Cytokine responses to lipopolysaccharide in vivo and ex vivo : Genetic polymorphisms and inter-individual variation

Schippers, E.F.

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When you get the choice to sit it out or dance
I hope you dance

(Mark D. Sanders/Tia Sillers, 1999)

Voor mijn ouders
Aan Jacqueline, Rianne en Eline

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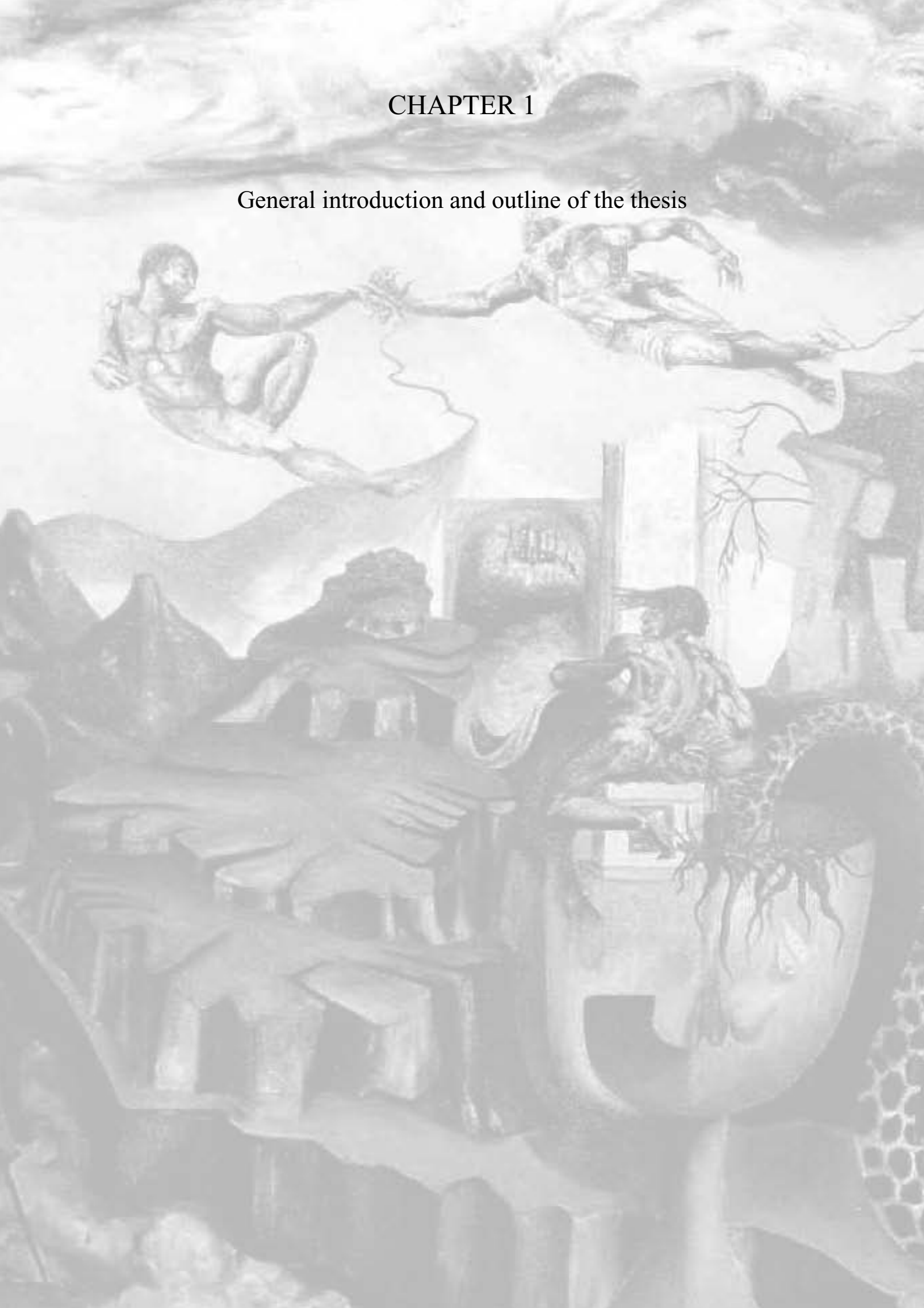
Contents

CHAPTER 1

General introduction and outline of the thesis



General background

It has been long realized that once a person acquires an infection, the clinical manifestations of disease may differ markedly between individuals. Some individuals may have an asymptomatic infection while others suffer life threatening consequences of the disease. This variability in clinical manifestation of infectious diseases has been attributed to differences in the ability of the causative microbial agent to cause disease ('virulence') and the premorbid health status of the patient ('predisposition'). Between microbial species, and even within species, differences exist in the ability of the agent to cause disease. Also, the port of entrée of the agent and the site of infection are important. For example, invasion of a microbial agent into the central nervous system or abdominal cavity might cause more severe disease as compared to invasion of the same agent into the lung. On the other hand, the predisposition of the patient is determined by concomitant disease, such as cancer or cardiovascular disease, and factors such as religious and cultural forces that shape the approach toward therapy. Finally, inherited differences ('genetic makeup') in the individual's ability to cope with an invading pathogen and mount an appropriate immune response play a role. This variability in response of the host applies to not only the clinical manifestations of infectious diseases, but also the response of an individual to treatment. It is important to appreciate that these multiple predisposing factors may influence both the incidence and the outcome in similar or conflicting ways. For example, a genetic make-up leading to an early and strong response to the invasion of a microbial agent, might decrease a person's risk of infection but at the same time increase that person's risk of an overly exuberant, and potentially harmful, inflammatory response. To help clarify an individual's response to infection and classify its severity, the inflammatory reaction to infection has been captured in a definition of 'sepsis', characterised by fever or hypothermia, tachycardia, tachypnea, and leucocytosis or leucopenia. This sepsis syndrome may be accompanied by hypotension ('septic shock'), hypotension refractory to fluid treatment ('refractory septic shock'), and single or multiple organ system failure, ultimately leading to death (1).

In the host response to infection, both cellular components and humoral components play a role. For instance, bacteria that breach the integrity of the mucosal lining of the respiratory or gastrointestinal tract are ingested ('phagocytised') by specialized host immune cells, the neutrophils and mononuclear phagocytes. In many instances, phagocytes are assisted in their task by another type of host cells, the lymphocytes, that may produce opsonising antibodies and thus help phagocytes recognize the invaders, or release substances, designated cytokines, that activate the phagocytes and enhance their phagocytic or antimicrobial activity. This complex process that constitutes the host

inflammatory response to invading pathogens (and is defined as "infection") is orchestrated both by cell-to-cell contact as well as by cytokines secreted by the various host immune cells. Locally, the cytokines that may either stimulate ('pro-inflammatory') or dampen ('anti-inflammatory') the host inflammatory reaction in a complex system of checks and balances, help contain and eliminate the invading pathogens. Some inflammatory mediators act systemically, by inducing an acute phase response and fever, one of the pivotal signs of the sepsis syndrome. It is not until immunostimulatory components of pathogens like the endotoxin of Gram-negative bacteria or the invading bacteria reach the bloodstream and are disseminated through the circulation that a cascade of inflammatory reactions are triggered within the bloodstream and soon may affect the integrity of the whole body, e.g., through activation of neutrophils within the circulation, activation of the coagulation cascade, and complex fluid rearrangements. The perfusion of critical organs may become compromised through blood coagulation in small vessels and the formation of microthrombi, at the same time intravascular fluids leak away into the tissues. Eventually, multiple organ failure ensues. Clearly, in such a disproportional response, the system that checks and balances the host' inflammatory reaction has become disrupted (2;3).

Any rational intervention rests on an understanding of the host factors that direct the inflammatory response to microbial products and thus contribute to the progression of sepsis into septic shock, multiple organ failure and eventually death. Today, the severe stages of sepsis constitute an important cause of morbidity and mortality in individual patients and have a great impact on hospital care and health costs in infectious diseases (4;5).

First contact

In the last decade many of the mechanisms by which the host responds to the presence of microbial pathogens on its mucosal surfaces or in the tissues, have been elucidated, most up to their molecular level. In the cascade from recognition to response, first the host must recognize the presence of a potentially pathogenic invader. The discovery of a group of receptors called Toll-like receptors (TLRs) and accessory proteins involved in their functioning has renewed interest in specificity of pathogen recognition (6). Because these receptors are highly specific to microbial components such as endotoxin, alone or in concert and each elicit a particular activation response in the host cells, already the very first reaction to pathogens exerts some specificity (7;8). This recognition results in cellular and humoral responses, the latter often made up of the release of a mix of pro-inflammatory and anti-inflammatory cytokines.

However, it is not only the pathogen that dictates the type and intensity of the cytokine release. Also an individual host's capacity to release such humoral factors must be taken into account. With the sequencing of the genome and recognition of the genetic variation within the human population, it became clear this variation is responsible for at least part of the inter-individual differences in cytokine release after challenge with microbial products (9). Soon it was recognized that such genetically determined variations in cytokine release could be relevant to explain the variability in susceptibility too, and outcome from, sepsis. In particular, interest focussed on genes that encode for proteins involved in the pathogenesis of sepsis including those involved in the initial recognition of bacterial products such as Toll-like receptors, and cytokines known to mediate the host inflammatory response, such as tumor necrosis factor (TNF)-α, interleukin(IL)-6 and IL-10. For many of these factors, polymorphic genes were discovered (10). A polymorphic gene is one in which a comparison of the DNA sequence of the gene in multiple individuals shows differences at a frequency of at least 1%. The sites within the genes that differ between individuals are called polymorphic sites and these may differ by insertions, deletions, or substitutions (e.g., 'single nucleotide polymorphisms' or SNPs) of one or more base pairs, or by the presence of a variable number of repeats of short, repetitive DNA sequences. Also, there may be one or more polymorphic sites within a single gene, and sites affected may pertain to coding regions of the gene or noncoding regions. The latter are important as well as they often affect regulation of gene expression. As of yet, only some of the variations observed have been linked to level and/or activity of the resulting protein, and therefore are known to affect cell function. Recent population association studies have indicated that polymorphisms in some of these genes indeed are associated with the severity of the response to infection.

Lipopolysacharide-mediated inflammatory response

The host response to bacterial invasion requires first the recognition of the presence of pathogens. The presence of a pathogen is detected by binding of pathogen-preserved molecules called 'pathogen-associated molecular patterns' or PAMPs on cell surface receptors known as 'pattern recognition receptors' or PRRs. Lipopolysaccharide (LPS; endotoxin), one of the major outer membrane components of the Gram-negative bacterial cell wall, is an important PAMP and thereby a powerful stimulator of the host inflammatory reaction. LPS elicits this response by binding to a cell surface receptor composed of at least three distinct proteins: CD14, Toll-like receptor-4 (TLR4), and MD-2 (Figure 1) (11;12).

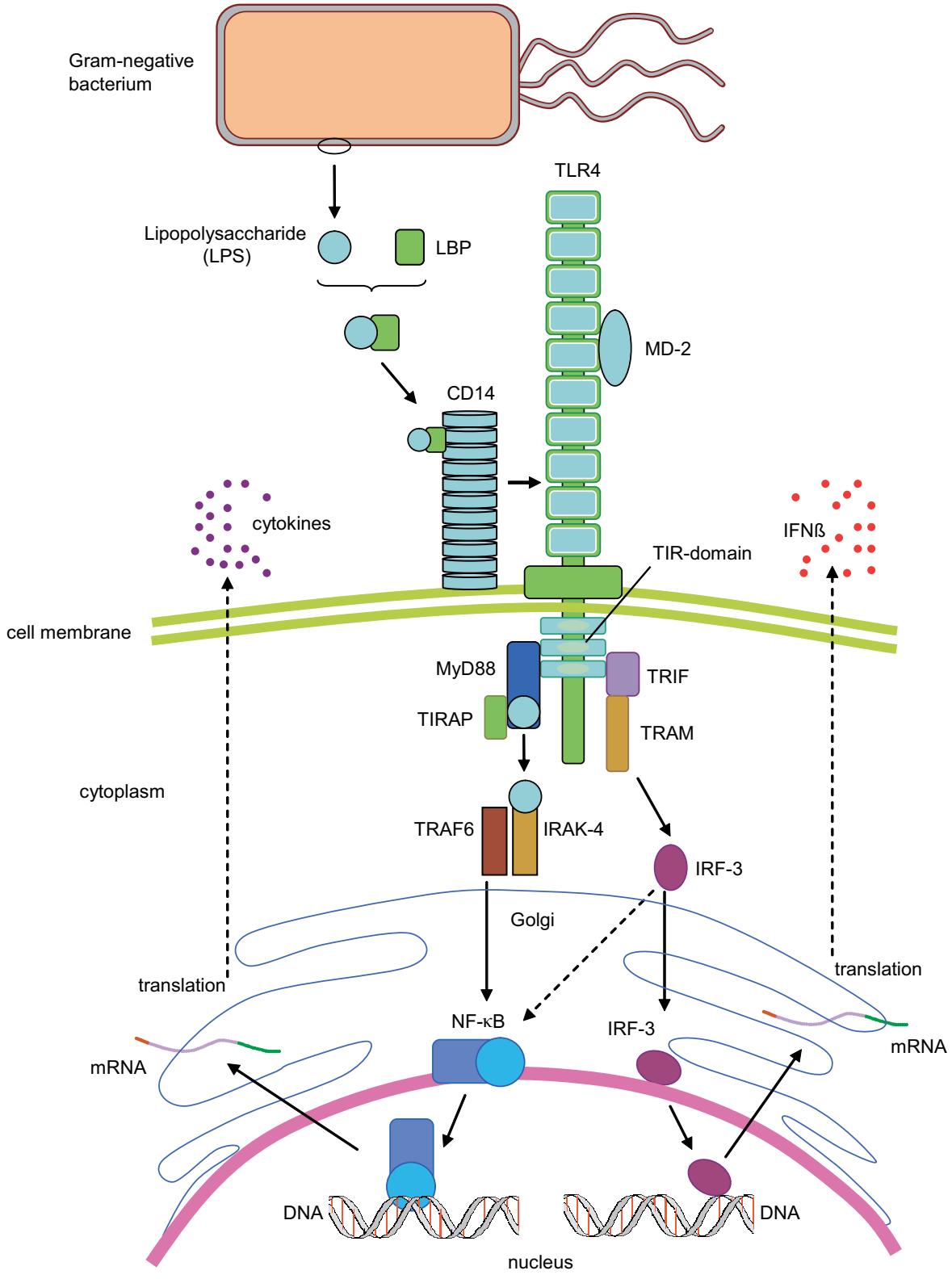


Figure 1: TLR4 signaling of LPS. LPS=lipopolysaccharide, LBP= lipopolysaccharide binding protein, TLR4=toll-like receptor-4, MD-2=myeloid differential protein, TIR-domain=Toll-Interleukin-1 resistance domain, MyD88=myeloid differentiation factor 88, TIRAP= TIR domain-containing adapter protein, IRAK-4=interleukin-1 receptor associated kinase-4, TRAF6=tumour necrosis factor-receptor associated factor 6, NF-κB=nuclear factor-κB, TRIF= TIR domain-containing adapter inducing interferon-ß, TRAM=TRIF-related adapter molecule, IRF-3=interferon-regulating factor, DNA=deoxyribonucleic acid, mRNA= messenger ribonucleic acid [Figure adapted from W.J.Wiersinga en T.van der Poll (108)].

At least two of these components, CD14 and TLR4, are polymorphic proteins and the various forms have been associated with altered functioning of the LPS receptor complex (13-18).

TLR4 is part of a family of transmembrane proteins. The receptor is expressed on monocytes and macrophages and to a lesser extent on lymphocytes and other cell types. Recently, a polymorphism in the coding region of TLR4 has been characterised (13;14). The single nucleotide polymorphism (SNP) was identified in the fourth exon of the TLR4 coding gene and results in replacement of a conserved aspartic acid at amino acid residue 299 with glycine. The SNP is in linkage disequilibrium with a second SNP at amino acid 399, which changes a threonine to an isoleucine. Both these amino acid replacements are located in the extracellular domain of the TLR4 receptor, and are thought to cause a functional defect in the receptors' ability to bind LPS (13-15). Studies in mice demonstrate that TLR4 is required for the response to LPS and that replacement of a single amino acid can significantly reduce the responsiveness to LPS and enhance susceptibility to infection by Gram-negative bacteria (6). *Ex vivo* studies using primary human epithelial cells heterozygous for the variant TLR4 or studies using a transfected cell system in which the variant TLR4 is expressed also suggest that the TLR4 299-variant is associated with a decreased responsiveness to LPS (13). However, the search for clinical correlates of this putative reduced LPS-responsiveness has been less definite. A number of studies have shown that this TLR4 variant was associated with reduced responsiveness to LPS, as assayed by airway reactivity or the systemic cytokine response to inhaled LPS (13-15). Other studies suggested that this TLR4 variant was associated with proneness to develop Gram-negative bacterial infections and even septic shock (19-21). It therefore was surprising that there was a lack of association between the TLR4 variant and susceptibility to, or severity of, meningococcal disease, notwithstanding that some rare TLR4 mutations have been implicated in meningococcal susceptibility (22;23). In addition, *ex vivo* studies of whole blood or isolated blood leukocytes failed to show an altered response to LPS in cells sampled from individuals heterozygous for the TLR4 variant (24-26).

Thus, it is still uncertain whether the *in vivo* response to LPS in humans having one copy of the TLR4 variant varies from that seen in individuals with two copies of wild-type TLR4. Clearly, additional studies in a large number of patients will be required to clarify whether the TLR4 variant indeed is associated with susceptibility to Gram-negative infection, and severity of the inflammatory host reaction, i.e., severity of sepsis.

CCR5

Another receptor that might function as a PRR in bacterial infection is CCR5. This seven-transmembrane spanning G protein-coupled receptor binds the CC chemokines macrophage inflammatory protein-1a (MIP-1a), MIP-1b, and RANTES. CCR5 is expressed on the cell surface of monocytes and CD45RO+ (memory) T-lymphocytes (27;28). Much of the interest in this molecule stems from its role as an HIV co-receptor, required for the infection of CD4-positive lymphocytes with macrophage-tropic HIV isolates. A genetic variant of this molecule exists due to a 32-nucleotide deletion (Δ32) in the region encoding the second external loop; the deletion causes a frame shift and premature stop codon. By consequence, the encoded protein is not translated (29). Homozygosity for this deletion-induced null mutation was reported as a mechanism of resistance to HIV infection (29). Similarly, individuals heterozygous for the Δ32 mutation lack about halve of the surface associated CCR5 and when HIV-infected display a slower progression to AIDS and a longer disease-free survival compared with CCR5-wild-type HIV-infected individuals (30;31). The frequency of the mutated CCR5 allele is about 10% in Caucasians. Carriers of the Δ32 allele are healthy and, no phenotype could be attributed to a heterozygous or homozygous mutated CCR5 genotype (32).

Studies in mice carrying a targeted disruption of this molecule presented evidence for a role of CCR5 in host defense against bacterial infection (33). For instance, after intraperitoneal challenge with lipopolysaccharide CCR5-deficient mice had a higher survival rate than mice carrying two CCR5 wild type genes. Also, the LD_{50} value of lipopolysaccharide was significantly lower in wild-type compared with the CCR5-deficient mice. Moreover, peritoneal macrophages of CCR5 knock-out animals differed from their wild-type littermates with regard to the cytokine production in response to LPS, by displaying reduced LPS-stimulated production of IL-1ß and IL-6. Of note, however, no difference in the release of TNF-α was found between knock-out and wild type mice. So far, it is uncertain whether heterozygosity for the Δ32 allele affects the cytokine response to lipopolysaccharide in humans.

Nod2

Another receptor reported to be involved in LPS responsiveness is Nod2, an intracellular protein that interacts with LPS through a C-terminal leucine-rich repeat (LRR) domain and is predominantly expressed within monocytes. The subsequent homodimerisation of Nod2 molecules induces NF-kB translocation, and cytokine production. A mutation in Nod2 that apparently diminishes the ability of the protein to interact with LPS is associated with

Crohn's disease (34). Studies thus far have focused on LPS responsiveness of recombinant Nod2. There is a lack of studies on LPS responsiveness of the natural Nod2 protein in humans.

Tumor necrosis factor-α encoding gene and its promoter

TNF-α is a pivotal member of the inflammatory cytokine cascade. It is responsible for the initial activation of the inflammatory response and if left unopposed plays an important role in the pathogenesis of septic shock, by mediating capillary leak, hypotension and coagulation events, resulting in acute respiratory distress syndrome (ARDS) and multiple organ system failure (35).

As previously mentioned, a dysbalance between pro-inflammatory (mainly TNF-α) and anti-inflammatory cytokines (such as IL-10) may underlie clinical manifestation of severe sepsis. The mechanism by which such an exaggerated pro-inflammatory response develops is area of intense research. A genetically-determined inclination to produce very high or very low rather than intermediate amounts of TNF-α could be one of the contributing factors in this respect.

TNF-α and genetic polymorphisms within the regulatory regions of the gene coding for TNF-α, are perhaps the most extensively studied of all cytokines and cytokine genes involved in sepsis (36-53). Upon stimulation by LPS human monocytes, the main producers of TNF-α, exhibit substantial inter-individual differences in TNF-α production (54). Studies have shown that inter-individual variation of TNF-α production capacity *ex vivo* may partly be explained by differences in genetic background (9;54). Because cytokine release is most likely transcriptionally regulated, polymorphisms in the regulatory region of the gene coding for TNF-α, the promoter region, is a significant target for this research. Several SNPs in the promoter region of the gene have been identified and were found to be associated with spontaneous and LPS-stimulated TNF-α production, assayed either *ex vivo* or *in vivo* (37;38;43;48;53;55). These SNPs include a G to A transition 308 base pairs upstream from the transcriptional start site of the TNF-α gene. *Ex vivo* studies have demonstrated that the rare TNF-α-308 A allele is associated with increased gene transcription as compared with the wildtype TNF-α-308 G allele (41). Furthermore, the TNF-α-308 A allele has been associated with increased secretion of TNF-α from macrophages after LPS stimulation *ex vivo* (38).

The promoter polymorphisms lie near putative DNA binding sites of transcription factors. For instance, by electrophoretic mobility shift assay it was demonstrated that nuclear proteins differ in their ability to bind to DNA fragments containing either an A or a G at the TNF-α-308 position (43). In a variety of infectious diseases, the TNF-α promoter

polymorphisms have been associated with clinical presentation and/or outcome of diseases. These include cerebral malaria, human immunodeficiency virus dementia in adults, meningococcal infections in children, and bacteraemia and septic shock, and community-acquired pneumonia (36;37;45;46;46-48;48-50). For example, among patients with septic shock, the TNF-α-308 A allele was found more frequently in those who died compared with those who survived, and the risk of an adverse outcome was about 3.5-fold higher in patients with at least one copy of the TNF-α-308 A allele (48). Furthermore, the TNF-α-308 A allele was observed more frequently in patients with fatal meningococcal disease as compared with patients surviving the disease (56). However, the distribution of TNF-α-308 alleles did not differ between healthy controls and an ethnically similar cohort of adults with severe sepsis, or between survivors and nonsurvivors in the severe sepsis group (51). In this and several other studies, no association was found between plasma TNF-α concentration and the above described polymorphism. Also, no increased risk of septic shock or mortality was observed in a cohort of consecutively admitted postoperative surgical patients. Although most evidence supports an association between genotypes in the regulatory region of the gene coding for TNF-α, levels of TNF-α production and mortality in patients with sepsis, there are a significant number of studies that could not confirm such an association. Additional studies in large groups of well defined patients are needed to quantify the role of these genetic polymorphisms in TNF-α production and outcome from infectious disease.

Interleukin-10 encoding gene and its promoter

IL-10 is an important immunoregulatory cytokine. It is involved in the regulation of the host inflammatory response, through inhibition of TNF-α production and other cellular processes. In Gram-negative infection, the presence in the circulation of lipopolysaccharide signals the release of IL-10. The cytokine is produced primarily by monocytes and down-regulates besides TNF-α the expression of other cytokines such as IL-1α and -ß, IL-6, and IL-8. By its role in the balance of pro-inflammatory and anti-inflammatory cytokines, IL-10 may codetermine the course of various infectious diseases. Be the relationship causal or not, the stage of severity to which meningococcal meningitis progresses is associated with serum IL-10 concentrations, such that high serum IL-10 levels are associated with poor or fatal outcome, whereas patients with mild disease and a good prognosis displayed much lower levels of IL-10 (57-59). Anti-inflammatory effects of IL-10 are evident in animal models of sepsis showing that neutralization of IL-10 results in an exaggerated pro-inflammatory response and death, while administration of IL-10 confers protection (60). In some models such as bacterial peritonitis, however, IL-10 may

induce a state of immunosuppression and may inhibit bacterial clearance (61). In these circumstances, administration of IL-10 is associated with exacerbation of bacterial sepsis and increased mortality (61).

