muizen. Bovendien ontdekten we dat deze effecten tot stand komen

via de β-adrenerge receptor. Ook leidde behandeling met fenylefrine voor verhoogde verspreiding van bacteriën bij muizen met sepsis, al waren de effecten wat minder sterk dan die van noradrenaline. Tenslotte werden deze bevindingen vertaald naar de mens, opnieuw gebruikmakend van het experimentele humane endotoxinemiemodel. Hierin verlaagde fenylefrine de bloedspiegels van pro-inflammatoire cytokines terwijl spiegels van het anti-inflammatoire IL-10 werden verhoogd. We concludeerden dat fenylefrine de afweerreactie remt en daardoor de gevoeligheid voor infecties bij patiënten die een operatie ondergaan kan vergroten.

DEEL III EFFECTEN VAN VASOPRESSORS OP DE BLOEDSOMLOOP TIJDENS EEN AFWEERREACTIE

Bij patiënten met sepsis is de doorbloeding van de hersenen geregeld verstoord. Tevens hebben deze patiënten vaak een lage bloeddruk door verstoringen in zowel kleine bloedvaten als grote bloedvaten (de centrale bloedsomloop). In het laatste deel van dit proefschrift hebben we de effecten van vasopressors op de doorbloeding van de hersenen, kleine bloedvaten en de centrale bloedsomloop onderzocht tijdens een afweerreactie, wederom gebruik makend van het experimentele endotoxinemiemodel. In **hoofdstuk 7** worden de effecten van noradrenaline, fenylefrine en vasopressine op de hersendoorbloeding beschreven. De *critical closing pressure* (CrCP) werd gebruikt als maat voor de hersendoorbloeding. CrCP is de ondergrens van de bloeddruk in de slagaders. Als de bloeddruk onder de CrCP komt, vallen de bloedvaten samen en stopt de bloedstroom naar de hersenen. We lieten zien dat toediening van noradrenaline en fenylefrine de bloeddruk in de slagaders vóór toediening van endotoxine verhoogt (wanneer er dus nog geen sprake is van een afweerreactie), terwijl vasopressine geen effect heeft. Bovendien verhoogde fenylefrine vóór toediening van endotoxine de CrCP, in tegenstelling tot noradrenaline en vasopressine. De afweerreactie na toediening van endotoxine zorgde voor een verlaagde CrCP. Geen van de drie vasopressors had echter een effect op deze verlaging van CrCP. In **hoofdstuk 8** evalueerden we de effecten van dezelfde drie vasopressors op kleine en grote bloedvaten (waarbij de eerste model staan voor de orgaandoorbloeding en de laatste model staan voor de centrale bloedsomloop). Vóór de toediening van endotoxine verhoogden noradrenaline en fenylefrine de bloeddruk in de grote vaten, maar er werden geen andere effecten op de centrale bloedsomloop waargenomen. Geen van de vasopressors beïnvloedde de doorbloeding van de kleine bloedvaten vóór toediening van endotoxine. De afweerreactie die werd opgewekt door toediening van endotoxine veroorzaakte een afname van de 'spanning' op de grote bloedvaten en daarmee daalde de bloeddruk. Deze afname werd niet beïnvloed door noradrenaline of fenylefrine, terwijl vasopressine dit wel deels voorkwam door de spanning op de grote bloedvaten en de werking van het hart te stabiliseren. Toediening van endotoxine veroorzaakte ook een vermindering van de doorbloeding van de kleine vaten, maar deze werd niet beïnvloed door één van de vasopressors. Concluderend

konden we in deze studies geen grote verschillen in effecten van de vasopressors op de doorbloeding van de hersenen of de kleine bloedvaten onderscheiden. Daarentegen waren er wel duidelijk verschillende effecten van de vasopressors op de grote bloedvaten.

Gezamenlijk geven de onderzoeken uit dit proefschrift aan dat de veelgebruikte vasopressors noradrenaline en fenylefrine de werking van het immuunsysteem sterk kunnen remmen en zo de afweer tegen infecties kunnen verminderen. Dit kan een belangrijke nadelige rol spelen bij verscheidene patiëntengroepen, zoals patiënten met sepsis of patiënten die een operatie ondergaan. Ook tonen we aan dat er een alternatief voorhanden is dat het immuunsysteem niet beïnvloedt, namelijk vasopressine. Tenslotte concluderen we dat toediening van vasopressors aan patiënten met sepsis niet automatisch zal leiden tot een verbeterde orgaandoorbloeding, terwijl de centrale bloedsomloop wel zal verbeteren. De bevindingen uit dit proefschrift geven aanleiding om de huidige behandeling van ernstig zieke patiënten met vasopressors kritisch onder de loep te nemen en nieuwe patiëntonderzoeken uit te voeren om deze behandeling te verbeteren.

APPENDICES

DANKWOORD
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RESEARCH DATA MANAGEMENT

The data obtained in this thesis are archived according to the Findable, Accessible, Interoperable and Reusable principles (1). Data generated in this thesis are part of published articles and files are available upon request. The human studies were conducted according to the principles of the declaration of Helsinki, including latest revisions and were approved by the Medical Ethics Committee of the Radboudumc. All participants gave written informed consent before participation. Case Report Forms were stored in Castor (2). Raw and processed *in vitro*, *ex vivo* and animal data were stored digitally on a local server of the department of intensive care, which is backed up daily on the Radboud Server.