Human monocytes exhibit substantial inter-individual differences in IL-10 production upon LPS stimulation *ex vivo*. A study suggested that differences in *ex vivo* IL-10 production capacity may to a large extent be explained by the genetic makeup of the individuals being tested (62). Of note, inter-individual differences in production of IL-10 could not be explained merely by corresponding changes in TNF-α production, thus implicating that the individual's ability to produce IL-10 is not simply reflecting the individual's ability to produce TNF-α (9). Because differences in IL-10 production are likely transcriptionally regulated, it has been suggested that differential transcription is the principle mechanism of inter-individual differences in IL-10 production. Indeed, IL-10 production was shown to be proportionally related to mRNA production and not to the half-life of IL-10 (63).

Located within the proximal 1.1 kb region upstream from the transcriptional start site which controls transcription of the IL-10 gene, four SNPs, at position -1330 (G or A), -1082 (G or A), -819 (C or T), -592 (C or A) have been identified (64-66). The -1082 G/A substitution occurs within a putative binding site of Ets, a transcription factor, whereas the -819 C/T lies within a putative positive regulatory region, and the -592 C/A polymorphism within a putative STAT 3 binding site and a negative regulatory region (67). Linkage disequilibrium between the alleles at the -1330 and -1082 sites on the one hand and -819 and -592 sites on the other hand has been demonstrated such that -1330 A is always associated with -1082 G, -1330 G with -1082 A, -819 C with -592 C and -819 T with -592 A. Therefore, only four haplotypes exist: AGCC, GACC, AGTA, and GATA. The influence of IL-10 promoter polymorphisms on IL-10 production was examined in several *ex vivo* stimulation studies and found associations between the production capacity of IL-10 *ex vivo* and haplotypes. For instance, the AGCC/AGCC genotype is associated with higher IL-10 production than the other haplotypes (68). Likewise, in a study in which only the IL-10-1082 site was examined, blood cells of individuals with the G allele released higher amounts of IL-10 compared with those of individuals with the A allele. In addition, the 5'-flanking regions from individuals homozygous for the three haplotypes AGCC, GACC, and GATA, were cloned into a luciferase vector and transiently transfected into cells. The AGCC construct demonstrated significantly increased transcriptional activity compared with the GATA construct, with the GACC construct demonstrated an intermediate transcriptional activity (67). In an *ex vivo* study in which the IL-10-592 site was examined, LPS-stimulated whole blood from healthy volunteers with the C allele produced higher amounts of IL-10 than individuals with the A allele at the -592 site. Taken

together, *ex vivo* evidence suggests that genetic variation in the promoter region of the IL-10 encoding gene influences the amount of IL-10 produced.

Next, the association between the IL-10 haplotypes and susceptibility to infectious diseases and severity of disease was investigated, yielding conflicting results. For instance, in a cohort of adults with community-acquired pneumonia, the "high secretor" G allele at position -1082 was associated with severe disease when compared with the "low secretor" A allele (69). In a cohort of patients with culture proven pneumococcal disease, 54% of the patients developing septic shock had the high secretor G allele, as compared with 16% of those patients who did not develop septic shock. With respect to the IL-10-592 site, in a cohort of critically ill adults no increased risk of sepsis was apparent in those patients with the low secretor A allele at the -592 site, but the mortality was higher in those patients with the A allele as compared with those with the C allele, regardless of whether they met the criteria for sepsis (70). In a cohort of intensive care unit patients with a wide variety of medical, surgical, and traumatic conditions, there was no association between IL-10 haplotypes and either serum IL-10 levels or mortality (71). Thus, clinical studies have been conflicting; on one hand, there appears to be an increased risk of more severe disease (development of septic shock) in those individuals who have the high secretor GG genotype at the -1082, yet there is a higher mortality in individuals with the low secretor genotype at the -592 site. The variable results in these studies may well be explained by the heterogeneity present among the groups studied.

In vivo model for lipopolysaccharide-mediated cytokine response

As illustrated in the previous paragraphs, clinical research into the role of genetic polymorphisms to susceptibility and outcome of lipopolysaccharide (LPS) mediated inflammatory disease, such as occurs in Gram-negative infection, is complex. Due to heterogeneity in underlying condition, type of infection, medication, etcetera, in the populations studied, inter-individual differences in release of cytokines may be caused by many factors besides genetic determinants. By consequence, background noise reduces the power of most studies to detect possible differences based on genetic background. Several approaches have been taken to circumvent this problem. First, small controlled studies were carried out in healthy subjects given low concentrations of LPS intravenously (72). However, the number of subjects and thereby ability to vary genetic background is limited and the injection of purified but chemically altered LPS directly into the bloodstream, without a local focus of inflammation represents an artificial model of severe sepsis. Another approach is to study cytokine release in reaction to naturally occurring endotoxemia in patients undergoing elective cardiac surgery (73-76). This approach

enabled us to study inter-individual differences in cytokine responses under controlled conditions of standardized surgery, and relate these to genotypes.

Cardiopulmonary bypass surgery is a frequently performed surgical procedure, and during reperfusion upon aorta declamping a perioperative endotoxemia occurs in about 70 percent of the patients (Figure 2).

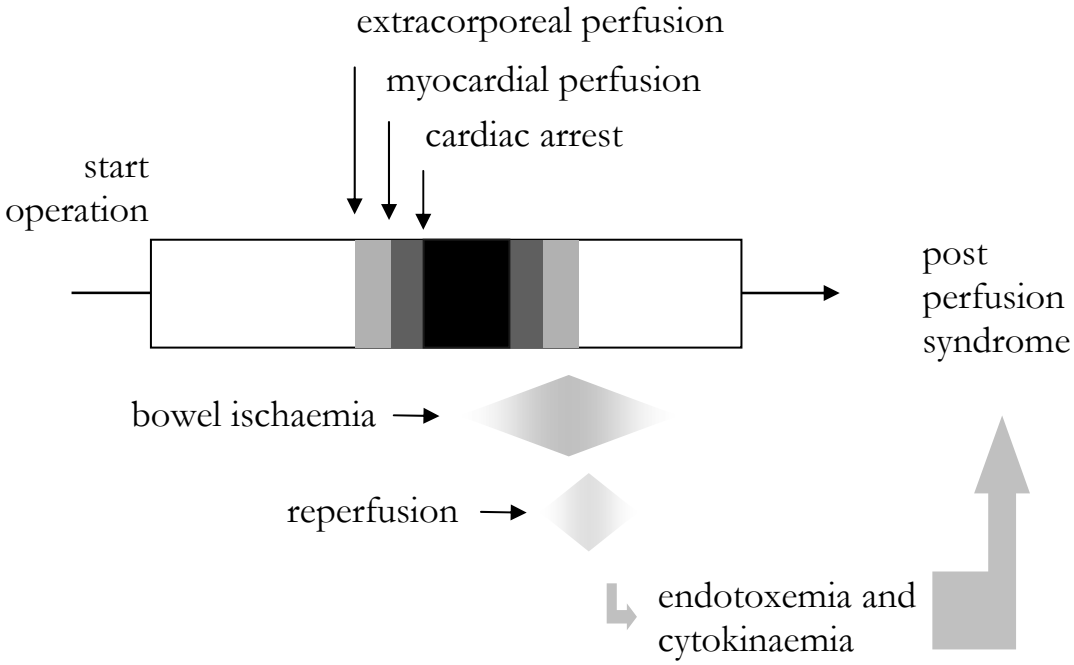


Figure 2. Schematic representation (time-line) of the *in vivo* model. In patients undergoing cardiac surgery, extracorporal perfusion is applied. In the first phase of the procedure, the heart is perfused and stops beating, circulation and oxygenation is maintained by an extracorporal system('Heart-Lung machine'). During this phase the bowel is in a state of relative hypoperfusion. Following the completion of the surgery, the heart is restarted and pulsatile circulation is resumed, resulting in reperfusion of the bowel. Accumulated metabolites and translocated intestinal products come into the circulation, leading to endotoxemia and subsequent cytokine release. In severe cases this might lead to the postperfusion syndrome.

Typically, during the phase of extracorporeal nonpulsatile circulation, the bowel undergoes relative hypo-perfusion, leading to splanchnic ischemia. Upon release of the aortic clamp and restart of pulsatile perfusion, gut reperfusion injury occurs frequently. The disturbance of the gut barrier function is marked by translocation of endotoxin from the gut into the systemic circulation, and leads to detectable levels of circulating endotoxin (74;75;77-81). The subsequent systemic inflammatory response varies from a relatively mild feverspike to a severe sepsis-like syndrome and potentially life threatening condition. The response is accompanied by complement activation, release of cytokines, leukocyte activation along with expression of adhesion molecules, and the production of substances such as oxygen-free radicals, arachidonic acid metabolites, platelet-activating factor (PAF), nitric oxide (NO), and endothelins. In more severe cases the inflammatory response contributes

to increased cardiac output in the presence of reduced systemic vascular resistance (SVR) and high oxygen consumption, generally referred to as ‘hyperdynamic instability’ or postperfusion (or “post-pump”) syndrome (75;76;82-87). This condition requires prompt fluid replacement and treatment with vasoactive agents. Ultimately, the derangement of hemodynamic variables may be complicated by lactic acidosis, impaired organ perfusion, and pulmonary dysfunction, which cause an extended intensive care unit stay and even fatal outcome.

Since the inflammatory response following cardiac surgery has similarities with sepsis, this condition of a natural occurring endotoxemia may serve as elegant model to study the host response in sepsis. Furthermore, the highly variable nature of the syndrome, both in its occurrence and in its severity, makes it clinically relevant to identify factors that help explain this variation, including possible genetic determinants. Currently, it is not known to what extend SNPs as those mentioned above contribute to the observed inter-individual variation in severity of the inflammatory response and development of the post-pump syndrome following cardiac surgery. Also, the standardized time course of the noxe enables study of the cytokine responses to the preceeding endotoxemia: in the model it is possible the sequentially measure the level of endotoxemia and the inflammatory response, making a quantitative mathematical approach to cytokine concentrations possible.

Ex vivo model for lipopolysaccharide-mediated cytokine response

The *ex vivo* cytokine production capacity of an individual is elegantly determined in the supernatant of whole blood after stimulation with LPS (54). This system provides a natural environment for cytokine producing cells, mainly being monocytes. The assay is generally accepted as gold standard of *ex vivo* assays to determine an individual’s ability to produce cytokines upon well defined stimuli. Day-to-day intra-individual variation and laboratory variation are small, i.e., both estimated at around 15% (54). The assay can distinguish ‘high’ and ‘low’ producing individuals of TNF-α and IL-10 based on the cytokine concentration found in the supernatant upon stimulation with one single concentration of LPS. In one study the aforementioned approach was able to detect differences in *ex vivo* cytokine production in relatives of patients with poor or fatal outcome of meningococcal disease as compared to relatives of patients with more favorable outcome (9). These observations led to the hypothesis that inter-individual variation in cytokine production can be explained to a large extend by genetic background. Most studies assessed the association between gene(- promoter) polymorphisms and (whole blood) stimulation phenotype (i.e. individuals categorized as 'high' as compared to 'low' producers) by measuring the cytokine release after stimulation of whole blood with only one single – and

between studies varying – concentration of LPS (38;41;43;51;55;63;64;67;88-105). Not surprisingly, the conclusions of these studies have been conflicting.

Categorizing individuals as 'high' as compared to 'low' producers based on the cytokine production upon stimulation by a single LPS concentration is not adequate. Firstly, the LPS concentrations applied always exceeded by far the concentrations of naturally occurring endotoxemia in patients with infectious diseases, hampering the translation of the *ex vivo* phenotype to the *in vivo* situation (106). Secondly, the cytokine concentrations found after stimulation with one single high concentration of LPS will at best reflect maximal production capacity, but will not necessarily provide insight in the cytokine response characteristics at lower LPS concentrations. Furthermore, aspects of the dose-response characteristics, for instance the LPS concentration at which 50% of the maximal cytokine concentration is reached and the maximal cytokine production capacity of the dose-response curve are not taken into account. Since the discovery of the TLR4, these aspects are of interest since this mode of LPS signaling is likely to follow the basic concepts of the receptor-ligand model used in other fields of research. Due to the elective nature of the surgical procedures included in our study, patients could be sampled before the operation and their *ex vivo* LPS-responsiveness assayed. Based on the dose response model know as the C_{max} model we calculated the dose response characteristics of individuals by using supernatant cytokine concentrations after stimulation with a wide range of LPS concentrations (107). We hypothesized that the intra-individual day-to-day variation in the dose-response parameters is small as compared to the variation found in the intra-individual day-to-day variation found in a single point measurement and have better discriminative properties in labeling an individuals phenotype. If these premises were met, it would be suitable to use these parameters as patient characteristics in future research.

OUTLINE OF THE THESIS

Part I

In **Chapter 1** a general introduction to the subject is given. In a prospective, randomized, placebo-controlled study described in **Chapter 2**, we examined whether preoperative selective gut decontamination aimed to eliminate the aerobic Gram-negative bowel flora would preclude perioperative endotoxemia, cytokine activation, and postoperative complications in elective cardiac surgery. In **Chapter 3** we discuss some of the details concerning the effect of preoperative selective gut decontamination on aerobic Gram-negative bacilli and fecal endotoxin concentrations. In **Chapter 8** we investigated the role of perioperative endotoxemia, and cytokine activation, and of polymorphic forms of TLR4 and SNPs in the promoter region of IL-10, on the postoperative response leading to hemodynamic stability, morbidity, complications and outcome in elective cardiac surgery.

Part II

In **Chapter 4**, based on the dose response model know as C_{max} model, we calculated lipopolysaccharide-elicited cytokine dose-response characteristics of individuals by determining TNF-α and IL-10 concentrations in the supernatant after stimulation of blood cells with a wide range of LPS concentrations. We quantified the influence of the laboratory and intra-individual day-to-day variation on these parameters. We evaluated which *ex vivo* parameter describing the lipopolysaccharide-stimulated cytokine release is most suitable for use as patient characteristic in future studies correlating *ex vivo* cytokine production to genetic factors, and determined how much of the inter-individual variation could be attributed to these genetic factors.

Part III

In **Chapter 5** we determined to what extent the lipopolysaccharide-induced TNF-α production capacity *in vivo* and *ex vivo* is determined by polymorphisms in Toll-like receptor-4 (TLR4), the TNF-α promoter region and Nod2. In **Chapter 6** we determined to what extent lipopolysaccharide-induced IL-10 production capacity *in vivo* and *ex vivo* is determined by polymorphisms in TLR4 and the IL-10 promoter region. In **Chapter 7**, finally, we evaluated to what extend a naturally occurring mutation of the CCR5 gene (a 32-basepair deletion, Δ32) is associated with a differential *in vivo* and *ex vivo* cytokine production.

The results are summarized and discussed in **Chapter 9**.

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CHAPTER 2

No effect of preoperative selective gut decontamination on endotoxemia and cytokine activation during cardiopulmonary bypass: a randomized, placebo-controlled study

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No effect of preoperative selective gut decontamination on endotoxemia and cytokine activation during cardiopulmonary bypass: A randomized, placebo-controlled study

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Background: **Cardiopulmonary bypass predisposes the splanchnic region to inadequate perfusion and increases in gut permeability. Related to these changes, circulating endotoxin has been shown to rise during cardiac surgery, and may contribute to cytokine activation, high oxygen consumption, and fever ("postperfusion syndrome"). To a large extent, free endotoxin in the gut is a product of the proliferation of aerobic Gram-negative bacteria and may be reduced by nonabsorbable antibiotics.**

Objective. **To evaluate the effect of preoperative selective gut decontamination (SGD) on the incidence of endotoxemia and cytokine activation in patients undergoing open heart surgery.**

Design: **Prospective, randomized, placebo-controlled double-blind trial.**

Setting: **Tertiary-care university teaching hospital.**

Intervention: **Preoperative administration for 5 to 7 days of oral nonabsorbable antibiotics (polymyxin B and neomycin) vs. placebo. The efficacy of SGD was assessed by culture of rectal swabs.**

Patients: **Forty-four patients (median age 65 yrs, 29 males) were included in a pilot study to establish the sampling points of perioperative measurements. Seventy-eight consecutive patients (median age 65 yrs, 55 males) were enrolled for the prospective study; of these, 51 were randomly allocated to take SGD (n = 24) or placebo (n = 27); 27 were included in a control group (no medication).**

Measurements and Results: **SGD but not placebo effectively reduced the number of rectal swabs that grew aerobic Gram-negative bacteria (27% vs. 93%, respectively; $p < .001$). SGD did not affect the occurrence of perioperative endotoxemia, nor did it reduce the tumor necrosis factor-α, interleukin-10, or interleukin-6 concentrations ($p > .20$), as determined before surgery, upon aorta declamping, 30 mins into reperfusion, or 2 hrs after surgery. Also, SGD did not alter the incidence of postoperative fever or clinical outcome measures such as duration of artificial ventilation and intensive care unit and hospital stay.**

Conclusion: **SGD effectively reduces the aerobic Gram-negative bowel flora in cardiac surgery patients but fails to affect the incidence of perioperative endotoxemia and cytokine activation during cardiopulmonary bypass and the occurrence of a postperfusion syndrome. (Crit Care Med 2002; 30:38–43)**

Key Words: selective gut decontamination; cardiac surgery; cardiopulmonary bypass; endotoxemia; cytokine; systemic inflammatory response syndrome

Cardiopulmonary bypass predisposes the intestines to inadequate perfusion, hypoxic injury, and increases in gut permeability (1, 2). During cardiopulmonary bypass, endotoxin that originates from the gut has been shown to rise in the circulation and may contribute to the cytokine and complement activation already caused by the exposure of blood to artificial surfaces of the oxygenator. The resulting proinflammatory reaction can cause fever and high oxygen consumption in the direct postoperative period, the so-called postperfusion (or "post-pump") syndrome (3–5). Because this inflammatory reaction has been associated with increased morbidity after cardiac surgery, clinicians have sought strategies to attenuate this response.

The reaction to infection can be manifested by a systemic inflammatory response syndrome progressing to septic shock and multiple organ dysfunction syndrome (6). This chain of events is triggered by bacterial components such as endotoxin, peptidoglycan, lipoteichoic acid, lipoprotein, and exotoxins, and executed through many mediators (7–9). In Gram-negative infection, the presence of endotoxemia plays a pivotal role in the morbidity and mortality associated with bacteremia caused by aerobic Gram-negative microorganisms (7, 10, 11). Evidence as to the central role of endotoxin in cytokine activation and the proinflammatory reaction in Gram-negative infection has stimulated interest in the translocation of aerobic Gram-negative bacteria and endotoxin during cardiopulmonary bypass. It has been hypothesized that reducing the number of aerobic Gram-negative microorganisms in the bowel at the time of cardiac surgery would diminish the gut ischemia-associated translocation of Gram-negative bacteria. Thus, presumably, circulating endotoxin during cardiopulmonary bypass would diminish as well (12, 13). This hy-

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pothesis followed the observation in mice that free endotoxin in the intestinal tract mainly is a product of the proliferation of aerobic Gram-negative bacteria (14), and may be amenable to treatment with antibiotics (15, 16). In rats, selective gut decontamination aimed at eradicating the aerobic Gram-negative microorganisms was effective in preventing bacterial translocation and reduced gut-derived endotoxemia and mortality after severe thermal injury (17). In man, a reduction in the aerobic Gram-negative bowel flora can be readily achieved by selective gut decontamination with nonabsorbable antibiotics (12, 18, 19), and thus may represent a relatively low-cost strategy to prevent postoperative complications in patients undergoing elective heart surgery. The aim of the present prospective, randomized, placebo-controlled study was to investigate in patients undergoing elective cardiac surgery with cardiopulmonary bypass, the effect of preoperative selective gut decontamination on perioperative endotoxemia, cytokine activation, and postoperative complications.

METHODS

The study was performed at the Leiden University Medical Center, a 800-bed secondary and tertiary referral hospital. Patients 18 yrs and older, undergoing cardiac surgery and consecutively referred to the Department of Cardiothoracic Surgery were enrolled following institutional approval by the local medical ethics committee (protocol P168/96). Each patient gave a written, informed consent.

Anesthesia protocol was standardized and consisted of premedication with lorazepam (0.05 mg/kg) per os and induction with a bolus sufentanil (1–2 μg/kg) and a bolus midazolam (0.1 mg/kg). Curarization was achieved with a pancuronium injection (0.1 mg/kg) at induction. Anesthesia was maintained with continuous infusion of sufentanil (1–3 μg/kg/hr) and midazolam (0.1 mg/kg/hr). The patient was ventilated with an air-oxygen mixture to obtain a $Paco_2$ of 4.7–5.3 kPa. Heparin sulfate was administered before start of cardiopulmonary bypass at a dose of 3 mg/kg and subsequently at doses adapted to maintain the activated clotting time above 400 secs. At the end of cardiopulmonary bypass, the heparin action was antagonized by protamine sulfate. Bypass priming included gelatin, mannitol, sodium bicarbonate, and heparin. After venous and arterial cannulation, cardiopulmonary bypass was commenced using a membrane oxygenator (Baxter, Uden, The Netherlands). Flow rates at about 2.4 L/min/m^2 with nonpulsatile flow were set to maintain a mean arterial pressure of 50–70 mm Hg, and a Pao_2 between 9 and 11 kPa, and a $Paco_2$ of 4.7–5.3 kPa. Next, antegrade cardioplegia with cold crystalloids was administered for myocardial preservation. During bypass, the core temperature was maintained at 28°C. A pulmonary artery catheter and three-way central venous catheter were inserted after induction of anesthesia. Standard antimicrobial surgical prophylaxis consisted of flucloxacillin.

Intervention. At the outpatient clinic, patients were randomly allocated to selective gut decontamination or placebo, and took this medication home. Randomization was performed by the hospital pharmacist using a computer-generated code for randomized allocation to medication or placebo; patients and investigators were blinded until completion of the study. On Thursday morning in the week before surgery, patients were notified by phone of the exact day of operation. Patients to be operated on Tuesday, Wednesday, or Thursday were instructed to take their medication for 5, 6, or 7 days, respectively; last medication was taken at the night before surgery. Patients received four times daily two tablets each containing neomycin 125 mg and polymyxin B 500,000 E or placebo. Leftover tablets were taken in and counted at admission. The tablets for selective gut decontamination were prepared by the hospital pharmacist and have been in use for many years in the hematology and bone marrow transplant unit of our hospital (18, 19). Patients to be operated on Monday or Friday were instructed not to take their medication, as logistic reasons concerning part of the perioperative blood sampling and microbiological culture precluded the inclusion of these patients; tablets were taken in and counted at admission.

Before the prospective selective decontamination study was started, a pilot study (including 44 consecutive patients) was done to determine the time-points for blood sampling.

Microbiological Surveillance. In all patients who took medication, microbiological surveillance was performed by culture of rectal swabs taken the evening before surgery and screening for aerobic Gram-negative microorganisms. Rectal swabs were used to swab McConkey 3 and sheep red blood cell-plates, and subsequently added in brain-heart infusion broth and cultured overnight at 37°C. Aerobic Gram-negatives were identified macroscopically and on the basis of growth characteristics and biochemically by API. The culture of aerobic Gram-negative microorganisms on the solid plates as well as in the fluid medium is categorized as "many bacteria present"; a positive culture in the brain-heart infusion broth only as "few bacteria present" (18, 19). In two patients, no rectal swabs were available; these patients were later found to be allocated in the selective gut decontamination group.

Endotoxin and Cytokine Sampling and Assay. Blood for endotoxin determination was collected in pyrogen-free tubes (Kabi-Vitrum, Amsterdam, The Netherlands) and platelet-rich plasma was prepared by centrifugation. Endotoxin was determined in platelet-rich plasma by a quantitative photometric assay with end-point measurement as described (11); the assay's lower detection limit for endotoxin is about 3.0 pg/mL; concentrations ≥5 pg/mL were considered to indicate endotoxemia (11, 20). Blood for determination of cytokines was collected in pyrogen-free ethylenediaminetetraacetic acid tubes (Chromogenics, Amsterdam, The Netherlands) and immersed in ice. Plasma was prepared by centrifugation at 3000 *g* for 5–10 mins at 4°C and stored at −70°C. Tumor necrosis factor (TNF)-α, interleukin (IL)-10, and IL-6 concentrations were determined with a standard enzyme-linked immunoassay technique (Central Laboratories for Bloodtransfusion, Amsterdam, The Netherlands; Medgenix Diagnostics, Floury, Belgium) (11); the lower detection limit was 0.5 pg/mL for TNF-α, and 5.0 pg/mL for IL-10 and IL-6. For the IL-6 determinations, samples were available from 22 of 24 patients receiving selective gut decontamination, and from 23 of 27 patients receiving placebo. All samples were determined in a blinded fashion; results were not available until completion of the study.

Analysis of Data. Analysis of variance, nonparametric testing, and logistic regression analysis were used to assess an association between medication (selective gut decontamination or placebo) and endotoxin, cytokines, clinical parameters (postoperative fever, and duration of artificial ventilation, cardiothoracic intensive care unit [ICU] and hospital stay) and outcome of hospitalization. Data for endotoxin and cytokine levels were used after log-transformation (11). When indicated, endotoxemia and cytokines were entered as categorical variable, using cutoff concentrations of 5 pg/mL for endotoxin and TNF-α, 100 pg/mL for IL-6, and 25 pg/mL for IL- 10. These cutoff values are somewhat arbitrarily but based on previous assessment in clinical studies (11, 20). Multiple organ dysfunction syndrome (MODS) was considered when a patient had three or more organ system failures during a 24-hr period in the postoperative phase.

Statistical significance was tested two-tailed, with the α set to 0.05.

RESULTS

Pilot Study. In 44 consecutive patients (median age 67 yrs, 29 male), a total of 247 measurements of endotoxin concentration were taken after anesthetic induction, on aorta declamping, at the end of and 30 mins after stopping the cardiopulmonary bypass (i.e., extracorporal perfusion), on surgical closure, and at the ICU about 2 hrs after surgery. In 64 of these samples (26%). the endotoxin concentration was ≥5 pg/mL.

None of the 44 patients had endotoxemia directly after anesthetic induction.

Twenty-four patients (55%) did not have a raised endotoxin concentration at any point during the operation; four patients in this group, however, had endotoxemia at admission to the ICU. Twenty patients (45%) had 52 episodes of endotoxemia during and after cardiopulmonary bypass. In 16 of these patients, at least two separate consecutive samples were positive, whereas 8 patients still had endotoxemia at admission to the ICU. On the basis of the pilot study, it was decided to measure the endotoxin concentrations in the prospective study after anesthetic induction, on aorta declamping, 30 mins into body reperfusion (i.e., 30 mins after stopping the extracorporal perfusion), and at the ICU about 2 hrs after surgery.

Allocation of Patients and Comparability of Groups. Seventy-eight patients were randomized to receive placebo or selective gut decontamination. Of these, 24 patients received selective gut decontamination and 27 patients took placebo. The other patients (n = 27) were operated upon on days outside the study protocol and were allocated in the nonintervention, control group. Patients receiving selective gut decontamination did not differ from those taking placebo or the nonintervention control group with respect to age, gender, smoking history, diabetes mellitus, and other co-morbidities like emphysema ($p > .20$), surgical procedure (coronary artery bypass graft and/or valve replacement) and ASA score (Table 1).

Aerobic Gram-Negative Gut Flora. In patients receiving placebo, aerobic Gram-negative bacteria were cultured from the rectal swab in 25 out of 27 patients (93%); in 3 of these, the microorganisms were cultured only from the broth. In patients given selective gut decontamination, the number of patients carrying aerobic Gram-negative bacteria was significantly ($p < .001$) decreased: in only 6 of 22 patients (27%) were such microorganisms isolated; in 2 of these patients the bacteria were present only in the broth (Table 2).

Perioperative Endotoxemia. In all but one patient (selective gut decontamination), endotoxin levels were <5 pg/mL directly after anesthetic induction. On aorta declamping and 30 mins into reperfusion, endotoxemia was common in both patients receiving selective gut decontamination and those given placebo: between 31% and 61% of the patients had ≥5 pg/mL endotoxin in the blood. Neither the percentage of patients with endotoxemia nor the actual plasma concentration of endotoxin differed significantly ($p > .20$) between patients receiving selective gut decontamination or placebo (Table 3; Fig. 1). Similarly, on arrival at the ICU 2 hrs after surgery, the number of patients with a significant endotoxin level was similar ($p > .20$) in the groups. Of note, the subgroup of patients receiving selective gut decontamination and showing no growth of aerobic Gram-negative microorganisms in the rectal swab had similar rates of endotoxemia as those in this group with such bacteria still present. Also, in the nonintervention control group, 37% to 65% of the patients had ≥5 pg/mL endotoxin in the blood on aorta declamping and during body reperfusion (data not shown), whereas the actual plasma concentration of endotoxin did differ significantly ($p > .20$) from those in patients receiving selective gut decontamination or placebo (Fig. 1).

Perioperative Cytokine Activation. Plasma concentrations of the cytokines rose significantly at the end of cardiopulmonary bypass and reached maximum values at 30 mins after start of reperfusion (TNF-α, IL-10) or at admission to the ICU (IL-6). Overall cytokine concentrations were highly similar ($p > .20$) in patients receiving selective gut decontamination and those given placebo (Table 3; Fig. 1). Also, if the TNF-α, IL-10, and IL-6 measurements were dichotomized at various different values, cytokine activation was similar ($p > .20$) in patients receiving selective gut decontamination and those given placebo, and, in addition, did not differ from those in the control group (Fig. 1). In the ICU, 2 hrs after surgery, TNF-α and IL-10 al-

Table 1. Comparability of study groups

Characteristic[a]	Intervention Group: Selective Gut Decontamination (n = 24)	Intervention Group: Placebo (n = 27)	Control Group (n = 27)	*p* Value
Age, yrs	64 (58–69)	65 (57–71)	65 (55–72)	>.20
Gender, male (%)	16 (67)	17 (63)	22 (81)	>.20
Weight, kg	75 (67–87)	79 (70–87)	80 (71–93)	>.20
Active smoker, n (%)	4 (17)	4 (19)	7 (26)	>.20
Type of operation				>.20
CABG	20	22	21	
Valve replacement	1	2	2	
CABG + valve replacement	1	1	1	
Other	2	2	3	
ASA score ≥3, %	80	76	90	>.20
Perioperative data				
Operation time, mins	255 (176–308)	264 (197–280)	222 (188–261)	>.20
Perfusion time, mins	120 (83–152)	111 (91–149)	104 (70–131)	>.20
Aorta clamp time, mins	58 (45–87)	69 (38–89)	66 (42–83)	>.20
Flow bypass, min-max, L/min	3.7–4.5	3.8–4.6	3.8–4.6	>.20
Lowest temperature, °C	28.1 (27–31)	30.2 (28–32)	30.2 (28–32)	.18
Postoperative data				
Hospital stay, days	11 (9–15)	10 (9–13)	9 (8–12)	.13
Fatalities, n (%)	1 (4)	1 (4)	2 (7)	>.20

CABG, coronary artery bypass graft.

[a]Median values and interquartile range are between parentheses.

Table 2. Efficacy of selective gut decontamination in intervention group

Preoperative Culture of Rectal Swab[a]	Intervention Group: Selective Gut Decontamination[b]	Intervention Group: Placebo	*p* Value
Aerobic Gram-negative bacteria			<.001
BHI broth and plates	4	22	
BHI broth only	2	3	
Absent	16	2	

BHI, brain-heart infusion.

[a]Culture of rectal swab, categorized as explained under the Methods section; [b]in two patients, no rectal swabs were available.

Table 3. Endotoxemia and cytokine activation in patients receiving selective gut decontamination or placebo, %

Perioperative Data	Intervention Group: Selective Gut Decontamination (n = 24)	Intervention Group: Placebo (n = 27)	*p* Value
Endotoxemia (>5 pg/mL)			>.20
Anesthetic induction	1/23 (4)	0/25 (0)	
Release of aortic clamp	14/23 (61)	15/26 (58)	
30-min reperfusion	11/23 (48)	12/26 (38)	
At admission to ICU	11/23 (48)	8/26 (31)	
TNF-α (>5 pg/mL)			>.20
Anesthetic induction	1/24 (4)	0/27 (0)	
Release of aortic clamp	6/24 (25)	6/27 (22)	
30-min reperfusion	13/24 (54)	13/27 (48)	
At admission to ICU	10/24 (42)	12/27 (44)	
IL-6 (>100 pg/mL)			>.20
Release of aortic clamp	5/22 (23)	2/23 (9)	
At admission to ICU	18/22 (82)	15/23 (65)	
IL-10 (>25 pg/mL)			>.20
Anesthetic induction	1/24 (4)	0/27 (0)	
Release of aortic clamp	13/24 (54)	11/27 (41)	
30-min reperfusion	12/24 (50)	11/27 (41)	
At admission to ICU	6/24 (25)	8/27 (30)	

ICU, intensive care unit; TNF, tumor necrosis factor; IL, interleukin.

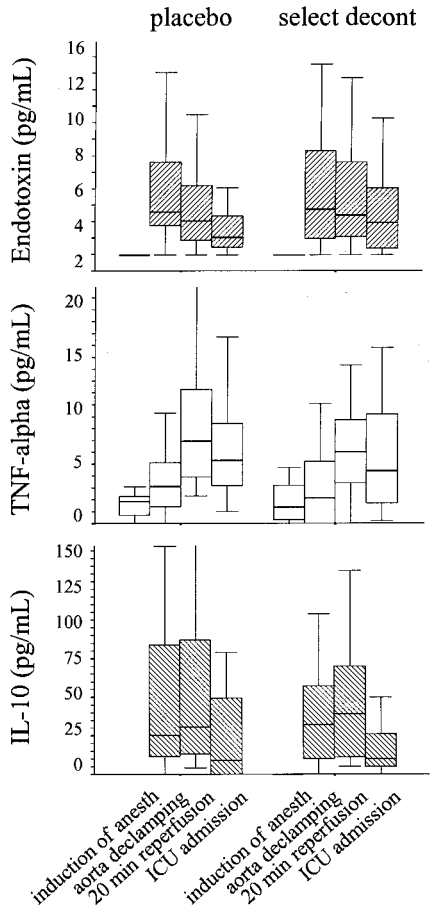

Figure 1. Plasma endotoxin concentrations in patients undergoing open heart surgery, and at the cardiothoracic intensive care unit (*ICU*). Patients were randomized to receive placebo or selective gut decontamination (*decont*). The box plots display the median, the 25th and 75th percentiles, and smallest and largest endotoxin values that are not outliers. Endotoxin was determined in platelet-rich plasma by a quantitative photometric assay with end-point measurement; the detection limit for endotoxin is about 3.0 pg/mL. Tumor necrosis factor (*TNF*)-α and interleukin (*IL*)-10 were measured by enzyme-linked immunosorbent assay; the detection limit is 0.5 pg/mL and 5.0 pg/mL, respectively.

ready were decreasing in all groups, whereas IL-6 increased further, as compared with the levels upon aorta declamping. In the patients who did not have endotoxemia, plasma TNF-α, and IL-6 rose significantly during surgery, albeit less than in patients with endotoxemia ($p < .015$); IL-10 levels were not different between patients with or without endotoxemia.

In four patients receiving selective gut decontamination and seven patients receiving placebo, but none in the control group, glucocorticosteroids were administered during the operation. Glucocorticosteroids raised the IL-10 concentrations significantly ($p = .01$) and led to a somewhat decreased IL-6 concentration at admission to the ICU (118 pg/mL, range 40–760; 350 pg/mL, range 40–23,000, respectively; $p = 0.02$), but the administration did not affect either TNF-α ($p > .80$) or endotoxin concentrations ($p > .50$).

Clinical Evaluation. No differences between the groups were observed regarding the length of stay in the ICU, the duration of artificial ventilation, and postoperative complications like reoperation (mostly for bleeding complications), multiple organ failure, or fatal outcome. Patients who received selective gut decontamination had a temperature peak above 38.5°C on the first postoperative day more often than the placebo or controls (Table 4). However, the percentage of patients with a temperature rise above 38.5°C that sustained at least 6 hrs did not differ significantly between the groups, and at 1 day after the operation, the temperature of the patients in the various groups was identical (Table 4). Furthermore, 1 day after the operation, the systolic and diastolic blood pressure, pulse rate, blood leukocyte count, and serum creatinine concentrations did not differ between the groups ($p > .15$; data for blood leukocyte count included in Table 2).

DISCUSSION

The association between morbidity in cardiac surgery patients and perioperative plasma endotoxin and cytokine activation is important in terms of the reduction in morbidity that would follow a description of the underlying pathophysiological mechanism and validation of a successful (pre- or perioperative) intervention strategy aimed at this mechanism. The present prospective, randomized, placebo-controlled study in patients undergoing cardiac surgery with cardiopulmonary bypass demonstrates that 1 wk of preoperative selective gut decontamination with nonabsorbable antibiotics has no effect on the occurrence of perioperative endotoxemia, cytokine activation, and the postperfusion systemic inflammatory response syndrome. Thus, given the effective reduction of aerobic Gram-negative microorganisms by the decontamination measure, our findings lend no support to the presumptive central role that the live, replicating microorganisms in the gut are thought to be the predominant source of circulating endotoxin during cardiopulmonary bypass and in the pathophysiology of the postperfusion syndrome (13, 21).

Our results show that selective gut decontamination with polymyxin B and neomycin given for 5–7 days is very effective in the reduction of aerobic Gram-negative flora from the gut: in most patients a complete eradication of these microorganisms was achieved, as was

Table 4. Clinical outcome in patients receiving selective gut decontamination or placebo

Postoperative Data[a]	Intervention Group: Selective Gut Decontamination (n = 24)	Intervention Group: Placebo (n = 27)	*p* Value
ICU stay, days	3 (2–6)	2 (2–4)	>.20
Artificial ventilator, days	1.0 (1–4)	1.0 (1–3)	>.20
Temperature, ≥38.5 °C (%)	23 (96)	15 (56)	.03
Temperature, ≥38.5 °C for ≥6 hrs (%)	17 (71)	11 (41)	.16
Temperature, at 24 hrs, °C	37.7 (37.4–37.8)	37.5 (37.2–37.7)	>.20
Leukocytes at 24 hrs	9.9 (8.2–12.3)	8.3 (7.4–8.7)	>.20
Reoperation (%)	4 (17)	5 (19)	>.20
Multiple organ failure (%)	1 (4)	0 (—)	>.20

ICU, intensive care unit.
[a]Median values and interquartile range are between parentheses.

Selective gut decontamination effectively reduces the aerobic Gram-negative bowel flora in cardiac surgery patients but fails to affect the incidence of perioperative endotoxemia and cytokine activation during cardiopulmonary bypass and the occurrence of a postperfusion syndrome.

previously shown in hematologic and bone marrow transplant patients during the extended episodes of granulocytopenia (18, 19). Studies in these patients had also demonstrated that, on average, selective gut decontamination needs to last for at least 5 days to be effective in the complete eradication of aerobic Gram-negative bowel flora (18). The present report extends these findings to patients undergoing elective cardiac surgery. The choice of polymyxin B in this regimen seems especially appropriate as this bactericidal antibiotic is well known for its endotoxin-binding properties (22).

Our findings add to previous studies showing that, during cardiopulmonary bypass, cytokine activation occurs in the absence of endotoxemia, and confirm that perioperative endotoxemia in itself is a weak predictor of clinical outcome in cardiac surgery patients. Another rich source of cytokines during cardiopulmonary bypass is the pulmonary circulation, and may lead to myocardial stunning (23). Some of our patients received a dose of glucocorticosteroids during surgery, and, in accordance with reported data, the plasma IL-10 concentrations were markedly increased in these patients (24). However, the administration did not affect the endotoxin and TNF-α levels, as previously shown for methylprednisolone, administration of which did not prevent or attenuate endotoxemia during cardiopulmonary bypass (25).

The present findings are in contrast to the efficacy of selective gut decontamination to lower endotoxin and cytokine concentrations in cardiac patients, as previously reported by Martinez-Pellús et al. (13, 21). In their open, randomized study, selective gut decontamination was administered for 3 days before operation, and succeeded in just over half of their patients in the elimination of fecal aerobic Gram-negative bacteria.

Nevertheless, plasma endotoxin, TNF-α, and IL-6 concentrations were significantly reduced in treated patients compared with controls, and in the subgroup of treated patients with no growth of aerobic Gram-negative microorganisms, endotoxin was undetectable in all samples. We are uncertain of how to explain the differences between their findings and ours in the effectiveness of selective gut decontamination to prevent or at least reduce perioperative endotoxemia and cytokine activation. The detection limit of our endotoxin assay, i.e., about 3 pg/mL, appears to be lower than theirs, and the fact that they considered all endotoxin values <5 pg/mL as 0 pg/mL for the statistical analysis may be relevant to explain some of the differences in reported endotoxin levels. Their assay may fail to adequately detect circulating endotoxin from anaerobic Gram-negative microorganisms such as the gut commensal *Bacteroides* spp., to which patients undergoing cardiac surgery are likely exposed (26) and may be responsible for up to 10% of the free endotoxin in the intestinal tract (15). On average, selective gut decontamination aimed at eradicating the live, aerobic Gram-negative coliforms results in a reduction of the endotoxin concentration of fecal supernatant to 10% of the untreated controls (15, 27), leaving an endotoxin pool of about 0.1–1.0 mg/g of feces (16, 27). The endotoxin measurements in subsequent samples in our patients were highly consistent, and the percentage of patients in whom we measured significant endotoxin concentrations (i.e., approximately 50%) is not different from that observed in studies by others (4, 21, 28–30). As neither the percentage of patients with a significant endotoxemia nor the actual circulating endotoxin concentrations during cardiac surgery were affected by preoperative selective gut decontamination, the present findings suggest that, at least in man, the size of the free endotoxin pool is not the key limiting factor in the pathophysiological mechanism that controls circulating endotoxin during cardiac surgery.

Our study was placebo-controlled and double-blinded, and performed in only one center, in contrast to the study by Martinez-Pellús et al (21, 31). Significantly, their finding that overall outcome was not very different between treated patients and controls—although precise clinical outcomes were not considered—is in accordance with our study that failed to demonstrate a positive effect of preoperative selective gut decontamination on the occurrence of postoperative fever and clinical outcome. In our study, patients screened because of elective cardiac surgery were eligible for inclusion. Although the population was heterogenous in terms of age, co-morbidity, etc., the mixture of patients in our study seems to represent that seen in many university and large community hospitals (4, 21, 28–30). Because we did not evaluate the use of selective gut decontamination in hospitalized patients, our findings do not exclude the possibility that preoperative selective gut decontamination may be of relevance in such a highly selected pa-

tient group. However, consistent with our findings, selective decontamination of the digestive tract in patients undergoing elective orthotopic liver transplantation failed to abolish perioperative endotoxemia or the development of organ system failure and did not enhance survival (32). Interestingly, the preoperative loading of patients with glutamine, aimed to protect the gut mucosal barrier during insufficient perfusion, did not prevent endotoxemia during and shortly after cardiac surgery (33).

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CHAPTER 3

Letter to the Editor

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E.F. Schippers, J.T. van Dissel, No effect of preoperative selective gut decontamination on endotoxemia and cytokine activation during cardiopulmonary bypass: a randomized, placebo-controlled study, Critical Care Medicine, 31(1):334-5, Copyright (2006), with permission from Lippincott Williams & Wilkins.

To the Editor:

We read with interest the comment by Dr. Oudemans-van Straaten and colleagues (1) on our trial investigating the effect of preoperative selective gut decontamination (SGD) on endotoxemia and cytokine activation during cardiopulmonary bypass (2). Their main point of criticism concerns the choice of neomycin over tobramycin in our SGD regimen. Furthermore, they claim that there is a lack of correlation between fecal aerobic Gram-negative bacilli and endotoxin concentration. Dr. Oudemans-van Straaten and colleagues (1) suggest that the use of neomycin instead of tobramycin is responsible for the absence of an effect of our SGD regimen on endotoxemia. We would like to counter their critical notes.

The SGD regimen used in our randomized controlled trail was highly effective in eradicating aerobic Gram-negative bacilli (AGNB) from the bowel; 73% of the patients taking the trial medication had no AGNB at all, whereas others had strongly reduced numbers in the culture of a rectal swab (2), confirming previous studies on the effectiveness of this regimen in hematology and bone marrow transplant patients (3;4). This finding implicates that the regimen has a very potent antimicrobial activity and thereby reputes the theoretical objection made by Dr. Oudemans-van Straaten and colleagues (1) that neomycin is "inactivated" by feces (1). Moreover, their proposition is based on rather artificial experiments that showed inactivation of several antibiotics by fecal material *in vitro*. These findings remain of undetermined significance since they have never been shown to correlate with the ability of the selected antibiotics to reduce AGNB or endotoxin in feces (5). Furthermore, the neomycin dose used in our study (250 mg every 6 hrs, i.e., 1000 mg/day) is very high. Even in the theoretical situation that <99% of the total amount of neomycin would be "inactivated" by feces, the remaining free concentration would still be well above the "minimal inhibitory concentration" of most target microorganisms (e.g., fecal amount is approximately 200 g/day, total concentration of neomycin is about 5000 µg/g feces, 1% of this equals about 50 µg free neomycin/g feces). Their remark that neomycin, as opposed to tobramycin, fails to show "anti-endotoxin" properties is not substantiated by the literature they refer to: In the study by Rogers et al. (6), neomycin was combined with streptomycin and amfotericin B and not with polymyxin as we did, making direct comparison impossible. The suggestion of Dr. Oudemans-van Straaten and colleagues(1) that tobramycin has strong "anti-endotoxin" properties is not substantiated by the study of Sjolin et al. (7), which showed no such property of tobramycin when added to cefuroxime-treated *Escherichia coli* cultures. The effect of aminoglycosiden in lowering endotoxin concentrations, although they might have a slightly different antibacterial spectrum, is most likely due to their mode of action being

rapid killing and inhibition of endotoxin synthesis and not an “anti-endotoxin” effect as such (8). Altogether we do not find it feasible to attribute “anti-endotoxin” properties to antibiotics as a separate entity apart from their primary mode of action being antimicrobial activity. Polymyxin is not an exception to this rule; the mode of action of this unique bactericidal polypeptide is binding to the bacterial outer membrane components, mainly being lipopolysaccharides and phospholipids. Not surprisingly, this compound is able to neutralize much of the toxic actions of endotoxin, that is, mainly *ex vivo* (9;10).

We do not believe that endotoxin and AGNB levels do not correlate. Several studies have shown this correlation (6;11;12).

We have no doubt that the SGD regime used in our study, like other studies showing correlation between reduction in AGNB and endotoxin concentrations, lowered fecal endotoxin concentrations. Therefore, our conclusion remains that reducing AGNB in the gut from patients undergoing elective cardiac surgery does not reduce the perioperative endotoxemia and subsequent inflammatory response. These findings suggest that, at least in humans, the total size of the free endotoxin pool is not the key limiting factor in the pathophysiological mechanism that controls circulating endotoxin during cardiac surgery (2). For instance, the lower endotoxin content due to Gram-negatives other than the AGNB could well account for a concentration of endotoxin that already saturates the underlying translocating mechanism in a reperfusing bowel.

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CHAPTER 4

TNF-α and IL-10 production upon whole blood stimulation with a wide range of LPS concentrations: estimating an individual's dose-response characteristic described by the underlying receptor-ligand model.

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Submitted

Abstract

Individuals are commonly described as 'low' or 'high' producers of cytokines based on ex vivo stimulation of their cells with various stimuli, most often lipopolysaccharide. This dichotomous characteristic is subsequently used as reference in clinical and genetic studies. Since this approach is not based on a physiological model and studies using this approach yielded conflicting results, we investigated whether it was possible to better describe an individuals cytokine production profile using a receptor-ligand interaction model that is fully characterized by two parameters, i.e., EC_{50}, the estimated LPS concentration at which half of the cytokine concentration is reached and the E_{max}, the estimated maximal concentration of cytokine released. Repeated measurements over several weeks disclosed that these two parameters were highly constant for individuals, yet differed between individuals. In contrast with this, only the TNF-α concentration measured after stimulation with the lowest LPS concentration (0.1 ng/mL) was different between the subjects, whereas IL-10 concentrations were only different between the subjects after stimulation with the lower LPS concentrations (i.e. 0.1 through 100 ng/mL). Our findings suggest that relevant information on cytokine release is lost, when the commonly applied approach is taken, i.e. testing the TNF-α and or IL-10 release after stimulation with a single, and often high, LPS concentration.