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2. Castor EDC. Castor Electronic Data Capture. 2019 August 28, 2019]. Available from: https://castoredc.com.

RIMLS PHD PORTFOLIO

Name PhD student:	Roeland F. Stolk	**PhD period:**	01-10-2015 until 01-03-2020
Department:	Intensive Care	**Promotor(s):**	Prof. dr. P. Pickkers,
Graduate school:	Radboud Institute for Molecular Life Sciences		Prof. Dr. H. van der Hoeven
			Dr. M. Kox,
		Co-promotor(s):	Dr. H.J. van Leeuwen

TRAINING ACTIVITIES	Year(s)	ECTS
Courses & Workshops		
- Introduction day Radboudumc	2015	0.5
- EndNote workshop	2015	0.1
- ICU internal writing course	2015	1.5
- BROK Course	2015	2
- Statistics introduction for PhD students	2016	3
- Workshop: monitoring van Laag Risico Studies	2018	0.1
- Scientific integrity course	2018	1
- BROK refresher course	2019	0.2
Subtotal		*8.4*
Seminars & Lectures		
- Radboud Research Rounds	2015-2019	0.6
- ICU Monthly Journal Clubs*	2015-2020	0.6
- ICU weekly research meeting*	2015-2020	2
- Multiple ICU scientific and educational lectures*	2015-2020	2
- Regional anesthesiology seminar *	2016	0.5
- World Sepsis Day online meeting	2018	0.3
- Radboud Anesthesiology Science day *	2019	0.3
Subtotal		*6.3*
(Inter)national Symposia & Congresses		
- Radboud Summer Frontiers Systems Biology of Innate Immunity	2016	0.75
- Nederlandse Internistendagen #	2017-2020	0.5
- World Sepsis Conference, Amsterdam	2017	0.4
- RIMLS PhD retreat ##	2016, 2018-2019	1.25
- Nederlandse Intensivisten dagen *#	2017-2020	1.5
- New Frontiers in Innate Immunity and Inflammation, Cluj-Napoca	2018	0.5
- European Society of Intensive care Medicine Lives, Paris*	2018	0.75
- Radboud Center for Infectious diseases annual symposium *#	2016-2020	1.25
- Radboud New Frontiers Innate immune Memory ##	2019	1.5
- European Society of Intensive Care Medicine Live, Berlin	2019	0.75
- American Thoracic Society. International Meeting, Philadelphia *#	2020	1
Subtotal		*10.15*
Other		
- Weekly cytokine meeting internal medicine (2 presentations/year) *	2015-2020	6
- Peer review of scientific papers	2016-2021	0.8
Subtotal		*6.8*
TEACHING ACTIVITIES	**Year(s)**	**ECTS**
- Supervision Medical master student internship (3 months)	2016	1
- Supervision BMW master students internships (3 students for 9 months each)	2016-2020	9
- Supervision of bachelor student for grant proposal writing project	2019	1.75
Subtotal		*11.75*
Other activities		
- Geneesmiddelencommissie Rijnstate hospital, monthly meeting	2018-2021	2
Total		**45.4**

LIST OF PUBLICATIONS

1. **R.F. Stolk**, C. Bakx, J. Mulder, HJ Timmers, J.W. Lenders. *Is the excess cardiovascular morbidity in pheochromocytoma related to blood pressure or to catecholamines?* **J Clin Endocrinol Metab. 2013 Mar**

2. **R.F. Stolk**, T. van der Poll, D.C. Angus, J.G. van der Hoeven, P. Pickkers, M. Kox. *Potentially in advertent immunomodulation: noradrenaline use in sepsis.* **Am Journal of Resp and Crit Care. 2016 Sep**

3. **R.F. Stolk,** M. Kox, P. Pickkers. *Noradrenaline drives immunoparalysis in sepsis: clinical consequences.* **Intensive Care Medicine. 2020 Apr**

4. **R.F. Stolk,** E. van der Pasch, F. Naumann, J. Schouwstra, S. Bressers, A.E. van Herwaarden, J. Gerretsen, R. Schambergen, M. Ruth, J.G. van der Hoeven, H.J. van Leeuwen, P. Pickkers, M. Kox. *Noradrenaline dysregulates the immune response and compromises host defense during sepsis* **Am Journal of Resp and Crit Care. 2020 May**

5. **R.F. Stolk,** F. Naumann, E. van der Pasch, J. Schouwstra, S. Bressers, A.E. van Herwaarden, J. Gerretsen, R. Schambergen, M. Ruth, J.G. van der Hoeven, H.J. van Leeuwen, P. Pickkers, M. Kox. *Phenylephrine impairs host defense mechanisms to infection: a combined laboratory study in mice an translational human study.* **Br J Anaesthesia. 2021 Jan**