Studies aimed at correlating in vitro with in vivo phenotypes and genetic polymorphisms should for this purpose use large dose-response ranges of whole blood stimulation with LPS to be able to closely estimate these dose-response characteristics. Such dose-response characteristics are more likely to be good estimates of an individual's in vitro cytokine production profile and therefore more suitable as a reference used in studies that investigate the genetic factors that make up for the large inter-individual variation found in cytokine production upon whole blood stimulation with LPS.

Introduction

Ex vivo cytokine production capacity of an individual is elegantly determined in the supernatant of whole blood after stimulation with lipopolysaccharide (LPS). The system provides a natural environment for cytokine producing cells, mainly being monocytes. Based on a rather small day-to-day intra-individual variation and laboratory variation, both estimated at around 15%, in combination with a rather large inter-individual variation, the system is able to distinguish 'high' and 'low' producing individuals of TNF-α and IL-10 based on the cytokine concentration found in the supernatant upon stimulation with one single concentration of LPS (1). In one study the aforementioned approach was able to detect differences in ex vivo cytokine production in relatives of patients with poor or fatal outcome of meningococcal disease as compared to relatives of patients with more favorable outcome (2). These observations led to the conclusion that the inter-individual variation found in these cytokine concentrations is to be explained to a large extend by genetic background (2;3). Many studies set out to search for genetic factors explaining this variation. Better understanding of the pathways involved in LPS sensing and intracellular signaling were essential in the search for these genes. Studies mainly focused on finding associations between specific gene(-promoter) polymorphisms and (whole blood) stimulation phenotype (i.e. individuals categorized as 'high' as compared to 'low' producers) based on a cytokine measurement after stimulation with one single – but between studies varying – concentrations of LPS (2-30). The conclusions of these studies have been conflicting. Part of the explanation for this might be differences in the handling of samples, differences in the various assays, cellular sources (whole blood, isolated cells), the LPS composition, incubation times, and, likely most importantly the differences in the LPS concentrations used to stimulate the cells. Categorizing individuals as 'high' as compared to 'low' producers based on cytokine production upon a single LPS concentration might not be adequate. First, the LPS concentrations used in these studies almost always exceeded the concentrations found in patients with infectious disease, hampering the translation of the in vitro phenotype to the in vivo situation. Secondly, the cytokine concentrations found after stimulation with a single high concentration of LPS will reflect the maximal production capacity, but might not necessarily provide insight in the cytokine response at lower LPS concentrations. Furthermore, aspects of the dose-response characteristics, for instance the LPS concentration at which 50% of the maximal cytokine concentration is reached and the maximal cytokine production capacity of the dose-response curve are not taken into account. Since the discovery of the toll-like receptor 4, the main extra cellular receptor for LPS, these aspects are of increased interest since this

mode of LPS signaling is likely to follow the basic concepts of the receptor-ligand model used in other fields of research (31). Based on the dose response model know as the C_{max} model we calculated the dose response characteristics of individuals by using supernatant cytokine concentrations after stimulation with a wide range of LPS concentrations. In order to be able to quantify the influence of the laboratory and the intra-individual day-to-day variation on these parameters we performed each measurement separately in duplicates and repeated the assay several times over several weeks. We hypothesize that the intra-individual day-to-day variation in the dose-response parameters is small as compared to the variation found in the intra-individual day-to-day variation found in a single point measurement and have better discriminative properties in labeling an individuals phenotype. If these premises were met, it would be suitable to use these parameters as patient characteristics in future research.

Materials and Methods

Subjects and blood sample collection.

We studied two healthy male subjects. Neither of the subjects used any prescription drugs, nor where they active smokers. No dietary restrictions were applied on the day of blood collection nor on the days prior. In the 3 weeks before and during the sampling the subjects did not experience any signs or symptoms of infection such as fever. Blood samples were drawn at 9:00 am using a Vacutainer® blood collecting system in four endotoxin-free collection tubes (Endo Tube ET, 4 mL, containing 120 IU sodium heparin, Chromogenix, Amsterdam, Netherlands), through puncture of a large antecubial vene. From each tube a separate whole blood stimulation assay, i.e. two whole blood stimulations for each cytokine, was performed without delay as described below. The whole procedure was repeated 7-9 times over a 3-week period.

Whole blood stimulation assay and LPS preparation.

Briefly, whole-blood samples were mixed 1:1 with RPMI 1640 (Gibco, Germany) and LPS (*Escherichia coli* O111, Sigma) diluted in RPMI 1640 was added to final concentrations of 0.1, 1, 10, 100, 1000 and 10,000 ng/mL and a total volume of 1 ml using 24-well microtiter plates. On each day fresh LPS dilutions were prepared from one single batch of LPS (5 mg/ml, stored at -80 ^{0}C). The stimulations were performed by incubation of the 1:1 diluted blood without LPS addition as a negative control. Cells were subsequently stimulated for 4 and 24 hours at 37 ^{0}C under 5% CO_2 for determination of TNF-α and IL-10, respectively. Supernatants (4 times 200 µl for each LPS concentration) were collected after

centrifugation of the microtiter plates at 600 x *g* at 4 ^{0}C and stored at -70 ^{0}C in 250 µl aliquots for final measurement of the cytokines.

TNF-α and IL-10 measurement.

Cytokine concentrations in the supernatants derived from the repeated stimulation assays were analysed on a single day using one ELISA batch. Tumor necrosis factor (TNF)-α and Interleukin-10 concentrations were determined with a standard ELISA technique (PeliKine Compact^{TM}, Central Laboratory of the Netherlands Red Cross Blood transfusion Service, Amsterdam, The Netherlands); the lower detection limit was 4.0 pg/mL. Each cytokine concentration was measured in duplicate. In case of laboratory failure, supernatants were retested using a new, previously unfrozen aliquot. In such case all supernatants derived from the two whole blood stimulations from that single vena puncture were retested.

Calculation of ex vivo TNF-α, IL-10 and LPS dose-response.

Dose-response characteristics for the mean of the whole blood stimulation for each cytokine (i.e. TNF-α or IL-10) were calculated for each subject using the ligand-receptor response model as previously described (31). The basis for this model is the principal that the interaction between a ligand (i.e., LPS) and its receptor is the first step leading to the response. The effect is correlated with the number of receptors occupied and is maximal when all receptors are occupied. The ligand concentration at which 50% of the maximal response is reached (EC_{50}) is a parameter of the affinity of the ligand for its receptor. Taking together, the assumptions lead to the E_{max} model, mathematically described as the Hill equation.

In the first approach we used a single step E_{max} equation as a model.

$$E_N = E_{N,max} \text{ x } C/(EC_{50} + C) \qquad \text{(model 1)}$$

Where E_N is the observed cytokine concentration at a given LPS concentration C, EC_{50} is the estimated LPS concentration at which 50% of the maximal cytokine concentration is reached, and $E_{N,max}$ is the estimated maximal cytokine concentration.

In a second approach, for instance if we assumed that the release of cytokine might be described better as a two-stage process; first, stimulation leads to the release of preformed cytokines, stored intracellular in granules; secondary, stimulation activates gene transcription and de novo production of cytokines. Furthermore, the measured amount of cytokine released upon stimulation with LPS is most likely a sum of cytokine released by stimulation of different pathways. Together, this would result in a two-step release, with

different time coefficients and possibly different regulatory set points, depending on the intensity of the stimulus. To fit for such a model, the second analysis was based on two Emax equations revealing four dose-response characteristics for the LPS-induced cytokine production (i.e. TNF-α or IL-10) in each subject.

$$E_N = E_{N,max\text{-}1} \text{ x } C/(EC_{50\text{-}1} + C) \quad + \quad E_{N,max\text{-}2} \text{ x } C/(EC_{50\text{-}2} + C) \qquad \text{(model 2)}$$

Where E_N is the observed cytokine concentration at a given LPS concentration C, $EC_{50\text{-}1}$ and $EC_{50\text{-}2}$ are the estimated LPS concentration at which 50% of the maximal cytokine concentration at the first stage and the second stage was reached, respectively, and $E_{N,max\text{-}1}$ and $E_{N,max\text{-}2}$ is the estimated maximal cytokine concentration at the first stage and the second stage, respectively.

The characteristics were calculated by non-linear regression using SPSS 12.0.1.

Statistical Analysis.

The spread in the TNF-α and IL-10 ELISA measurements was estimated by calculating the mean of the paired differences of each duplicate measurement divided by the mean of these measurements. This coefficient was calculated for each LPS concentration.

For the whole blood assay the coefficient of variation (the standard deviation (SD) divided by the mean) was determined for each LPS concentration. For this calculation the mean of the duplicate measurements were used. The coefficients of variation were calculated pair wise in repeated experiments (yielding the mean of individual coefficients of variation). Because the coefficient of variation is less subject to scaling effects as compared to the SD, values of the coefficient of variation for different experimental conditions were pooled to assess overall variation. Comparison of means was performed by ANOVA or its non-parametric alternative (Mann-Whitney *U* test) where appropriate. Furthermore, we performed an analysis of variance to estimate day-to-day and inter-individual variance, adjusted for LPS concentrations and the individual tested.

Calculations of the correlation coefficient between duplicate measurements were assessed non-parametrically using Spearman correlation test.

Results

Within ELISA variation.

For each of the LPS concentrations thirty-six duplicate ELISA measurements for TNF-α and thirty for IL-10 were available. The spread of the TNF-α and IL-10 ELISA

measurement was estimated as described in the methods. Briefly, the paired differences of each duplicate measurement were divided by their mean. For each LPS concentration the mean of these coefficients (eighteen for TNF-α and fifteen for IL-10) was calculated, revealing the mean spread relative to the mean for both TNF-α and IL-10 at each of the LPS concentrations. For TNF-α this mean spread relative to mean, after stimulation with 0.1, 1, 10, 100, 1000 and 10,000 ng/mL LPS, was 10.9, 11.9, 13.2, 11.9, 14.5 and 15.1%, respectively. Since these percentages were not statistically different, the data were pooled revealing an overall spread relative to the mean of 12.9%. For IL-10 the spread relative to the mean after stimulation with 0.1, 1, 10, 100, 1000 and 10,000 ng/mL LPS was 37.5, 11.2, 17.0, 17.5, 19.1 and 13.6%, respectively. The percentage after stimulation with 0.1 ng/mL was high as compared to the other percentages, likely due to low concentrations of IL-10 (i.e. close to the level of detection) leading to a relatively high impact of even small differences in the test result (i.e. scaling effect). For this reason in the calculation of the overall spread relative to the mean, the data after stimulation with 0.1 ng/ml LPS were disregarded, leading to an overall spread relative to the mean of 15.7%.

TNF-α and IL-10 within whole blood stimulation variation.

The variation in the whole blood stimulation assay was investigated by performing the assay in duplicate; for each assay a single tube, drawn from one subject through a single vena puncture, was used as described in the methods. The cytokine measurements were performed on a single day, using a single ELISA batch. In each supernatant the cytokine measurement was performed in duplicate and the mean of the two ELISA measurements was used for this analyses.

The TNF-α concentration found with the first whole blood stimulation correlated with the second with r = 0.812, 0.676, 0.664, 0.773, 0.827 and 0.767 after stimulation with 0.1, 1, 10, 100, 1000 and 10,000 ng/mL LPS, respectively. Correlation between the two TNF-α measurements after stimulation with all LPS concentrations combined was very high (r = 0.961). The same procedure was done for the IL-10 assay. The IL-10 concentration found with the first whole blood stimulation correlated with the second with r = 0.959, 0.946, 0.693, 0.782, 0.776, 0.979 after stimulation with 0.1, 1, 10, 100, 1000 and 10,000 ng/mL LPS, respectively. Correlation between the two IL-10 measurements after stimulation with all LPS concentrations combined was very high (r = 0.979). A plot of the differences against the means, to estimate the repeatability of the assay, is given in Fig. 1 for TNF-α and Fig. 2 for IL-10.

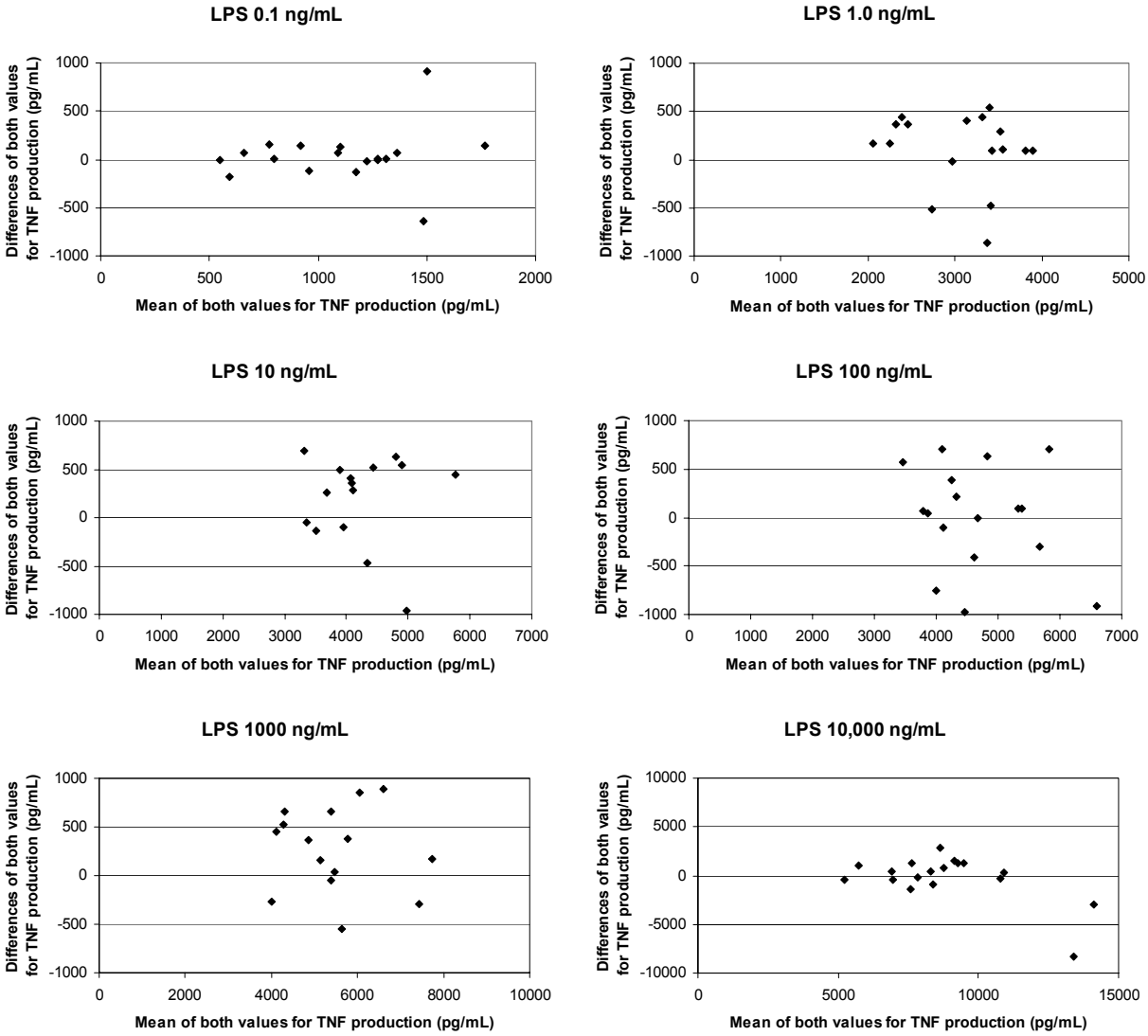

Figure 1. Ex vivo production of TNF-α in 18 whole blood samples in two subjects stimulated in duplicate with 0.1, 1, 10, 100, 1000 and 10.000 ng/mL lipopolysaccharide. Each dot represents the difference between both measurements (first minus second) dependent on the mean of both measurements.

For TNF-α the mean of the paired differences using all data was 17.2 pg/mL (95% confidence interval around the mean difference -2346 to 1367 pg/mL). The coefficient of variation (the standard deviation (SD) divided by the mean) was 8.4, 15.6, 10.1, 10.7, 3.2 and 11.1% after stimulation with 0.1, 1, 10, 100, 1000 and 10,000 ng/mL LPS, respectively. Taking together this leads to an overall mean coefficient of variation of 9.9%. For IL-10 the mean of the paired differences using all data was 136.7 pg/mL (95% confidence interval around the mean difference -392 to 1610 pg/mL). The coefficient of variation (the standard deviation (SD) divided by the mean) was 30.5, 9.1, 3.0, 3.0, 3.7 and 2.0% after stimulation with 0.1, 1, 10, 100, 1000 and 10,000 ng/mL LPS, respectively. Taking these percentages taking together, ignoring the data from the stimulation with 0.1 ng/mL LPS for previously mentioned reasons, the overall mean coefficient of variation is 4.2%.

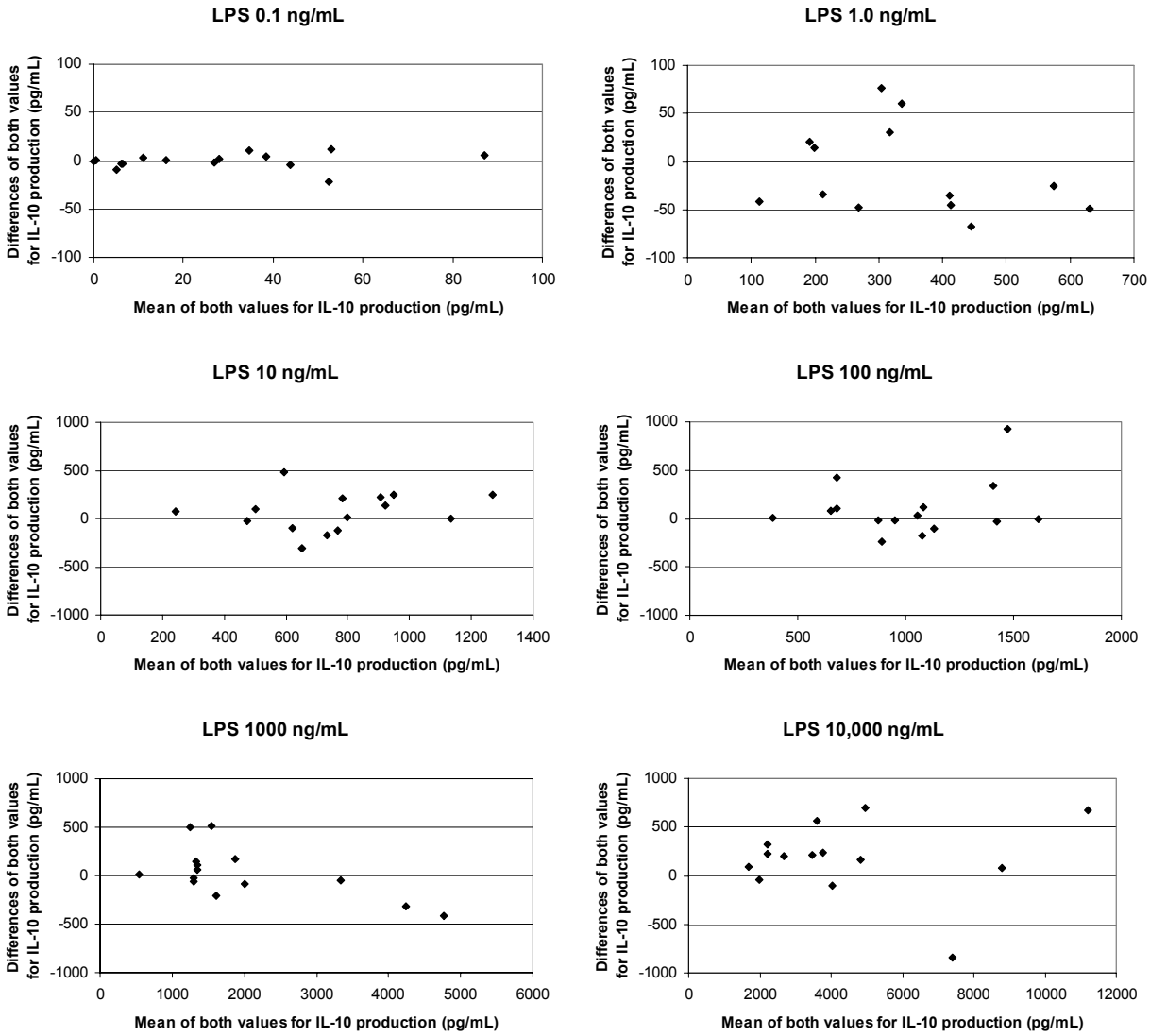

Figure 2. Ex vivo production of IL-10 in 15 whole blood samples in two subjects stimulated in duplicate with 0.1, 1, 10, 100, 1000 and 10.000 ng/mL lipopolysaccharide. Each dot represents the difference between both measurements (first minus second) dependent on the mean of both measurements.

Day-to-day variation in whole blood stimulation.

As described in the methods the whole blood stimulation was performed in two subjects over a period of three weeks. To investigate the day-to-day variation in the assay the mean cytokine concentration from two whole blood stimulations performed on the same day were used for each subject separately. The data are shown in Table 1. The coefficients of variation for TNF-α measurements were around 20% at each of the LPS concentrations in both subjects. For IL-10 the coefficients of variation were higher, i.e. around 35%. Coefficients for the IL-10 measurements were especially high in the extremes of the LPS concentrations used (Table 1).

Table 1. Intra-individual variation over 3 weeks in ex vivo production of TNF-α and IL-10 upon whole blood stimulation (performed in duplicate) with LPS in various concentrations

Subject	LPS (ng/mL)	TNF-α				IL-10			
		No. of observations	Mean (pg/mL)	SD (pg/mL)	CV (%)	No. of observations	Mean (pg/mL)	SD (pg/mL)	CV (%)
1	0.1	9	916	318	34.7	7	6.5	5.6	84.8
	1	9	2861	631	22.1	7	229	72	31.4
	10	9	4507	752	16.7	7	568	183	32.2
	100	9	4948	1096	22.2	7	822	332	40.4
	1000	9	5898	1513	25.7	7	1737	1180	67.9
	10,000	9	9293	2049	22.0	7	3895	1245	32.0
2	0.1	9	1286	256	19.9	8	45.5	19.5	42.9
	1	9	3282	422	12.9	8	502	116	23.1
	10	9	4106	544	13.2	8	921	206	22.4
	100	9	4598	776	16.9	8	1203	266	22.1
	1000	9	5524	1054	19.1	8	2652	1701	64.1
	10,000	9	8387	2617	31.2	8	5106	3555	69.6

Observations are means of two whole blood stimulation performed on a single day. CV, coefficient of variation expressing relative spread of ex vivo cytokine production over 3 weeks. LPS, lipopolysaccharide.

Multivariate analysis of variance.

Next, we examined in a multivariate model the variance in the TNF-α and IL-10 concentrations released within and between experiments. In this analysis TNF-α and IL-10 concentrations were analyzed after logtransformation. For TNF-α, there was a LPS dose-dependent ($p < 0.0001$) increase in the cytokine concentration measured. After adjusting for the LPS concentration, there was no significant difference in the concentration of TNF-α measured within separate runs (i.e. duplicate whole blood stimulations) performed on a single day; between days, the concentration of TNF-α measured differed significantly ($p = 0.005$), but this accounted for only about 4% of the total variance observed. Adjusting for the between day variance and LPS concentrations, the concentration of TNF-α measured did not differ between the two subjects. For IL-10, similarly to TNF-α, there was a LPS dose-dependent ($p < 0.0001$) increase in the cytokine concentration measured. After adjusting for the LPS concentration, there was no significant difference in the concentration of IL-10 measured within separate runs (i.e. duplicate whole blood stimulations) performed on single day; between days, the concentration of IL-10 measured differed somewhat, i.e., on average for about 2% of the total variance observed, but this inter-day difference did not reach a level of significance ($p = 0.066$). However, after

adjusting for the between day variance and LPS concentrations, the concentration of IL-10 measured did differ significantly between the subjects ($p < 0.001$), accounting for about 27% of the total between subjects variance observed.

Variation in dose-response characteristic using Hill-equation.

As described in the methods the results from the whole blood stimulation measurements using the wide range of LPS concentrations was used to calculate the dose-response characteristics using the receptor-ligand model, i.e. the E_{max} model using the Hill equation. For these calculations the means of the two whole blood stimulation assays performed on the same day were used (Table 1). The results are shown in Table 2.

Table 2. Intra-individual variation over 3 weeks of dose response characteristics calculated from TNF-α and IL-10 production upon whole blood stimulation (performed in duplicate) with LPS in various concentrations

Model	Charac-teristic	Subject 1				Subject 2				*p**
		No. of Observa-tions	Mean	SD	CV (%)	No. of Observa-tions	Mean	SD	CV (%)	
TNF-α										
1	EC_{50}	9	2.498	1.508	60.4	9	2.180	3.482	159.7	0.063
	E_{max}	9	6636	1416	21.3	9	6016	1614	26.8	0.489
2	EC_{50-1}	9	0.605	0.163	26.9	9	0.279	0.0924	33.1	<0.001
	E_{max-1}	9	4721	858.5	18.2	9	4297	615.3	14.3	0.340
	EC_{50-2}	9	$9.7x10^9$	$2.5x10^9$	257.7	9	4285	2420	56.5	0.605
	E_{max-2}	9	$3.2x10^9$	$8.4x10^9$	260.9	9	5808	3236	55.7	0.136
IL-10										
1	EC_{50}	7	1986	1135	57.2	8	721.5	839.6	116.4	0.029
	E_{max}	7	4630	1517	32.8	8	5418	4414	81.5	0.867
2	EC_{50-1}	7	2.017	0.805	39.9	8	1.090	0.439	40.3	0.014
	E_{max-1}	7	627.6	218.5	34.8	8	969.0	176.4	18.2	0.006
	EC_{50-2}	7	13629	20537	150.7	8	6204	11503	185.4	0.152
	E_{max-2}	7	6557	3036	46.3	8	6126	4830	78.8	0.779

Observations are means of two whole blood stimulation performed on a single day. CV, coefficient of variation expressing relative spread of ex vivo cytokine production over 3 weeks. LPS, lipopolysaccharide. EC_{50}, LPS concentration at which 50% of the maximal cytokine concentration is reached. E_{max}, maximal cytokine concentration. *Mann-Whitney *U* test.

For the TNF-α measurements nine measurements were available. As compared to model 1, model 2 revealed a good estimate of the EC_{50-1} as well as E_{max-1}. The coefficient of variation for TNF-α EC_{50-1} was 26.9 and 33.1 for subject 1 and 2, respectively. For the TNF-α E_{max-1}, these values were 18.2 and 14.3 for subject 1 and 2, respectively.

For IL-10, seven measurements were available for the first subject and eight were available for subject two. As for TNF-α model 2 performed best in a way that a rather precise estimation of EC_{50-1} as well as E_{max-1} could be made. The coefficient of variation for EC_{50-1} was 39.9 and 40.3 for subject 1 and 2, respectively. For the E_{max-1}, these values were 34.8 and 18.2, respectively (Table 2).

Finally for each of the subjects a best estimation of the dose response characteristics was calculated, using the same measurements as described above, for each of the cytokines. The results of this analysis are shown in Table 3.

Table 3. Dose response characteristics of TNF-α and IL-10 calculated from repeated whole blood stimulation assays with LPS in various concentrations in two subjects

Model	Characteristic	Subject 1		Subject 2	
		No. of observations	Value	No. of observations	Value
TNF-α					
1	EC_{50}	9	1.903	9	0.8816
	E_{max}	9	6565	9	5855
2	EC_{50-1}	9	0.6033	9	0.2613
	E_{max-1}	9	4780	9	4280
	EC_{50-2}	9	4981	9	3284
	E_{max-2}	9	6761	9	5452
IL-10					
1	EC_{50}	7	1279	8	829
	E_{max}	7	4350	8	5421
2	EC_{50-1}	7	2.212	8	1.014
	E_{max-1}	7	692.1	8	981.2
	EC_{50-2}	7	2961	8	1952
	E_{max-2}	7	4152	8	4932

Observations are means of two whole blood stimulation performed on a single day. CV, coefficient of variation expressing relative spread of ex vivo cytokine production over 3 weeks. LPS, lipopolysaccharide. EC_{50}, LPS concentration at which 50% of the maximal cytokine concentration is reached. E_{max}, maximal cytokine concentration.

Using these characteristics, the cytokine concentrations were calculated using model 1 and model 2 for each of the LPS concentrations used. The results for both subjects are shown in Fig. 3A and B (for TNF-α) and Fig. 3C and D (for IL-10).

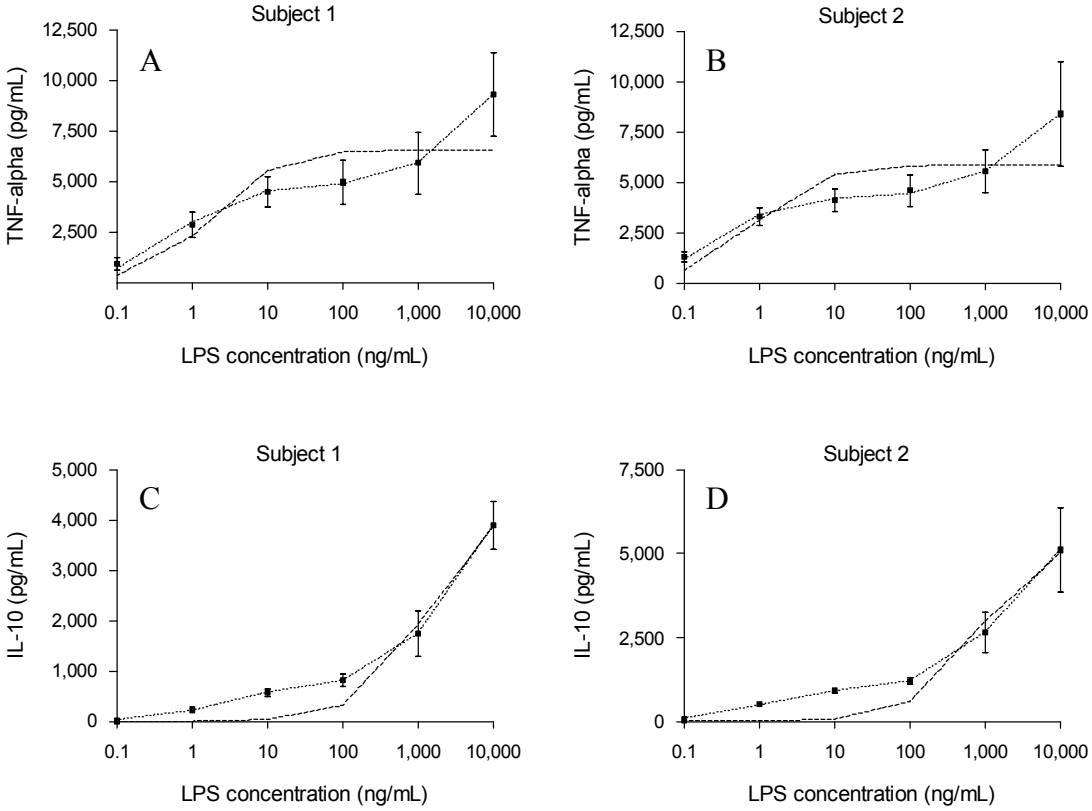

Figure 3. Ex vivo production of TNF-α in subject 1 (A) and subject 2 (B), IL-10 in subject 1 (C) and subject 2 (D). Whole blood stimulation was performed nine times over a 3 week period in duplicate. Each data point represents the mean of the two whole blood stimulations performed on the same day and the standard deviation (SD). Lines represent the fitted concentrations predicted by model 1 (---) and model 2 (…), respectively.

Comparison of TNF-α and IL-10 measurements between the subjects.

First, at each of the LPS concentrations the cytokine concentrations were compared between the two subjects. For TNF-α there was a significant difference in the concentrations found after stimulation with 0.1 ng/mL ($p = 0.019$, Mann-Whitney *U* test). After stimulation with the other LPS concentration no statistically difference differences between the two subjects were found (ANOVA and Mann-Whitney *U* test). Secondly, the dose response characteristics were compared between the two subjects. The results are shown in Table 2. Both with model 1 and model 2 the $EC_{50\text{-}1}$ values were higher in subject 1 as compared to subject 2, however the difference was only statistically different using model 2 ($p < 0.001$, Mann-Whitney *U* test, Table 2). For IL-10 the concentrations were different between the two subjects after stimulation with 0.1, 1, 10 and 100 ng/mL LPS ($p < 0.001$, <0.001, 0.004 and 0.021, Mann-Whitney *U* test, respectively, data not shown). The $EC_{50\text{-}1}$ values derived from model 1 and model 2 were statistically different between the two subjects ($p = 0.029$ and 0.014, respectively, Mann-Whitney U test). Furthermore, the $E_{max\text{-}1}$ derived from model 2 was statistically different between the two subjects ($p = 0.006$, Mann-Whitney *U* test, Table 2).

Discussion

The main finding of the present study is that the dose-response characteristics of TNF-α and IL-10 release by human peripheral blood cells, upon stimulation with a wide range of LPS concentrations, can be described adequately by a receptor-ligand interaction model that is fully characterized by two parameters, i.e., EC_{50}, the estimated LPS concentration at which half of the cytokine concentration is reached and the E_{max}, the estimated maximal concentration of cytokine released. Repeated measurements made over several weeks disclosed that these two parameters were highly constant for individuals, yet differed between individuals. These differences in dose-response characteristics would have been discarded however, if only one or two LPS concentrations had been used to test the cytokine response of these individuals. For instance, only the TNF-α concentration measured after stimulation with the lowest LPS concentration (0.1 ng/mL) was different between the subjects, whereas IL-10 concentrations were only different between the subjects after stimulation with the lower LPS concentrations (i.e. 0.1 through 100 ng/mL). These findings suggest that relevant information on cytokine release is lost, when the commonly applied approach is taken, i.e. testing the TNF-α and or IL-10 release after stimulation with a single, and often high, LPS concentration. Such an approach yields one value that doesn't represent a physiological model of release and often shows variation over time. By contrast, we estimated two parameters that fully characterize an underlying model and are intrinsic parameters that are insensitive to day-to-day variation.

Two examples may illustrate the relevance of our approach. First, the TNF-α-308 AA or GA genotypes are generally being termed as the high TNF-α producing genotypes and the TNF-α-308 GG as the low producing genotype. A study using reporter gene constructs demonstrated convincingly that the -308A allele is a much stronger transcriptional activator as compared to the more frequently occurring -308G allele (13). However, studies using ex vivo stimulation assays using various stimuli, cells and incubation times revealed conflicting results (8;14;17;18;20;23;25-29;32). In one study a difference between the two TNF-308 genotypes was detected at the lowest LPS concentration (1 ng/mL), whereas at the high concentration (100 ng/mL), no difference was detected (8). Although comparison of the studies is hampered by differences in the assays used, studies using low LPS concentrations or other rather poor stimuli are more likely to detect (non-significant) differences between the two TNF-308 genotypes (17;18;26). Although we did not determine genetic factors in our study, we detected significant differences between the two subjects at the lowest LPS concentration used, and more importantly the dose-response parameters based on the whole range of LPS concentrations remained significantly different between the subjects.

Secondly, The IL-10-1082 GG and GA genotypes are generally termed as high IL-10 producing and the IL-10-1082 AA as the low producing genotype. In a previous study in cardio-thoracic surgery patients, we were only able to confirm this finding in carriers of the GATA allele after stimulation with low LPS concentrations (10 and 100 ng/mL), whereas no differences were detected at the highest LPS concentrations (1000 ng/mL) (30). More interestingly the EC_{50} value, estimated from the measurements of IL-10 after stimulation with the whole range of LPS concentrations, was significantly higher in carriers of the GATA haplotype, indicating lower LPS sensitivity. Furthermore, carriers of the AGCC allele were only distinguishable from the other individuals based on the estimated EC_{50}, whereas IL-10 concentrations produced upon stimulation with a range of LPS concentrations (10, 100 and 1000 ng/mL LPS) were not different. Similarly to this example, we expect that discrepancies found in studies results could well be explained by differences in the LPS stimulus used in the ex vivo assay. Either the concentration or the composition of the LPS used could be different in the way that the cytokine concentrations measured are at different levels of the dose-response curve. Our approach could to some extend normalize these differences. By calculating the dose-response characteristic based on a wide range of LPS concentrations the intrinsic dose-response characteristics could be calculated, making comparison between studies possible.

The whole blood stimulation we used is similar to the assays used by others. In a previous study similar variation coefficients were found in repeated whole blood stimulation measurements over a smaller LPS range as used in our study. Similar to our results this study showed slightly higher variation in IL-10 production as compared to TNF-α. Due to the small range of LPS concentrations used in this study (ranging from 10-1000 ng/mL) calculation of dose-response characteristics was not possible.

So far, no other studies used the approach of estimating dose-response characteristics for an individual based on cytokine production upon a wide range of LPS concentrations in a whole blood stimulation system. In previous studies, based on the observed cytokine production ex vivo, individuals were designated as 'high' or 'low' producers. Also, individuals with specific polymorphisms of their cytokine genes, were given the same labels, after the presumed relationship between that genotype and the corresponding in vitro cytokine production profile.

In summary, we conclude that labeling subjects as ‘high’ or ‘low’ cytokine producers based on the in vitro production of cytokines after stimulation with one single, supraphysiological LPS concentration, might proof to be to rough a measure to describe a subjects cytokine production profile. Based on our result we expect that characterization of an individual's cytokine production profile by means of dose-response characteristics, calculated from in vitro cytokine production upon a wide range of LPS concentrations, is a

more suitable approach. Studies aimed at correlating in vitro with in vivo phenotypes and genetic polymorphisms should for this purpose use large dose-response ranges of whole blood stimulation with LPS to be able to closely estimate these dose-response characteristics. These dose-response characteristics are more likely to be good estimates of an individual's in vitro cytokine production profile and therefore more suitable as a reference used in studies that investigate the genetic factors that make up for the large inter-individual variation found in cytokine production upon whole blood stimulation with LPS.

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CHAPTER 5

TNF-α promoter, Nod2 and toll-like receptor-4 polymorphisms and the in vivo and ex vivo response to endotoxin

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TNF-α promoter, Nod2 and toll-like receptor-4 polymorphisms and the in vivo and ex vivo response to endotoxin

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Abstract

Humans exhibit substantial inter-individual differences in TNF-α production upon endotoxin stimulation. To determine to what extent the lipopolysaccharide-induced TNF-α production capacity in vivo and ex vivo is determined by polymorphisms in toll-like receptor-4 (TLR4), the TNF-α promoter region and Nod2, we screened for two TLR4 polymorphisms, a Nod2 polymorphism and the TNF-α promoter polymorphisms. We measured the perioperative endotoxemia and TNF-α production and the TNF-α production capacity of each patient in a whole-blood stimulation assay using blood drawn before anesthesia, using various LPS concentrations, in patients undergoing elective cardiac surgery. This operation represents a major surgical trauma associated with ischemia–reperfusion injury and triggers an endotoxemia and profound inflammatory response. In vivo TNF-α production was positively correlated with the level of endotoxemia after aortic declamping; thus TNF-α levels were higher in patients having endotoxemia compared to patients without endotoxemia. This correlation was observed in patients with any of the genotypes studied, and did not differ between the various genotypes. In vivo TNF-α levels correlated best with those ex vivo after stimulation with 1000 ng/mL LPS, and the estimated maximal TNF-α release capacity. Subjects with the wild-type TLR4 gene had similar levels of TNF-α upon LPS stimulation ex vivo as compared with patients carrying Asp299Gly and/or the Thr399Ile TLR4 polymorphism. Our results indicate that polymorphisms in the TLR4 receptor, Nod2 and TNF-α promoter region are not strongly associated with in vivo and ex vivo TNF-α production capacity upon endotoxin stimulation. This suggests that in this model of natural LPS release, the variation between individuals in TNF-α release can only modestly be determined by genetic background (TNF-α promoter, Nod2 and TLR4) of the individual.

Keywords: Cardiopulmonary bypass; Endotoxemia; Lipopolysaccharides; TNF-α promoter; Toll-like receptor-4

1. Introduction

Sepsis-induced organ failure (and death) appears to be due to the activation of a mediator cascade initiated by microbial components. TNF-α is a central cytokine in this inflammatory cascade and is believed to play an important role in the pathogenesis of septic shock [1]. In Gram-negative infection, the presence of lipopolysaccharide (LPS) in the circulation plays a pivotal role in the release of TNF-α. Human monocytes, the main producers of TNF-α, exhibit substantial inter-individual differences in TNF-α production. Genetic studies have shown that inter-individual variation of TNF-α production capacity ex vivo may partly be explained by differences in genetic background [2]. These genetic differences are likely to be located in genes involved in the innate immune system. For instance, the single nucleotide polymorphism (SNP) in the TNF-α promoter at position −308 has been correlated with ex vivo TNF-α production capacity in one study [3]. So far,

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other known SNPs in the TNF-α promoter have not been evaluated in this way.

Toll-like receptor-4 (TLR4) is part of a large family of transmembrane proteins and is believed to be crucial in mediating LPS effects. TLR4 is expressed on monocytes and macrophages, and to a lesser extent on lymphocytes and other cell types [4,5]. The common, co-segregating missense mutations (Asp299Gly and Thr399Ile) affecting the extracellular domain of the TLR4 receptor are associated with a blunted response to inhaled LPS in humans [6]. The recently described association between the Asp299Gly polymorphism in TLR4 and Gram-negative septic shock suggests a functional defect in TLR4 leading to increased susceptibility to Gram-negative bacteremia [7]. Another receptor that recently has been reported to be involved in LPS responsiveness is Nod2. Nod2 is an intracellular protein that interacts with LPS by a C-terminal leucine-rich repeat (LRR) domain [8]. Subsequent homodimerisation of Nod2 molecules induces NF-κB translocation [8,9]. A Nod2 mutation that diminishes the LPS responsiveness of the protein is associated with Crohn's disease [10]. Studies thus far have been focused on the LPS responsiveness of recombinant Nod2 and are lacking on the LPS responsiveness of the natural Nod2 protein, which is predominantly expressed by monocytes.

Cardiopulmonary bypass surgery leads to perioperative endotoxemia in most patients, and this procedure may serve as a model to study the association of genetic polymorphism and endotoxin-mediated TNF-α production. The aims of the current study were to assess the intersubject variation in TNF-α production upon whole-blood stimulation with LPS and to determine the correlation between production rates and various SNPs in the TNF-α promoter region and the TLR4 coding region. We also studied the intersubject variation in in vivo TNF-α production following cardiopulmonary bypass surgery in the same way.

2. Results

2.1. Patients

We studied 159 consecutive patients undergoing elective cardiac surgery with cardiopulmonary bypass (Table 1). There was a predominance of male patients (66%), 35 patients were active smokers (22%) whereas 20 patients (12%) had diabetes mellitus. Surgical procedures were extensive; 20 patients (12%) underwent CABG combined with valve replacement, 49 patients (30%) underwent valve replacement only, whereas 89 patients (54%) underwent CABG only. Eight patients underwent other surgical procedures, mainly aortic surgery.

Table 1
Characteristics of 159 patients undergoing elective cardiac surgery with cardiopulmonary bypass[a]

Preoperative data	
Age (years)	65 (56–72)
Weight (kg)	78 (68–88)
Male, *n* (%)	106 (67)
Active smoker, *n* (%)	31 (20)
Diabetes (types I and II), *n* (%)	19 (12)
ASA score, *n* (%)	
≤2	12 (8)
3	88 (55)
≥4	9 (6)
Unknown	50 (32)
Type of operation	
CABG only, *n* (%)	87 (55)
Valve replacement only, *n* (%)	47 (30)
CABG and valve replacement, *n* (%)	19 (12)
Other, *n* (%)	6 (4)
Perioperative data	
Operation time (min)	240 (183–285)
Perfusion time (min)	116 (85–141)
Aorta clamp time (min)	71 (49–90)
Lowest temperature (°C)	29 (28–31)
Postoperative data	
Days on ventilator	1 (1–2)
ICU stay (days)	3 (2–5)
MOF, *n* (%)	6 (3.8)
Hospital stay (days)	11 (9–15)
Fatalities, *n* (%)	14 (9)

CABG: coronary artery bypass graft, ICU: intensive care unit, MOF: multiple organ dysfunction.
[a] Median values and interquartile range in parentheses.

2.2. TNF-α promoter mutation analysis

Typing for the TNF-α single nucleotide polymorphisms was successful in most patients; in a minority of the samples no definite typing could be established (Table 2).

2.3. TLR4 mutation analysis

The TLR4 Asp299Gly and Thr399Ile substitutions were successfully determined in all patients. The Asp299Gly mutation was found in 17 patients (10.7%); 16 patients were heterozygous and one was homozygous. Except for that in one patient the TLR4 Asp299Gly mutation was accompanied by the TLR4 Thr399Ile mutation. Two patients were identified with isolated TLR4 Thr399Ile mutations (Table 2). The patient homozygous for the Asp299Gly mutation was also homozygous for the Thr399Ile mutation.

2.4. Nod2 mutation analysis

Determination of the Nod2 insertion mutation was successful in 143 patients. The insertion was found in

Table 2
TNF-α promoter polymorphism, TLR4 and Nod2 genotype/haplotype in 159 patients undergoing elective cardiac surgery with cardiopulmonary bypass

TNF-α promoter genotype, *n* (%)		
−238	GG	123 (95)
	GA	6 (5)
−308	GG	99 (67)
	GA	48 (32)
	AA	1 (1)
−376	GG	133 (96)
	GA	5 (4)
TLR4 haplotype, *n* (%)		
299+/399+		140 (88)
299−/399+		1 (1)
299−/399−[a]		16 (10)
299+/399−		2 (1)
Nod2 genotype, *n* (%)		
3020insC present[b]		10 (7)
3020insC absent		133 (93)

[a] One out of 16 patients homozygous, all others heterozygous.
[b] All patients carrying the Nod2 insertion were heterozygous.

10 patients (7%, see Table 2). All patients carrying the Nod2 insertion were heterozygous.

2.5. Ex vivo measurements

Blood samples for ex vivo LPS stimulation were available from 127 patients. In general, we found a dose-dependent TNF-α production upon stimulation of whole blood with increasing concentrations of LPS. Mean TNF-α levels upon stimulation with 0, 10, 100 and 1000 ng/mL LPS were 5.1 (SD 5.9), 9766 (SD 4782), 13,462 (SD 6688) and 18,070 (SD 7721) pg/mL, respectively (Table 3). For each patient we estimated the dose−response characteristics (i.e. EC_{50} and E_{max}), as described in Section 4. No differences were found in the TNF-α levels between the patient groups carrying the different TNF-α promoter polymorphisms at positions −238, −308, and −376 at any of the LPS concentrations used, or between their EC_{50} and E_{max} (all $p > 0.2$, Table 3). The −308AA homozygous patient could not be included in this analysis since no data from this patient were available. Neither the TNF-α production at any of the LPS concentrations, nor the E_{max} or EC_{50} was different in patients carrying TLR4 mutations versus the wild-type group (all $p > 0.085$, Table 3).

2.6. Endotoxin measurements

Perioperative blood samples for endotoxin measurements were available for most patients. Endotoxin levels were ≥ 5 pg/mL directly before anesthetic induction in four out of 152 patients (2.6%). On aorta declamping (time-point 2), 30 min into reperfusion (time-point 3) and on arrival at the ICU (approximately 2 h after surgery, time-point 4) 74 out of 142 patients (52.1%), 71 out of 143 patients (49.7%) and 54 out of 151 patients (35.8%) had endotoxin levels ≥ 5 pg/mL, respectively (Table 4). In 85 out of 143 patients (59.4%) the endotoxin level at either time-point 2 and/or 3 was ≥ 5 pg/mL. Median endotoxin levels increased to 5.0 and 4.9 pg/mL at aorta declamping and 30 min into reperfusion, respectively, and decreased afterwards to 4.2 pg/mL on arrival at the ICU.