6. J.M.D. van den Brule, **R.F. Stolk**, E.J. Vinke, L.M. van Loon, P. Pickkers, J.G. van der Hoeven, M. Kox, C.W.E. Hoedemaekers. *Vasopressors Do Not Influence Cerebral Critical Closing Pressure During Systemic Inflammation Evoked by Experimental Endotoxemia and Sepsis in Humans.* **Shock. 2018 May**

7. D. Rao, **R.F. Stolk**, M.H. de Blauw, M.M.C. Hovens, RJ. Hassing. *Ancient bruises: a case of skin lesions due to vitamin C deficiency.* **EJCRIM 2015 Oct.**

8. J.M.D. van den Brule, C.R. van Kaam, G.P. Leijte, **R.F. Stolk,** P. Pickkers, J.G. van der Hoeven, M. Kox, C.W.E. Hoedemaekers. *Dynamic Cerebral Autoregulation and Critical Closing Pressure in Experimental Human Endotoxemia and Sepsis Patients*. **Med One. 2019 May**

9. L.M. van Loon, **R.F. Stolk**, J.G. van der Hoeven, P.H. Veltink, P. Pickkers, J. Lemson, M. Kox. *Effect of Vasopressors on The Macro- and Microcirculation During Systemic Inflammation In Humans in vivo.* **Shock. 2019 Apr**

10. **RF Stolk**, HJ van Leeuwen, M Kox, M van Borren, H de Boer, P Pickkers. *The chicken or the egg: low testosterone predisposes for COVID-19 or COVID-19 induces a decrease in testosterone?* **Crit Care. 2021 Jul**

11. Q.L. Habes, P. Konstanti, H.D. Kiers, R.M. Koch, **R.F. Stolk**, C. Belzer, M. Kox, P. Pickkers. *No interplay between gut microbiota composition and the lipopolysaccharide-induced innate immune response in humans in vivo.* **Clin Transl Immunology. 2021 Apr**

12. **R.F. Stolk**, N. Bruse, A Jansen, I. Ricaño Ponce, J. Gerretsen, J.G. van der Hoeven, V. Kumar, P. Pickkers, M. Kox. *Common β2-adrenergic receptor polymorphisms do not affect noradrenaline-induced immunosuppression ex vivo or the systemic inflammatory response in vivo.* **Submitted 2021**

13. **R.F. Stolk**, H.J.P. Fokkenrood, J.E.M. Sybrandy, JW.H.P. Lardenoije, J. van Vliet, H.J. van Leeuwen. *A case series of ruptured abdominal aortic aneurysm in octogenarians : what Is the impact of frailty?* **Submitted 2021**

CURRICULUM VITAE

Roel Stolk werd op 15 mei 1989 geboren in Tilburg. In 1992 verhuisde hij naar Wijchen waar in 2001 aan het Maaswaal college met het VWO begonnen werd. Het atheneum diploma behaalde hij in 2007 aan het Marnix College Ede. Hij studeerde Geneeskunde aan de Radboud Universiteit Nijmegen van 2007 tot 2013. Tijdens de bachelor werkte hij als student-assistent onder supervisie van professor Jacques Lenders op de afdeling interne geneeskunde en voerde een onderzoek uit naar patiënten met feochromocytoom. Hieruit ontstond een interesse voor de interne geneeskunde. Eind 2013 werd het geneeskunde diploma behaald (*cum laude*) mede na een seniorcoschap op de Intensive care in het Canisius Wilhelmina Ziekenhuis Nijmegen waarin de interesse in acute en intensieve zorg werd aangewakkerd. Van begin tot medio 2014 werkte hij als arts beschouwende specialismen in het Ziekenhuis Gelderse Vallei Ede. De opleiding tot internist werd gestart in oktober 2014 in het Rijnstate Ziekenhuis Arnhem. Na een jaar opleiding kwam hij in aanraking met de onderzoekslijnen naar sepsis van dr. Matthijs Kox en professor Peter Pickkers en kon beginnen aan een promotietraject op de Experimentele Intensive Care van het Radboudumc. Hierbij werden dr. Henk van Leeuwen en professor Hans van der Hoeven aan het begeleidend team toegevoegd. Het onderzoek besloeg laboratoriumstudies, dierexperimenten, studies in proefpersonen en patiëntgebonden onderzoek. Het resultaat van dit onderzoek is beschreven in dit proefschrift. Met het werk uit dit proefschrift werd onder andere in 2018 de abstractprijs van de Nederlandse Vereniging voor Intensive Care en European Society of Intensive Care Medicine gewonnen, alsmede in 2020 een American Thoracic Society International trainee scholarship.

Het onderzoekstraject en de opleiding interne geneeskunde wisselden elkaar enkele jaren af. In maart 2020 werd de opleiding weer volledig hervat in het Rijnstate Ziekenhuis Arnhem en vanaf april 2021 in het Radboudumc. Eind 2021 tot begin 2022 volgde tevens een stage infectieziekten in het Academisch Ziekenhuis Paramaribo. Vanaf juni 2022 zal gestart worden met de enkelvoudige differentiatie tot internist-intensivist aan het Radboudumc.

Roel is getrouwd met Charlotte en zij zorgen met veel plezier voor hun hond Lisa.

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