Table 3
TNF-α production ex vivo on whole-blood stimulation with LPS[a] according to phenotype/genotype[b]

		10 ng/mL	100 ng/mL	1000 ng/mL	TNF_{max}	$EC_{50[LPS]}$
All patients ($n = 127$)		9766 ± 4782	13,462 ± 6688	18,070 ± 7721	16,891 ± 7359	15.4 ± 33.4
TNF-α promoter genotype (*n*)						
−238	GG (100)	9537 ± 4962	13,293 ± 6951	17,832 ± 8131	16,735 ± 7759	17.3 ± 37.4
	GA (5)	6151 ± 3355	8140 ± 3757	12,307 ± 6775	11,059 ± 5750	10.7 ± 4.70
−308	GG (81)	9928 ± 4537	13,609 ± 6514	18,142 ± 7385	16,902 ± 7140	12.1 ± 17.9
	GA (41)	9561 ± 5436	13,296 ± 7226	18,085 ± 8695	17,032 ± 8067	22.6 ± 52.8
−376	GG (108)	9567 ± 4911	13,235 ± 6809	17,764 ± 7917	16,581 ± 7472	15.9 ± 35.3
	GA (3)	11,076 ± 4421	15,533 ± 3400	16,982 ± 3690	16,777 ± 3421	6.10 ± 3.78
TLR4 haplotype (*n*)						
299+/399+	(111)	9754 ± 4934	13,238 ± 6724	17,700 ± 7776	16,414 ± 7320	12.6 ± 18.6
299−/399+	(1)	10,830 ± −	12,723 ± −	28,575 ± −	30,168 ± −	90.8 ± −
299−/399−[c]	(13)	9306 ± 3440	14,183 ± 5822	19,321 ± 6155	18,733 ± 5852	34.9 ± 87.0
299+/399−	(2)	12,868 ± 6247	21,572 ± 10,573	25,274 ± 12,483	24,747 ± 12,205	9.63 ± 0.20
Nod2 genotype						
3020insC present[d]	(7)	10,133 ± 4946	12,335 ± 4936	16,755 ± 6393	15,226 ± 5618	7.3 ± 4.2
3020insC absent	(107)	9629 ± 4898	13,379 ± 6880	18,032 ± 7888	16,880 ± 7538	16.8 ± 36.1

[a] Levels of TNF-α without LPS stimulation were below 50 ng/mL in all cases.
[b] Values are expressed as mean ± SD. Incomplete data due to incomplete sampling.
[c] One out of 13 patients homozygous, all others heterozygous.
[d] All patients carrying the Nod2 insertion were heterozygous.

Table 4
Perioperative endotoxemia[a]

	n/total (%)
Anesthetic induction	4/152 (2.6)
Release of aortic clamp	74/142 (52.1)
30 min reperfusion	71/143 (49.7)
On ICU admission	54/151 (35.8)

[a] Endotoxemia ≥ 5 pg/mL.

2.7. TNF-α measurements

Plasma TNF-α concentrations before anesthetic induction, on aorta declamping, 30 min into reperfusion and on arrival at the ICU were 1.5 (IQR: 0.0–2.6), 2.6 (IQR: 0.6–5.0), 5.3 (IQR: 3.3–9.5) and 4.3 (IQR: 2.3–7.5) pg/mL, respectively. TNF-α levels at 30 min into reperfusion were higher in patients having endotoxemia (defined as having an endotoxin level >5 pg/mL at aorta declamping and/or 30 min into reperfusion) compared to TNF-α levels in patients without endotoxemia ($p = 0.027$). At other time-points the median TNF-α levels were higher in patients having endotoxemia; however, this was not statistically significant. TNF-α production at the different time-points was similar in all TNF-α promoter genotypes and TLR4 haplotype subgroups (all $p > 0.2$, Table 5). The −308AA homozygous patient could not be included in this analysis since no data from this patient were available. We found no differences in TNF-α levels in patients carrying the Asp299Gly or the Thr399Ile TLR4 polymorphisms as compared to wild-type patients.

2.8. Correlations between endotoxemia and TNF-α levels

A significant positive correlation between endotoxin level and TNF-α concentration was found at 30 min into reperfusion ($r = 0.210$; $p = 0.040$). On aortic declamping and ICU arrival no significant correlation was found ($p > 0.4$). Furthermore, no significant differences were found in the correlation between endotoxin level and TNF-α concentrations between the TNF-α promoter genotypes and TLR4 haplotypes.

2.9. Correlations between ex vivo TNF-α production and in vivo TNF-α concentrations

A significant positive correlation between TNF-α concentration at 30 min into reperfusion with ex vivo TNF-α production using 1000 ng/mL LPS and TNF_{max} was found ($r = 0.241$; $p = 0.032$ and $r = 0.237$; $p = 0.035$). At other LPS concentrations a positive correlation was also observed; however, this did not reach level of statistical significance ($r = 0.172$; $p = 0.128$ and $r = 0.106$; $p = 0.349$, at 10 and 100 ng/mL, respectively). No other significant correlations were found between ex vivo and in vivo measured TNF-α levels.

3. Discussion

The main findings of the present study are that the TNF-α levels measured in vivo after cardiac surgery with cardiopulmonary bypass correlate significantly with the intensity of endotoxemia during the reperfusion phase upon aortic declamping, and with the maximal TNF-α release ex vivo upon stimulation with lipopolysaccharide in whole blood drawn before anesthesia. Although the aforementioned correlation is statistically significant, the extent of its scope seems to be rather limited due to the relatively low correlation coefficient. None of the inter-individual variation in endotoxin-stimulated TNF-α responses, however, could be explained by the known TLR4 and TNF-α promoter polymorphisms, including the −308 G/A substitution.

The model we choose, i.e. measuring naturally occurring endotoxemia and subsequent pro-inflammatory cytokine release in vivo in patients undergoing cardiac surgery, enables us to study inter-individual differences in these responses and relate these to genotypes. We found significant endotoxemia in over half of the patients undergoing cardiac surgery with cardiopulmonary bypass which is in accordance with previous studies [11,12]. The endotoxemia probably derives from the gut and reperfusing bowel after a phase of ischemia during cardiopulmonary bypass and occurs when the systemic circulation is restored. We found the highest plasma concentration of endotoxin immediately after aortic declamping. Most patients already had lower levels of circulating endotoxin 2–3 h later, upon arrival at the ICU. As endotoxin triggers the production of TNF-α within minutes to 1 h, we sampled the patients 30 min after peak endotoxemia (time-point 3) and 2–3 h later (time-point 4). Indeed, 30 min into reperfusion, shortly after the beginning of endotoxemia, we observed a significant positive correlation between circulating endotoxin levels and TNF-α production, indicating the importance of the role of endotoxin in the perioperative release of TNF-α. However, although we found a significant correlation, its magnitude is limited, suggesting that many other factors apart from endotoxin play a role in the total TNF-α production in these patients.

The in vivo TNF-α release was significantly correlated with the maximal TNF-α release ex vivo upon stimulation with lipopolysaccharide. Thus, the ex vivo LPS-stimulation assay is a predictor for the in vivo release of TNF-α during endotoxemia after cardiac surgery. Since we used different concentrations of LPS we were able to calculate descriptive parameters of the dose–response relation for each patient. Also, to estimate spread relative to the mean, the coefficients of variation (standard deviation [SD] divided by the mean) were calculated for all LPS concentrations. The values were 48.9%, 49.7% and 42.7% for 10, 100 and 1000 ng/mL LPS, respectively. This variation is similar to the

Table 5
Perioperative TNF-α production according to phenotype/genotype[a]

		Anesthetic induction	Aorta declamping	30 min into reperfusion	On arrival at the ICU
All patients		1.5 (0.0–2.6) $n = 112$	2.6 (0.6–5.0) $n = 106$	5.3 (3.3–9.5) $n = 108$	4.3 (2.3–7.5) $n = 110$
Endotoxemia[b]					
Negative		1.5 (0.2–2.7) $n = 38$	2.5 (0.6–4.1) $n = 38$	4.5 (2.5–6.8) $n = 37$	4.0 (2.4–5.5) $n = 37$
Positive		1.3 (0.1–2.5) $n = 70$	2.7 (0.6–6.2) $n = 68$	5.6 (3.6–12.0) $n = 70$	4.6 (2.2–8.2) $n = 70$
TNF-α promoter genotype					
−238	GG	1.7 (0.2–2.6) $n = 83$	2.5 (0.3–5.1) $n = 78$	5.3 (3.3–10.9) $n = 79$	4.3 (2.4–7.6) $n = 81$
	GA	0.3 (0.0–1.8) $n = 6$	2.1 (1.3–6.5) $n = 6$	4.6 (1.4–6.6) $n = 6$	2.0 (1.1–4.6) $n = 6$
−308	GG	1.5 (0.2–2.7) $n = 72$	2.0 (0.4–5.9) $n = 68$	5.2 (3.1–9.3) $n = 69$	4.2 (2.1–7.0) $n = 70$
	GA	1.6 (0.2–2.7) $n = 33$	3.0 (1.2–5.1) $n = 31$	6.3 (4.6–12.8) $n = 32$	5.9 (3.3–10.1) $n = 33$
−376	GG	1.6 (0.2–2.6) $n = 88$	2.3 (0.4–4.5) $n = 82$	5.4 (2.8–9.8) $n = 84$	4.2 (2.3–7.5) $n = 87$
	GA	1.1 (0.0–2.8) $n = 5$	0.0 (0.0–2.1) $n = 5$	4.9 (4.1–5.1) $n = 5$	6.7 (5.6–7.3) $n = 5$
TLR4 haplotype					
299+/399+		1.4 (0.0–2.6) $n = 100$	2.6 (0.7–4.5) $n = 95$	5.2 (3.4–10.0) $n = 96$	4.3 (2.4–7.8) $n = 98$
299−/399+		2.8 (−) $n = 1$	8.3 (−) $n = 1$	8.1 (−) $n = 1$	7.5 (−) $n = 1$
299−/399−[c]		1.7 (0.4–3.2) $n = 9$	4.4 (0.5–7.7) $n = 8$	6.4 (3.0–7.8) $n = 9$	4.3 (0.9–7.3) $n = 9$
299+/399−		1.9 (0.6–3.3) $n = 2$	0.0 (0.0–0.0) $n = 2$	3.2 (0.6–5.8) $n = 2$	1.4 (0.3–2.4) $n = 2$
Nod2 genotype					
3020insC present[d]		1.0 (0.2–2.6) $n = 7$	2.6 (0.7–3.3) $n = 7$	4.0 (2.3–16.1) $n = 7$	2.4 (1.0–8.9) $n = 7$
3020insC absent		1.5 (0.0–2.6) $n = 94$	2.7 (0.5–6.0) $n = 89$	5.4 (3.4–10.0) $n = 91$	4.4 (2.5–7.6) $n = 92$

[a] Median values and interquartile range in parentheses. Lower detection limit for TNF-α and endotoxin was 0.1 and 3.0 pg/mL, respectively.
[b] Positive defined as having an endotoxin level ≥5 pg/mL at aorta declamping and/or 30 min into reperfusion, negative defined as an endototoxin level <5 pg/mL at both time-points.
[c] One out of 13 patients homozygous, all others heterozygous.
[d] All patients carrying the Nod2 insertion were heterozygous.

variation found in other populations and exceeds the variation that can be explained by the laboratory variation only, which is estimated at 7.5–12.3% [13]. This indicates that the variations we found between subjects are to be explained by factors other than laboratory variation only. Since we find it feasible to assume that the LPS concentration needed for the release of 50% of the maximally evoked TNF-α, the TNF-EC_{50}, is a marker of LPS sensitivity we evaluated to what extent this parameter could predict the in vivo response to endotoxin. Theoretically, important inter-individual differences in the dose–response characteristics of TNF-α production by LPS can be overlooked by using measurements derived from one single (supra-physiological) LPS concentration. We found a significant correlation between the TNF-α production ex vivo using the highest LPS concentration (i.e. 1000 ng/mL) and in vivo TNF-α levels, but no significant correlation between TNF-EC_{50} and in vivo measured TNF-α levels. This indicates that the maximal TNF-α production capacity measured ex vivo is at present the best predictor of in vivo TNF-α levels upon cardiac surgery. To our knowledge no other study investigated the correlation between endotoxin-mediated TNF-α production ex vivo and in vivo.

Since several studies indicated the role of genetics in innate immunity we investigated the influence of various known polymorphism in the TNF-α promoter, the TLR4 and Nod2 genes. We found no correlation between the level of perioperative TNF-α production and TNF-α promoter SNPs, TLR4 polymorphisms and the presence of the Nod2 insertion. Also, we found no

correlation between ex vivo TNF-α production capacity and TNF-α promoter SNPs, TLR4 polymorphisms and the presence the Nod2 insertion. The frequencies of the various genotypes were similar to those found in other populations (Table 2) [3,10,14–16]. Although a type II error could be responsible for overlooking the differences in TNF-α production capacity between the various polymorphisms studied, our data suggest that their role, if present, is rather limited, and cannot be attributed to the large inter-individual differences found in TNF-α production capacity. Therefore, we conclude that the various polymorphisms studied play no clinically significant role in the cardiac surgery associated pro-inflammatory reaction in response to endotoxemia. The demographic characteristics of the cardiac surgery patients were similar to the patients of other groups described in the literature [17,18], making direct extrapolation of our findings possible.

Many studies have dealt with the question to what extent genetic variants affect regulation of the innate immune response. The results have been conflicting; to a large extent this can probably be explained by various models that have been used. Several studies evaluated the role of TNF-α promoter SNPs in susceptibility and severity of infections and septic shock [16,19–22]. Although some of the studies found increased susceptibility and/or severe outcome of sepsis or septic shock in carriers of a common TNF-α promoter polymorphism (i.e. −308 G/A) [16,19–22], in none of the studies was the in vivo TNF-α response positively correlated with this increased risk. These findings suggest that this polymorphism does not exert its effect on sepsis susceptibility and/or outcome by differences in gene expression and/or TNF-α production. One study investigated the response to endotoxin in a more controlled fashion. Recently, Fijen et al. found no differences in TNF-α levels between the TNF-(−308) genotypes in an experimental human endotoxemia model in healthy subjects [23]. These findings confirm our observation.

Only few studies investigated the association of SNPs within the TNF-α promoter and the capacity of TNF-α production upon LPS stimulation ex vivo. Results from studies using reporter constructs for TNF-α gene expression have been conflicting [24–27]. Also, data from several studies using ex vivo stimulation assays indicated an effect at some LPS concentrations at some of the incubating times, while no effect was found at other LPS concentrations and different incubating times [3,15,28].

Three studies investigated the role of TLR4 polymorphisms in susceptibility and outcome of Gram-negative infections in patients carrying either the Asp299Gly and/or the Thr399Ile mutations [7,29,30]. Only two studies indicated higher susceptibility [7,29], none of the studies found higher mortality. So far, only one study investigated the role of the common TLR4 polymorphisms in in vitro LPS stimulation of isolated monocytes [31]. This study used LPS derived from various microorganisms at a large range of concentrations. No differences were found between heterozygote carriers of the co-segregating Asp299Gly/Thr399Ile polymorphisms compared to the wild-type subjects. Although in this study isolated monocytes were used, our results are consistent with its results.

In a study conducted by Ogura et al. it was demonstrated that human embryonic kidney cells transfected with the Nod2 gene including the 3020insC mutation showed much less NF-κB activity in response to LPS, indicating the importance of the intact Nod2 protein in response to LPS [10]. A recent study, however, suggests that Nod2 is a general sensor of both Gram-negative and -positive peptidoglycan (PGN) and not so much LPS itself [32]. The unpurified LPS used by Ogura et al. might have been contaminated by PGN, leading to a misinterpretation of their observation. In our study no effect was found from the presence of this mutation on in vivo and ex vivo TNF-α production capacity upon endotoxin, confirming the conclusion by Girardin that endotoxin is not the ligand for Nod2.

In summary, based on our results and the largely conflicting findings in the literature, we propose that it is unlikely that the TNF-α promoter polymorphisms, the presence of the common TLR4 polymorphisms or the Nod2 insertion play an important role in LPS-mediated TNF-α production capacity in vivo and ex vivo. Thus, it remains unclear which and to what extent the additional genetic and environmental factors (e.g. sCD14, MD-2, LBP, lipoproteins) determine the large inter-individual variation found in in vivo and ex vivo responses to LPS.

4. Materials and methods

4.1. Patients

The study was performed at the Leiden University Medical Center, an 800-bed secondary and tertiary referral hospital. To be eligible for enrolment, the patients had to be 18 years or older and scheduled for elective cardiac surgery with cardiopulmonary bypass between July 1, 1998 and December 30, 1999. We obtained institutional approval from the local medical ethics committee (protocol #P168/96). Each patient gave a written, informed consent. The patients studied were 159 consecutive patients undergoing elective cardiac surgery with cardiopulmonary bypass. Of these, 122 had been included in a previously published study on the effect of selective gut decontamination (SGD) on endotoxemia and cytokine activation [33]. Of these 122 patients 24 received preoperative SGD consisting of polymyxin B and neomycin. In the previously mentioned study perioperative endotoxemia and subsequent

cytokine activation were found not to be influenced by the SGD regimen. Also, the results were not different when these patients were excluded from the analysis. Patients were followed-up throughout their stay in the hospital. Demographic characteristics were systematically collected for all patients entering the study.

4.2. Study design

On the day of surgery blood samples for ex vivo whole-blood LPS stimulation, using pyrogen-free tubes (Kabi-Vitrum, Amsterdam, The Netherlands), and DNA extraction were drawn before anesthesia. Blood samples for the determination of in vivo endotoxin and cytokine levels were collected from each patient before anesthetic induction, on aorta declamping, 30 min into body reperfusion (i.e. 30 min after stopping the extra corporal perfusion), and at the ICU admission (approximately 2 h after surgery). In a previous pilot study, we found that endotoxemia occurs most likely at these time-points [33].

4.3. Whole-blood LPS stimulation

Cytokine production was determined in whole-blood samples ex vivo as previously described [34]. Briefly, whole-blood samples were mixed 1:1 with RPMI 1640 (Gibco, Germany) and lipopolysaccharide (*Escherichia coli* 0111:B4, Difco, Detroit, MI) was added to the final concentrations of 10, 100 and 1000 ng/mL and cells were stimulated for 4 h at 37 °C under 5% CO_2 atmosphere. Tumor necrosis factor-α (TNF-α) concentrations were measured in the supernatant as described later.

4.4. Endotoxin measurements

Blood for endotoxin determination was collected in pyrogen-free tubes (Kabi-Vitrum, Amsterdam, The Netherlands) and platelet-rich plasma was prepared by centrifugation. Endotoxin was determined by a quantitative photometric assay with end-point measurement as described elsewhere [35]. The assay's lower detection limit for endotoxin was about 3.0 pg/mL [35].

4.5. TNF-α measurements

Blood for determination of TNF-α was collected in pyrogen-free ethylenediaminetetraacetic acid (EDTA) tubes (Chromogenics, Amsterdam, The Netherlands) and immersed in ice. Plasma was prepared by centrifugation at 3000*g* for 5–10 min at 4 °C and stored at −70 °C. TNF-α concentrations in the supernatants of the ex vivo whole-blood assay and the samples from the in vivo TNF-α production were analyzed at completion of the study. Tumor necrosis factor (TNF)-α concentrations were determined with a standard ELISA technique (Central Laboratories for Bloodtransfusion, Amsterdam, The Netherlands; Medgenix diagnostics, Floury, Belgium); the lower detection limit for TNF-α was 0.1 pg/mL [35].

4.6. DNA isolation

DNA was isolated from citrate blood from each patient, red blood cells were lysed with three volumes of lysis buffer (1.55 M NH_4Cl, 0.1 M $KHCO_3$, 0.01 M EDTA, pH 7.4); the remaining cells were incubated for 17 h at 37 °C with SDS and proteinase K. The DNA was extracted using phenol and chloroform as described by Maniatis et al. [36].

4.7. TNF-α promoter mutation analysis

Typing for the TNF-α single nucleotide polymorphisms −238, −308 and −376 was performed by PCR amplification. Forward and reverse primers used are given in Table 6. Primers were purchased from Biosource International (Foster City, CA). PCR amplifications were performed using the Thermolyne Amplitron II Thermal Cycler (Barnstead/Thermolyne, Dubuque, IA). The PCR conditions were as follows: 10 min at 95 °C followed by 35 cycles of 60 s at 95 °C, 60 s at 58 °C, 60 s at 72 °C for −238 and −376, and for −308 the conditions were 60 s at 94 °C, 60 s at 60 °C, and 60 s at 72 °C. The PCR-amplified products were electrophoresed on a 10% polyacrylamide gel to detect the size differences of the fragments between the polymorphisms, following digestion with a specific restriction enzyme (Table 6).

4.8. TLR4 mutation analysis

The TLR4 Asp299Gly mutation was determined by allele-specific PCR amplification of the involved region using sense primer 5′-CTT AGA CTA CTA CCT CGA TGA-3′ (wild-type) or 5′-CTT AGA CTA CTA CCT CGA TGG-3′ (mutant) in combination with the antisense primer 5′-TAA GCC TTT TGA GAG ATT TGA-3′. PCR was performed on ~300 ng DNA by 12 cycles of 10 s denaturing at 95 °C and 60 s at 65 °C, followed by 20 cycles of 10 s at 95 °C, 50 s at 60 °C, and 30 s elongation at 72 °C. Beta-actin primers (sense 5′-TCT GGC ACC ACA CCT TCT ACA A-3′ and antisense 5′-GTG GTG GTG AAG CTG TAG CCG-3′, amplified product is 334 bp) were included as positive control. Generation of the amplified products was evaluated with 1.2% agarose gel electrophoresis and ethidium bromide staining. Detection of the mutant 896G allele was verified by sequence analysis. The TLR4 Thr399Ile mutation was determined by PCR amplification of the involved region with sense primer 5′-GCT GTT TTC AAA GTG ATT TTG GGA GAA-3′ to

Table 6
Primer sequences and restriction enzymes used for detection of the polymorphisms in the TNF-α gene promoter and size of the PCR products and restriction fragments

Polymorphism	Primer sequence (5′→3′)	PCR product (bp)	Enzyme	Size	Allele
−238	CAGACCACAGACCTGGTC	165	*BamH*I	123 + 42	GG
	AAGGATACCCCTCACACTCCCCATCCTCCCGGATC			165	AA
−308	GAGGCAATAGGTTTTGAGGGCCAT	147	*Nco*I	121 + 26	GG
	GGGACACACAAGCATCAAG			147	AA
−376	CCTCAGGACTCAACACAGC	148	*Hpa*I	118 + 30	GG
	GGGGACCAGGTCTGTGGTCTGTTTCCTGTTAA			148	AA

create a *Hinf*I cleavage site when the mutant allele is amplified. After PCR amplification in combination with anti-sense primer 5′-CAC TCA TTT GTT TCA AAT TGG AAT G-3′ the amplicon was incubated with *Hinf*I as recommended by the supplier and cleavage of the product was evaluated by 2% agarose gel electrophoresis and ethidium bromide staining.

4.9. Nod2 mutation analysis

The Nod2 3020insC mutation that renders truncation of the Nod2 at position 1007 was determined using the allele-specific multiplex PCR assay described by Ogura et al. [8].

4.10. Calculation of ex vivo LPS/TNF-α dose–response

For each patient two characteristics of the LPS-induced TNF-α release were calculated by non-linear regression with the dose–response model according to the Hill equation (Eq. (1)).

$$E_N = E_{N,max} \times C/(EC_{50} + C) \quad (1)$$

where E_N is the observed TNF-α production at a given LPS concentration C, EC_{50} is the estimated LPS concentration at which 50% of the maximal TNF-α response is reached, and $E_{N,max}$ is the estimated maximal TNF-α production. In a previous pilot study we found that measuring the TNF-α production at the LPS concentration we used (i.e. 10, 100 and 1000 ng/mL) was sufficient to calculate the dose–response characteristics EC_{50} and $E_{N,max}$ (E.S., unpublished observation, report in preparation).

4.11. Statistical analysis

For statistical analysis blood samples with TNF-α and endotoxin levels below the limit of detection were entered as half of the value of the lower detection limit (0.05 and 1.5 pg/mL, respectively). To determine whether polymorphisms in the TLR4 gene, the presence of the Nod2 insertion and the TNF-α promoter were associated with levels of in vivo TNF-α production, repeated measurement models (SPSS 11.0, mixed) were used to determine whether the patterns of TNF-α production over time were different between polymorphisms. For this analysis log-transformed TNF-α levels were used. The ex vivo TNF-α production characteristics between the various genotypes were compared using a Mann–Whitney *U* test. Correlations were assessed non-parametrically using Spearman correlation test. Statistical significance was tested two-tailed, with the α set to 0.05.

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CHAPTER 6

IL-10 and toll-like receptor-4 polymorphisms and in vivo and ex vivo response to endotoxin

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IL-10 and toll-like receptor-4 polymorphisms and the in vivo and ex vivo response to endotoxin☆

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Abstract

To determine to what extent lipopolysaccharide-induced IL-10 production capacity is determined by polymorphisms in toll-like receptor-4 (TLR4) and the IL-10 promoter region, we measured in vivo IL-10 and TNF-α production in patients undergoing elective cardiopulmonary bypass surgery, a major surgical trauma associated with ischemia-reperfusion injury that triggers an endotoxemia and profound inflammatory response in most patients. Ex vivo the IL-10 and TNF-α production was measured in a whole blood stimulation assay, using 3 LPS concentrations. Positive correlations were found between TNF-α and IL-10 production ex vivo, upon stimulation with each of the LPS concentrations. Also, the estimated TNF-α and IL-10 EC_{50}, and TNF-α_{max} and IL-10_{max} were positively correlated ($r = 0.203$; $p = 0.023$ and $r = 0.287$; $p = 0.001$, respectively), indicating that these parameters describing LPS sensitivity and maximal production capacity, respectively, can be estimated by measuring either TNF-α or IL-10. Interleukin-10 concentrations in patients experiencing endotoxemia in vivo negatively correlated with the IL-10 levels produced upon stimulation with 1000 ng/mL LPS as well as the estimated IL-10_{max} ex vivo. In vivo, a positive correlation between the TNF-α concentration at time-point 2 and the IL-10 concentration at time-point 3 was found, consistent with an important contribution of the magnitude of TNF-α release upon the subsequent IL-10 production. Carriers of the IL-10 promoter −1330G, −1082A, −819T, −592A (GATA) haplotype had lower IL-10 production ex vivo upon stimulation with 10 and 100 ng/mL LPS and higher EC_{50} values (the estimated LPS concentration at which 50% of the maximal IL-10 response is reached) as compared to carriers of the other haplotypes combined, indicating decreased LPS sensitivity ex vivo. These individuals did not differ from the others in interleukin-10 production capacity upon stimulation with a high LPS concentration (i.e., 1000 ng/mL) and the estimated IL-10_{max} values, were similar, indicating unimpaired maximal IL-10 production capacity ex vivo. Carriers of the IL-10 promoter AGCC haplotype had lower EC_{50} values as compared to carriers of the other haplotypes combined, indicating increased LPS sensitivity ex vivo. In accordance with this finding, carriers of the AGCC haplotype had higher circulating IL-10 levels in vivo. The common TLR4 polymorphisms (Asp299Gly and Thr399Ile) were associated with slightly higher IL-10 production capacity ex vivo and in vivo, however, this was not statistically significant. Our results indicate that polymorphisms in the proximal IL-10 promoter region are associated with in

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vivo and ex vivo LPS sensitivity. The contribution to the inter-individual variation, however, is limited since the variation between individuals in LPS sensitivity and IL-10 production capacity can only partly be attributed to these IL-10 promoter polymorphisms.

Keywords: Cardiopulmonary bypass; Endotoxemia; Lipopolysaccharides; Toll-like receptor-4; IL-10 promoter

1. Introduction

Interleukin-10 (IL-10) is an important immunoregulatory cytokine. It is involved in the regulation of inflammatory responses through direct influence over tumor necrosis factor (TNF)-α production. Also, IL-10 plays an important role in the course of infectious diseases, for instance, the severity to which meningococcal meningitis progresses is associated with serum IL-10 concentrations, such that a high serum IL-10 level was observed in patients with a poor or fatal outcome, whereas patients with mild disease and a good prognosis had lower serum IL-10 levels [1−3]. In Gram-negative infection, the presence of lipopolysaccharide (LPS) in the circulation plays a pivotal role in the release of IL-10. Human monocytes, the main producers of IL-10, exhibit substantial inter-individual differences in IL-10 production upon LPS stimulation ex vivo [4]. A study investigating the inter-individual variation of IL-10 production following LPS stimulation of whole blood cultures ex vivo suggested that the differences in IL-10 production capacity can to a large extent be explained by differences in genetic background [5]. The inter-individual differences in produced IL-10 could not simply be explained by corresponding differences in TNF-α production, implicating that the differences in the individual's ability to produce IL-10 are not simply reflecting differences in the individual's ability to produce TNF-α [5]. It has been suggested that differential transcription is the principle mechanism of the inter-individual differences in IL-10 production [6]. Indeed, the IL-10 production is proportionally related to mRNA production and not to the half-life of IL-10 [7]. Given the fact that the differences in IL-10 production are most likely transcriptionally regulated, the IL-10 promoter region has been an important target for investigation [6]. In the IL-10 promoter, 11 single nucleotide polymorphisms (SNPs) have been described. Studies of SNPs in the proximal 1.1 kb have yielded the existence of a relative small number of haplotypes [8−10]. The dimorphic polymorphisms (G or A at position −1330, G or A at position −1082, C or T at position −819, C or A at position −592) are in preferential allelic association, namely AGCC, GACC and GATA haplotypes. It has been shown that the differences in the production capacity of IL-10 ex vivo are associated with IL-10 haplotypes [6]. Furthermore, the SNP in the IL-10 promoter at position −2849 was associated with differences in IL-10 production ex vivo upon stimulation with 1000 ng/mL LPS and transcriptional activity [11−13]. So far, other known SNPs in the IL-10 promoter have not been evaluated in this way.

Toll-like receptor-4 (TLR4) is part of a large family of transmembrane proteins and is believed to be crucial in mediating LPS effects. TLR4 is expressed on monocytes and macrophages, and to a lesser extent on lymphocytes and other cell types [14,15]. The recently described association between the Asp299Gly polymorphism in TLR4 and Gram-negative septic shock suggests a functional defect in TLR4 leading to increased susceptibility to Gram-negative bacteremia [16].

Cardiopulmonary bypass surgery leads to perioperative endotoxemia in most patients, and this procedure may serve as a model to study the association of genetic polymorphism and endotoxin mediated IL-10 production in vivo. The most immunoreactive reactive component of endotoxin is lipid A, being a structural element of LPS.

The aims of the current study were to assess the inter-individual variation in IL-10 production upon whole blood stimulation with LPS, to determine the correlation between production rates and various SNPs in the IL-10 promoter region as well as the TLR4 coding region, and to determine how much of the inter-individual variation could be attributed to these genetic factors. We also studied the inter-individual variation in the in vivo IL-10 production following cardiopulmonary bypass surgery in the same way.

2. Results

2.1. Patients

We studied 159 consecutive patients undergoing elective cardio-thoracic surgery with cardiopulmonary bypass. The patient characteristics have been described previously [17]. Briefly, there was a predominance of male patients (66%), the median age for males and females was 65 and 67, respectively ($p = 0.091$). Active smoking occurred in 35 patients (22%) whereas 20 patients (12%) had diabetes mellitus. Surgical procedures were extensive; 20 patients (12%) underwent coronary artery bypass surgery (CABG) combined with valve replacement, 49 patients (30%) underwent valve replacement only, whereas 89 patients (54%) underwent CABG only. Eight patients underwent other surgical procedures, mainly aortic surgery.

2.2. IL-10 promoter genotyping

Typing for the IL-10 single nucleotide polymorphisms was successful in most patients; in a minority of the samples no definite typing could be established (Table 1). As previously described the 4 defined proximal dimorphic polymorphisms (A or G at position −1330, G or A at position −1082, C or T at position −819, C or A at position −592) resulted in three allelic associations, namely AGCC, GACC, and GATA haplotypes, resulting in six subsequent biallelic haplotypes (Table 1). The haplotype frequencies were similar to the frequencies described previously [9].

2.3. TLR4 genotyping

The TLR4 Asp299Gly and Thr399Ile substitutions were successfully determined in all the patients. The Asp299Gly substitution was found in 17 patients (10.7%), 16 patients were heterozygous and 1 patient was homozygous. Except for 1 patient the TLR4 Asp299Gly substitution was accompanied by the TLR4 Thr399Ile substitution. Two patients were identified with an isolated TLR4 Thr399Ile substitution (Table 1). The patient homozygous for the Asp299Gly substitution was also homozygous for the Thr399Ile substitution.

2.4. Ex vivo IL-10 measurements

Blood samples for ex vivo LPS stimulation were available from 125 patients. In general, we found a dose dependent IL-10 production upon stimulation of whole blood with increasing concentrations of LPS. Median IL-10 levels upon stimulation with 0 ng/mL LPS were below or at the lower level of detection in the majority of patients [median 3.0 pg/mL (IQR: 3.0−3.0)], and no differences were found between the various groups (data not shown). Median IL-10 levels upon stimulation with 10, 100 and 1000 ng/mL LPS were 801 (IQR: 506−1221), 1482 (IQR: 996−1974) and 3493 (IQR: 2534−4673) pg/mL, respectively (Table 2). For each patient we estimated the dose−response characteristics EC_{50} and E_{max} (i.e., EC_{50} is the estimated LPS concentration at which 50% of the maximal IL-10 response is reached, and E_{max} is the estimated maximal IL-10 production), as described in the methods.

We first investigated whether any association was present between the IL-10 promoter polymorphisms and the levels of IL-10 production upon stimulation with the different LPS concentrations, and the estimated dose−response characteristics (i.e. EC_{50} and E_{max}) ex vivo. As shown in Table 2, the level of IL-10 produced following ex vivo LPS stimulation was not statistically different in the six biallelic genotypes of the proximal IL-10 promoter and the distal SNPs. However, carriers of the GATA haplotype (homozygous and heterozygous carriers combined), as compared to carriers of the other haplotypes combined, had lower IL-10 levels after stimulation with 10 and 100 ng/mL LPS [619 (IQR: 431−1011) versus 826 (IQR: 575−1142), $p = 0.041$ and 1253 pg/mL (IQR: 808−1707) versus 1651 (IQR: 1194−2042), $p = 0.006$, respectively, Fig. 1). Furthermore, these patients had significantly higher EC_{50} values [159 (IQR: 97−293) versus 122 ng/mL (IQR: 67−190), $p = 0.027$, Fig. 2], indicating decreased LPS sensitivity. More interestingly, the IL-10 concentration after stimulation with the highest LPS concentration (i.e. 1000 ng/mL), and the estimated E_{max} were not different in the two groups [3357 (IQR: 2111−4517) versus 3529 (IQR: 2634−4738), $p = 0.377$ and 3966 pg/mL (IQR: 2174−5345) versus 3737 (IQR: 2844−5585), $p = 0.767$, respectively]. However, homozygous carriers of the GATA haplotype, as compared to all other patients combined, had higher EC_{50} and E_{max} values [230 (IQR: 141−502) versus 134 ng/mL (IQR: 78−196), $p = 0.013$ and 5122 pg/mL (IQR: 3758−7555) versus 3605 (IQR: 2533−5242), $p = 0.035$, respectively]. On the other hand, patients carrying the AGCC haplotype (homozygous and heterozygous carriers combined), as compared to carriers of the other haplotypes combined, had slightly lower EC_{50} values [125 (IQR: 66−204) versus 159 ng/mL (IQR: 106−230), $p = 0.041$], indicating higher LPS sensitivity (Fig. 2).

Patients carrying the Asp299Gly polymorphism as compared to carriers of wild-type alleles, had somewhat higher IL-10 concentrations upon stimulation with 100 and 1000 ng/mL LPS [1799 (IQR: 1237−2058) versus

Table 1
IL-10 promoter haplotype/genotype in 159 patients undergoing elective cardiac surgery with cardiopulmonary bypass

IL-10 promoter genotype, *n* (%)		
−1330, −1082, −819, −592/−1330, −1082, −819, −592		
AGCC/AGCC		31 (20)
AGCC/GACC		42 (27)
AGCC/GATA		30 (20)
GACC/GACC		16 (10)
GATA/GATA		15 (10)
GACC/GATA		20 (13)
IL-10 promoter genotype, *n* (%)		
−3575	AT	70 (51)
	TT	50 (37)
	AA	16 (12)
−2849	GG	72 (55)
	AG	56 (42)
	AA	4 (3)
−2763	CC	61 (46)
	AC	61 (46)
	AA	11 (8)
TLR4 haplotype, *n* (%)		
299+/399+		140 (88)
299−/399+[a]		1 (1)
299−/399−		16 (10)
299+/399−		2 (1)

[a] One out of 16 patients homozygous, all others heterozygous.

Table 2
IL-10 production ex vivo on whole blood stimulation with LPS according to IL-10 and TLR4 genotype/haplotype[a]

			10 ng/mL	100 ng/mL	1000 ng/mL	IL-10_{max}	$EC_{50[LPS]}$
All patients ($n = 125$)			801 (506–1121)	1482 (996–1974)	3493 (2534–4673)	3817 (2712–5445)	139 (82.6–226)
IL-10 promoter genotype (n)							
−1330/−1082/−819/−592							
AGCC/AGCC		(28)	819 (518–1195)	1476 (921–2042)	3341 (2411–4259)	3526 (2454–4823)	145 (60.4–195)
AGCC/GACC		(30)	922 (630–1129)	1666 (1309–2161)	3722 (2856–5353)	3985 (3100–6056)	105 (75.8–161)
AGCC/GATA		(24)	646 (409–1081)	1129 (787–1734)	2765 (1898–4216)	3352 (1986–4694)	134 (67.0–268)
GACC/GACC		(12)	800 (698–1001)	1799 (1189–2025)	3680 (2826–4540)	3959 (3124–5225)	144 (71.8–192)
GATA/GATA		(12)	741 (478–963)	1446 (1043–1520)	4088 (3185–5906)	5122 (3758–7555)	230 (141–502)
GACC/GATA		(17)	508 (393–982)	1108 (691–1869)	3493 (1694–4985)	3965 (2064–6092)	145 (109–254)
IL-10 promoter genotype (n)							
−3575	AT	(55)	826 (516–1188)	1492 (968–2036)	3386 (2531–4840)	3542 (2348–5793)	117 (75.2–223)
	TT	(40)	757 (490–1009)	1378 (989–1878)	4075 (2819–5012)	4792 (3176–6403)	167 (132–300)
	AA	(13)	813 (482–1179)	1680 (760–2089)	3228 (1951–3852)	3470 (2406–4431)	67 (23.9–145)
−2849	GG	(60)	665 (489–1027)	1482 (979–1992)	3868 (2704–5498)	4627 (3135–6685)	165 (103–262)
	AG	(42)	832 (445–1221)	1360 (760–1866)	3044 (1812–3826)	3330 (1960–4425)	113 (42.1–235)
	AA	(3)	869 (813–1135)	1814 (1783–1887)	4130 (3528–4259)	4693 (3587–4849)	144 (59.8–147)
−2763	CC	(48)	678 (478–1009)	1412 (973–1992)	3798 (2363–5025)	4213 (2700–6403)	150 (95.9–224)
	AC	(49)	855 (510–1164)	1430 (1005–1834)	3444 (2553–4243)	3966 (2798–4727)	139 (79.8–267)
	AA	(9)	813 (370–1269)	1680 (664–1950)	3341 (1562–4735)	3470 (1767–5393)	144 (89.3–221)
TLR4 haplotype (n)							
299+/399+		(109)	801 (490–1148)	1450 (930–1940)	3395 (2411–4673)	3587 (2373–5345)	133 (76.8–226)
299−/399+		(1)	1080 (−)	2251 (−)	7381 (−)	9582 (−)	301 (−)
299−/399−[b]		(13)	716 (526–950)	1783 (1228–1979)	4130 (3345–4511)	4693 (3627–5813)	144 (109–241)
299+/399−		(2)	852 (664–1040)	1887 (1633–2140)	4476 (3722–5229)	5130 (4230–6031)	152 (143–160)

[a] IL-10 concentrations are in pg/mL. Values are expressed as median values and interquartile range between parentheses. Data on 0 ng/mL LPS not shown, the majority of IL-10 levels were below or at lower detection limit, and now differences were found between the various groups.
[b] One out of 13 patients homozygous, all others heterozygous.

1458 pg/mL (IQR: 957–1956), $p = 0.174$ and 4171 (IQR: 3351–4856) versus 3457 pg/mL (IQR: 2425–4685), $p = 0.083$, respectively]. The same trend was observed when comparing carriers of the Thr399Ile polymorphism with carriers of wild-type alleles [1783 (IQR: 1245–2006) versus 1451 pg/mL (IQR: 944–1969), $p = 0.177$ and 4130 (IQR: 3357–4567) versus 3426 pg/mL (IQR: 2418–4690), $p = 0.111$, respectively]. In

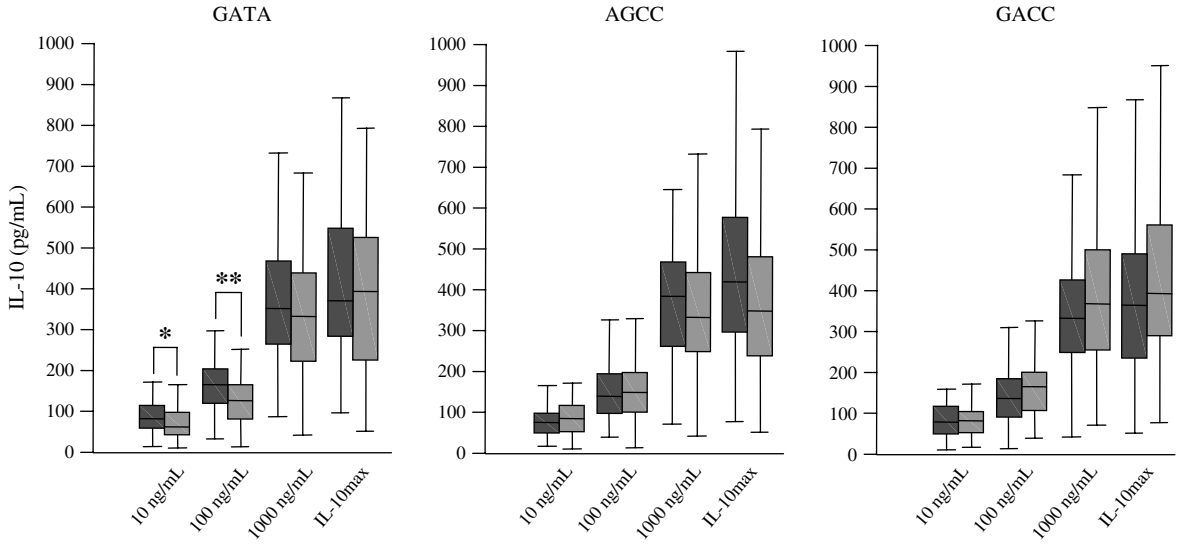

Fig. 1. IL-10 production upon whole blood stimulation with 10, 100 and 1000 ng/mL LPS respectively, and the estimated IL-10_{max}. Left panel represents carriers of the GATA haplotype (lighter box plots) as compared with all others. Middle panel represents carriers of the AGCC haplotype (lighter box plots) as compared with all others. Right panel represents carriers of the GACC haplotype (lighter box plots) as compared with all others. The box plots display the median, the 25th and 75th percentiles, and the smallest and largest IL-10 concentrations that are not outliers. IL-10 was measured by enzyme-linked immunosorbent assay; the detection limit was 4 pg/mL. $*p = 0.041$, $**p = 0.006$.

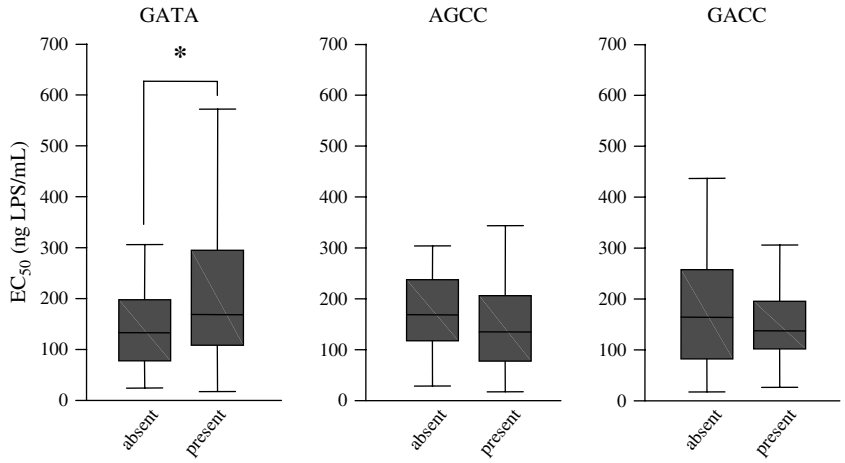

Fig. 2. Estimated EC_{50} values, calculated as described in the methods. Left panel represents carriers of the GATA haplotype (right box plot) as compared with all others. Middle panel represents carriers of the AGCC haplotype (right box plot) as compared with all others. Right panel represents carriers of the GACC haplotype (right box plot) as compared with all others. The box plots display the median, the 25th and 75th percentiles, and the smallest and largest EC_{50} values that are not outliers. $*p = 0.027$.

accordance with these findings, the estimated IL-10 E_{max} was higher in the carriers of either the Asp299Gly or the Thr399Ile polymorphism as compared to wild-type allele carriers [4771 (IQR: 3682–6506) versus 3604 pg/mL (IQR: 2399–5404), $p = 0.064$ and 4693 (IQR: 3738–6031) versus 3596 pg/mL (IQR: 2386–5425), $p = 0.085$, respectively]. Although none of the described differences reached the level of statistical significance, the consistency in the observations suggest an increased in vitro IL-10 production capacity at high concentrations of LPS in patients carrying either the Asp229Gly or the Thr399Ile polymorphism. No differences in EC_{50} value were found between the various groups (all $p > 0.38$).

2.5. In vivo endotoxin measurements

Perioperative blood samples for endotoxin measurements were available from most patients and have been described in detail previously [17]. Briefly, endotoxin levels were ≥ 5 pg/mL directly before anesthetic induction in 4 out of 152 patients (2.6%). On aorta declamping (time-point 2), 30 min into reperfusion (time-point 3) and on arrival at the ICU (approximately 2 h after surgery, time-point 4) 74 out of 142 patients (52.1%), 71 out of 143 patients (49.7%) and 54 out of 151 patients (35.8%) had endotoxin levels ≥ 5 pg/mL, respectively. In 85 out of 143 patients (59.4%) the endotoxin level at either time-point 2 and/or 3 was ≥ 5 pg/mL. Median endotoxin levels increased from below the level of detection to 5.0 and 4.9 pg/mL at aorta declamping and 30 min into reperfusion, respectively, and decreased afterwards to 4.2 pg/mL on arrival at the ICU.

2.6. In vivo IL-10 measurements

Plasma IL-10 concentrations before anesthetic induction were below or at the lower level of detection in the majority of patients [median 2.0 pg/mL (IQR: 2.0–4.0)]. On aorta declamping, 30 min into reperfusion and on arrival at the ICU were 32 (IQR: 10–88), 37 (IQR: 11–87) and 8.0 (IQR: 4.0–26) pg/mL, respectively (Table 3). No differences in IL-10 levels at any of the time-points were found between the six biallelic genotypes of the proximal IL-10 promoter and the distal SNPs, or the TLR4 haplotypes and genotypes (all $p > 0.17$). However, carriers of the AGCC haplotype (homozygous and heterozygous carriers combined), as compared to carriers of the other haplotypes combined, had slightly higher IL-10 levels at time-points 2 and 3 [35.0 (IQR: 11.0–88.5) versus 19.5 pg/mL (IQR: 6.5–49.0), $p = 0.053$ and 43.0 (IQR: 13.0–112) versus 24.0 pg/mL (IQR: 10.0–62.3), $p = 0.038$, respectively]. Furthermore, when combining measurements from consecutive time-points (i.e. time-point 2 and 3 or 2, 3 and 4 combined, respectively) the differences were more clear [43.0 (IQR: 12.0–106) versus 23.5 pg/mL (IQR: 10.0–55.3), $p = 0.005$ and 24.0 (IQR: 7.0–72.0) versus 16.0 pg/mL (IQR: 5.0–39.3), $p = 0.016$, respectively]. Also, homozygous carriers of the GATA haplotype, as compared to all other patients combined, had lower IL-10 at time-points 2 and 3 [17.0 (IQR: 4.3–28.3) versus 35.0 pg/mL (IQR: 11.0–89.0), $p = 0.026$ and 20.0 (IQR: 10.0–33.0) versus 39.0 pg/mL (IQR: 11.0–105), $p = 0.065$, respectively]. Furthermore, when combining measurements from consecutive time-points (i.e. time-point 2 and 3 or 2, 3 and 4 combined, respectively) the differences were more clear [18.0 (IQR: 5.5–32.0) versus 37.5 pg/mL (IQR: 11.0–106), $p = 0.04$ and 13.5 (IQR: 4.3–26.8) versus 23.0 pg/mL (IQR: 7.0–67.5),

Table 3
Perioperative IL-10 production according to IL-10 and TLR4 genotype/haplotype[a]

		Anesthetic induction	Aorta declamping	30 min into reperfusion	On arrival at the ICU
All patients		2.0 (2.0–4.0)	32 (10–88)	37 (11–87)	8.0 (4.0–26)
		$n = 150$	$n = 137$	$n = 138$	$n = 148$
Endotoxemia[b]					
Negative		2.0 (2.0–4.0)	19 (8.0–68)	33 (11–92)	5.5 (4.0–25)
		$n = 56$	$n = 55$	$n = 54$	$n = 56$
Positive		2.0 (2.0–4.0)	34 (11–103)	41 (12–89)	7.0 (2.0–24)
		$n = 82$	$n = 80$	$n = 82$	$n = 81$
IL-10 promoter genotype					
−1330, −1082, −819, −592/−1330, −1082, −819, −592					
AGCC/AGCC		2.0 (2.0–4.0)	48 (14–207)	44 (16–203)	9.0 (2.0–30)
		$n = 31$	$n = 29$	$n = 28$	$n = 31$
AGCC/GACC		2.0 (2.0–4.0)	32 (8.3–80)	37 (11–88)	6.0 (2.0–29)
		$n = 40$	$n = 36$	$n = 37$	$n = 39$
AGCC/GATA		2.0 (2.0–4.0)	53 (13–87)	45 (16–83)	9.0 (4.0–22)
		$n = 29$	$n = 24$	$n = 25$	$n = 28$
GACC/GACC		2.0 (2.0–2.5)	14 (6.0–46)	11 (6.0–63)	5.0 (2.0–17)
		$n = 14$	$n = 15$	$n = 15$	$n = 15$
GATA/GATA		2.0 (2.0–4.0)	17 (4.3–28)	20 (10–33)	5.0 (4.0–22)
		$n = 15$	$n = 14$	$n = 15$	$n = 15$
GACC/GATA		2.0 (2.0–3.0)	35 (17–168)	36 (20–177)	6.5 (2.5–25)
		$n = 17$	$n = 15$	$n = 14$	$n = 16$
IL-10 promoter genotype					
−3575	TA	2.0 (2.0–4.0)	43 (10–102)	50 (11–159)	7.0 (2.8–30)
		$n = 67$	$n = 60$	$n = 59$	$n = 66$
	TT	2.0 (2.0–4.0)	25 (8.0–50)	24 (10–60)	9.0 (4.0–25)
		$n = 46$	$n = 43$	$n = 43$	$n = 45$
	AA	2.0 (2.0–3.5)	23 (9.8–209)	28 (11–190)	6.0 (2.0–26)
		$n = 16$	$n = 14$	$n = 15$	$n = 16$
−2849	GG	2.0 (2.0–4.0)	32 (8.0–76)	34 (11–81)	9.0 (4.0–26)
		$n = 68$	$n = 62$	$n = 61$	$n = 67$
	AG	2.0 (2.0–4.0)	49 (11–128)	41 (11–140)	7.0 (2.0–31)
		$n = 54$	$n = 49$	$n = 50$	$n = 53$
	AA	3.0 (2.0–5.5)	11 (4.0–73)	12 (8.0–55)	7.0 (2.8–20)
		$n = 4$	$n = 3$	$n = 3$	$n = 4$
−2763	CC	2.0 (2.0–4.0)	28 (8.0–57)	33 (11–63)	9.0 (4.0–26)
		$n = 57$	$n = 51$	$n = 51$	$n = 56$
	AC	2.0 (2.0–4.0)	49 (11–135)	42 (11–194)	8.0 (2.0–28)
		$n = 59$	$n = 54$	$n = 54$	$n = 58$
	AA	2.0 (2.0–4.0)	39 (9.0–212)	36 (11–249)	5.0 (4.0–30)
		$n = 11$	$n = 10$	$n = 10$	$n = 11$
TLR4 haplotype					
299+/399+		2.0 (2.0–4.0)	32 (10–90)	33 (11–109)	8.0 (2.0–27)
		$n = 132$	$n = 122$	$n = 122$	$n = 130$
299−/399+		2.0 (−)	80 (−)	42 (−)	8.0 (−)
		$n = 1$	$n = 1$	$n = 1$	$n = 1$
299−/399−[c]		4.0 (2.0–4.0)	43 (10–50)	43 (21–67)	9.0 (5.0–17)
		$n = 15$	$n = 12$	$n = 13$	$n = 15$
299+/399−		2.0 (2.0–2.0)	9.0 (2.0–16)	21 (5.0–37)	7.5 (6.0–9.0)
		$n = 2$	$n = 2$	$n = 2$	$n = 2$

[a] IL-10 concentrations are in pg/mL. Median values and interquartile range between parentheses. Lower detection limit for IL-10 and endotoxin were 4.0 and 3.0 pg/mL, respectively.
[b] Positive defined as having an endotoxin level of ≥ 5 pg/mL at aorta declamping and/or 30 min into reperfusion, negative defined as an endotoxin level of <5 pg/mL at both the time-points.
[c] One patient out of 15, 12, 13 and 15 homozygous, respectively, all others heterozygous.

$p = 0.010$, respectively]. To determine whether the patterns of IL-10 production over time were different between carriers the proximal IL-10 promoter haplotypes or their six subsequent biallelic genotypes, we used a repeated measurements model as described in the methods. For this analysis log-transformed IL-10 levels were used as dependent variable. Since the time-point proved to be the most significant variable in this model

we used this variable as a fixed-factor, leaving baseline measurements (time-point 1) out of the analysis. To investigate the influence of other relevant variables (i.e. TNF-α and endotoxin concentrations) they were introduced in the model. We did not find statistically significant differences in the pattern of IL-10 production over time between any of the proximal IL-10 promoter haplotypes and the distal SNPs.

The median IL-10 levels were lower at all post-operative time-points in carriers of the 299 and 399 wild-types alleles as compared to carriers of the Asp299Gly or the Thr399Ile polymorphisms respectively, however, this was not statistically significant (all $p > 0.37$). The patterns of IL-10 production over time were not different between carriers of the substitutions as compared to the wild-types.

2.7. Correlations between endotoxin and IL-10 levels

A positive correlation between endotoxin and IL-10 levels was observed when analysing all paired samples ($n = 572$, $r = 0.433$; $p = <0.001$). After excluding either all paired samples at time-point 1 or all paired samples with endotoxin and/or IL-10 level below the level of detection (i.e., endotoxin and IL-10 level below 3.0 and 4.0 pg/mL, respectively) or both, the correlation decreased but remained statistically significant ($n = 424$, $r = 0.115$; $p = 0.018$, $n = 395$, $r = 0.333$; $p = <0.001$ and $n = 350$, $r = 0.132$; $p = 0.013$, respectively). Furthermore, median IL-10 levels, at all post-operative time-points were higher in patients having endotoxemia (defined as having an endotoxin level of >5 pg/mL at aorta declamping and/or 30 min into reperfusion) as compared to patients without endotoxemia, however, this difference did not reach the level of statistical significance (all $p > 0.14$, Table 3).

2.8. Correlations between ex vivo IL-10 production and in vivo IL-10 concentrations

Since in vivo IL-10 concentrations were found to correlate with the level of endotoxemia after aorta declamping, suggesting a causative role for endotoxin in the IL-10 production, it seems plausible to investigate whether a correlation could be found between the in vitro IL-10 production upon LPS stimulation and the in vivo IL-10 concentrations during endotoxemia. A significant negative correlation between IL-10 concentration at aorta declamping and 30 min into reperfusion with ex vivo IL-10 production using 1000 ng/mL LPS was found ($r = -0.303$; $p = 0.001$ and $r = -0.210$; $p = 0.030$, respectively). Also, a significant negative correlation was found between IL-10 concentration at aorta declamping with ex vivo IL-10 production using 100 ng/mL LPS ($r = -0.216$; $p = 0.024$).

Furthermore, we found a negative correlation between IL-10 concentration at aorta declamping and 30 min into reperfusion with the estimated IL-10_{max} ($r = -0.305$; $p = 0.001$ and $r = -0.225$; $p = 0.018$, respectively). After dividing the patients into two groups according to the presence or the absence of endotoxemia (endotoxemia positive patients, defined as patients having an endotoxin level $>$ 5 pg/mL at aorta declamping and/or 30 min into reperfusion or endotoxemia negative patients defined as patients having an endotoxin level $<$ 5 pg/mL at both the time-points) the correlations were only significant in the endotoxemia positive patients. In the patients having endotoxemia, a negative correlation between IL-10 concentration at aorta declamping and 30 min into reperfusion with ex vivo IL-10 production upon stimulation with 1000 ng/mL LPS was found ($r = -0.389$; $p = 0.001$ and $r = -0.282$; $p = 0.023$, respectively). In accordance with this finding the same negative correlation was observed between IL-10 concentration at aorta declamping with ex vivo IL-10 production upon stimulation with 100 ng/mL LPS ($r = -0.280$; $p = 0.023$). Moreover, a significant negative correlation between IL-10 concentration at aorta declamping and 30 min into reperfusion with the estimated IL-10_{max} was found ($r = -0.377$; $p = 0.002$ and $r = -0.279$; $p = 0.022$, respectively).

2.9. Correlations between IL-10 and TNF-α production ex vivo and in vivo

Data on ex vivo TNF-α production and in vivo TNF-α concentrations have been published previously [17]. Herein, we investigated whether any correlations existed between the ex vivo IL-10 and TNF-α production and in vivo concentration were present (Table 4).

We observed positive correlations between TNF-α and IL-10 production ex vivo upon stimulation with all the LPS concentrations tested (Table 4, r ranging from 0.305 to 0.563, all $p < 0.007$). Furthermore, the estimated TNF-α EC_{50} and IL-10 EC_{50}, as well as the TNF-α_{max} and IL-10_{max} were positively correlated ($r = 0.203$; $p = 0.023$ and $r = 0.287$; $p = 0.001$, respectively). The observed positive correlation between TNF-α and IL-10 EC_{50} values, being markers of LPS determined by ex vivo TNF-α and IL-10 production, respectively, indicates that LPS sensitivity influences ex vivo TNF-α and IL-10 production in the same direction.

In vivo no significant correlations were observed between TNF-α and IL-10 concentrations at any of the time-points (Table 4). We find this remarkable since one might expect a positive correlation, especially at the time-points at which endotoxemia occurs most significantly. We hypothesized that the correlation might be absent due to the fact that TNF-α is released early upon endotoxemia, whereas IL-10 is released later and peaks when TNF-α levels already decrease. This hypothesis

Table 4
Correlations between in vivo and in vitro IL-10 and TNF-α production

			IL-10								
			In vivo				In vitro				
			Anesthetic induction	Aorta declamping	30 min into reperfusion	On arrival at the ICU	10 ng/mL	100 ng/mL	1000 ng/mL	IL-10_{max}	$EC_{50[LPS]}$
TNF-α	In vivo	Anesthetic induction	$r = 0.358$ $p = <0.001$	$r = 0.144$ $p = 0.139$	$r = 0.172$ $p = 0.077$	$r = 0.208$ $p = 0.029$	$r = 0.163$ $p = 0.127$	$r = 0.084$ $p = 0.436$	$r = 0.127$ $p = 0.240$	$r = 0.117$ $p = 0.274$	$r = -0.018$ $p = 0.866$
		Aorta declamping	$r = 0.166$ $p = 0.091$	$r = 0.018$ $p = 0.854$	$r = 0.073$ $p = 0.463$	$r = 0.166$ $p = 0.091$	$r = 0.135$ $p = 0.220$	$r = 0.069$ $p = 0.534$	$r = 0.186$ $p = 0.093$	$r = 0.166$ $p = 0.128$	$r = -0.006$ $p = 0.955$
		30 min into reperfusion	$r = 0.222$ $p = 0.021$	$r = -0.069$ $p = 0.479$	$r = -0.072$ $p = 0.457$	$r = 0.258$ $p = 0.007$	$r = 0.215$ $p = 0.049$	$r = 0.129$ $p = 0.243$	$r = 0.168$ $p = 0.128$	$r = 0.131$ $p = 0.230$	$r = -0.042$ $p = 0.703$
		On arrival at the ICU	$r = 0.189$ $p = 0.049$	$r = -0.087$ $p = 0.375$	$r = -0.109$ $p = 0.265$	$r = 0.078$ $p = 0.416$	$r = 0.257$ $p = 0.016$	$r = 0.128$ $p = 0.236$	$r = 0.115$ $p = 0.293$	$r = 0.093$ $p = 0.389$	$r = -0.105$ $p = 0.332$
	In vitro	10 ng/mL	$r = -0.413$ $p = <0.001$	$r = -0.237$ $p = 0.012$	$r = -0.220$ $p = 0.021$	$r = 0.046$ $p = 0.613$	$r = 0.563$ $p = <0.001$	$r = 0.480$ $p = <0.001$	$r = 0.305$ $p = 0.001$	$r = 0.213$ $p = 0.017$	$r = -0.269$ $p = 0.002$
		100 ng/mL	$r = -0.400$ $p = <0.001$	$r = -0.211$ $p = 0.026$	$r = -0.186$ $p = 0.051$	$r = 0.077$ $p = 0.399$	$r = 0.491$ $p = <0.001$	$r = 0.468$ $p = <0.001$	$r = 0.242$ $p = 0.007$	$r = 0.153$ $p = 0.089$	$r = -0.342$ $p = <0.001$
		1000 ng/mL	$r = -0.381$ $p = <0.001$	$r = -0.288$ $p = 0.002$	$r = -0.250$ $p = 0.008$	$r = 0.068$ $p = 0.458$	$r = 0.498$ $p = <0.001$	$r = 0.493$ $p = <0.001$	$r = 0.390$ $p = <0.001$	$r = 0.318$ $p = <0.001$	$r = -0.174$ $p = 0.052$
		TNF-α_{max}	$r = -0.363$ $p = <0.001$	$r = -0.271$ $p = 0.004$	$r = -0.225$ $p = 0.018$	$r = 0.066$ $p = 0.471$	$r = 0.478$ $p = <0.001$	$r = 0.482$ $p = <0.001$	$r = 0.360$ $p = <0.001$	$r = 0.287$ $p = 0.001$	$r = -0.208$ $p = 0.020$
		$EC_{50[LPS]}$	$r = -0.153$ $p = 0.094$	$r = 0.057$ $p = 0.551$	$r = 0.078$ $p = 0.414$	$r = 0.014$ $p = 0.875$	$r = -0.387$ $p = <0.001$	$r = -0.239$ $p = 0.007$	$r = -0.122$ $p = 0.123$	$r = -0.055$ $p = 0.540$	$r = 0.203$ $p = 0.023$

was substantiated by the observation of a positive correlation between TNF-α concentration at time-point 3 and IL-10 production at time-point 4 (i.e. on average 2 h later, $r = 0.258$; $p = 0.007$), indicating some contribution of the TNF-α release magnitude on the subsequent IL-10 production.

3. Discussion

The main findings of the present study are that the circulating IL-10 concentrations following cardiopulmonary bypass in patients undergoing cardio-thoracic surgery correlate positively with the intensity of endotoxemia during the reperfusion phase upon aortic declamping. Patients carrying the AGCC allele had slightly higher post-operative IL-10 levels as compared to carriers of all other haplotypes combined. Homozygous carriers of the GATA allele had lower post-operative IL-10 levels as compared to all other patients. Furthermore, AGCC allele carriers had lower IL-10 EC_{50} values (i.e. higher LPS sensitivity ex vivo) as compared to patients carrying the other alleles, whereas carriers of the GATA allele showed the opposite (i.e. higher EC_{50} values; lower LPS sensitivity). For both the AGCC and the GATA allele carriers, the maximal induced IL-10 production upon ex vivo LPS stimulation was similar as compared to the other groups. The in vivo IL-10 release during reperfusion (i.e. at aorta declamping and 30 min into reperfusion) in patients experiencing endotoxemia was significantly correlated with the IL-10 production ex vivo upon stimulation with 1000 ng/mL and the estimated maximal IL-10 production capacity (the $IL\text{-}10_{max}$), no correlation was found between the IL-10 EC_{50} ex vivo with in vivo IL-10 levels.

The model we choose, i.e. measuring naturally occurring endotoxemia and subsequent cytokine release in vivo in patients undergoing cardio-thoracic surgery, enables us to study the inter-individual differences in these responses and relate these to genetic factors. In a previous publication we described the TNF-α response in the same way. We found significant endotoxemia in over half of the patients undergoing cardiac surgery with cardiopulmonary bypass which is in accordance with previous studies [18,19]. At the peak of endotoxemia, upon aortic declamping and 30 min into reperfusion, we observed a significant positive correlation between circulating endotoxin levels and IL-10 production, indicating a role of endotoxin in the perioperative release of IL-10. However, although we found a significant correlation, it's magnitude is limited, suggesting that many other factors apart from endotoxin play a role in the variation of IL-10 production in these patients.

Since we used 3 different concentrations of LPS in our ex vivo whole blood assay we were able to calculate descriptive parameters of the dose–response relation for each patient. Also, to estimate spread relative to the mean, the coefficients of variation (standard deviation [SD] divided by the mean) were calculated for all LPS concentrations. The values were 50.4%, 46.4% and 94.8% for 10, 100 and 1000 ng/mL LPS, respectively. This variation is similar to the variation found in other populations and exceeds the variation that can be explained by the laboratory variation only, which is estimated at 9.9–12.3% [4]. This indicates that the variations we found between subjects are to be explained by other factors than laboratory variation only. Since we find it feasible to assume that the LPS concentration needed for the release of 50% of the maximally evoked IL-10, the EC_{50}, is a marker of LPS sensitivity we evaluated to what extent this parameter could presage the in vivo response to endotoxin. Theoretically, important inter-individual differences in the dose–response characteristics of IL-10 production upon LPS stimulation can be overlooked by measuring IL-10 production upon stimulation with only one single (supra-physiological) LPS concentration.

We found that the level of ex vivo IL-10 production upon stimulation with 1000 ng LPS/mL was significantly correlated with the in vivo production upon significant endotoxemia. Thus, the ex vivo LPS stimulation assay is a predictor of the in vivo release of IL-10 during endotoxemia after cardiac surgery. Remarkably, however, the correlation is inverse; high ex vivo IL-10 production capacity is associated with low in vivo circulating IL-10 levels. The explanation of this finding is puzzling. To our knowledge no other study investigated the correlation between endotoxin mediated IL-10 production ex vivo and in vivo. With respect to the validity of the comparison of the ex vivo and in vivo IL-10 production, and the conclusions drawn, several factors should be taken into account. In our analysis we correlated IL-10 concentrations in vivo with in vitro production capacity. In these analyses the time factor is rather artificially ignored to a certain extent. In vivo the time between exposure to the stimulus (endotoxin) and measurement of the cytokine was several hours at the maximum. In vitro, however, we measured the IL-10 concentration at 24 h after adding LPS. In a previous study it was demonstrated that after 18 h of ex vivo stimulation the IL-10 mRNA levels reached the highest level making measurement of IL-10 after 24 h as indicator of maximal IL-10 production capacity logical [7]. Day-to-day intra-individual variation of IL-10 concentrations after 24 h of stimulation was low (about 10%). Giving the wide range of IL-10 production levels between individuals this measurement seems a good basis to demonstrate genetically defined variation [6]. We measured the ex vivo IL-10 production in a blood sample drawn several hours before the in vivo production capacity was measured, probably making the variation

due to day-to-day variation even smaller. Although in all patients a standardized anesthesia protocol was followed, differences in the use of certain medical devices and medications might have influenced our results. Also, not all patients experienced the same amount of endotoxin stimulus in vivo. Although most patients had significant endotoxemia at the later time-points, some did not. To be able to study the relationship between endotoxin and IL-10 release we specifically studied their relation in patients with significant endotoxemia. We found even stronger correlations in the patients experiencing endotoxemia, indicating the relevance of endotoxin as stimulus in the in vivo IL-10 production. Furthermore, for the in vitro stimulation assay we used crude *Escherichia coli* LPS rather than purified lipid A, the ligand for TLR4. Recent studies indicate that these preparations can be contaminated with relevant traces of other bacterial products, i.e. lipoproteins, rather than lipid A, that signal through other receptors deriving from the TLR family, especially TLR2 [20,21]. This might have complicated our conclusions on the role of the TLR4 genotype. However, our in vivo model reflects a physiological exposure to naturally occurring endotoxemia. Also, in Gram-negative bacterial infection it is likely that the innate immune system is confronted with a mixture of endotoxins. One study reported post-operative down-regulation of TLR2 and TLR4 receptors on PBMC's after abdominal surgery [22]. No data exist on TLR receptor down-regulation in cardio-thoracic surgery with extracorporal bypass.

Since several studies indicated a role of genetics in cytokine release we investigated the influence of the various known polymorphism in the IL-10 promoter. The frequencies of the various genotypes were similar to those found in other populations (Table 1) [9,10,13,23–28]. We found that the carriers of the GATA haplotype had lower LPS sensitivity and IL-10 production ex vivo, furthermore, homozygous GATA carriers also had lower IL-10 production in vivo. On the other hand, carriers of the AGCC haplotype had higher sensitivity to LPS. In a study of twins by Reuss et al. the AGCC haplotype was associated with higher (around 20%) transcriptional activity in a reporter gene assay using luciferase as reporter [23]. The study emphasizes the importance of the SNP at position −1082 in the transcriptional activity of the proximal promoter. It is suggested that an A at position −1082 confers optimal binding affinity for the transcriptional factor PU.1 that inhibits gene expression, resulting in an increased transcriptional activity when a G is present at position −1082. However, in the study by Reuss et al. levels of produced IL-10 upon whole blood stimulation using 100 ng/mL LPS were not different between the various haplotypes [23]. An explanation for this finding could be that the LPS concentration used in their assay was too high to pick up the differences we found at the lower LPS concentrations. As mentioned before, we found no differences in IL-10 levels using the highest LPS concentration (1000 ng/mL). The phenotype of increased LPS sensitivity we found ex vivo in AGCC haplotype carriers, is further supported by the significantly higher circulating IL-10 levels following cardiopulmonary bypass in the AGCC haplotype carriers as compared to carriers of the other haplotypes combined. At this time-point the endotoxin levels peaked in most patients. In a study conducted by Suárez et al. constitutive IL-10 levels were measured in peripheral blood of healthy volunteers and were correlated to SNPs in the proximal promoter region. In that study, the GCC genotype was found significantly more often in the group with high IL-10 levels ($\geq$ 2 pg/mL), again indicating the importance of a G at position −1082 [24]. Constitutive IL-10 gene expression (measured by IL-10 mRNA detection from whole blood samples) was higher in subjects carrying the GCC/GCC genotype as compared to the non-GCC carrying genotypes [24]. In a study performed by Fijen et al. no differences were found in IL-10 production between the −1082G/A genotypes in an experimental human endotoxemia model in healthy subjects [29]. However, looking more closely to their findings in a rather small group of healthy subjects, a trend is visible towards higher IL-10 levels in carriers of a G at position −1082. No other study investigated the role of IL-10 polymorphisms in endotoxemia mediated IL-10 production capacity in vivo.

The common TLR4 polymorphisms (Asp299Gly and Thr399Ile) are associated with slightly higher IL-10 capacity ex vivo, however, this did not reach the level of statistical significance. The frequencies of the various genotypes were similar to those found in other populations (Table 1) [30–34]. To our knowledge no other study investigated the correlation between TLR4 polymorphisms and the ex vivo or the in vivo endotoxin mediated IL-10 production. The common TLR4 polymorphisms (Asp299Gly and Thr399Ile) are not associated with differences in IL-10 production capacity ex vivo and in vivo.

Although a type II error could be responsible for overlooking differences in IL-10 production capacity between the other IL-10 promoter and the TLR4 polymorphisms studied, our data suggest that their role, if present, is limited, and cannot explain the large inter-individual differences found in IL-10 capacity, which was recently estimated at around 50% [23]. The demographic characteristics of the cardiac surgery patients were similar to other groups of patients described in the literature [35,36], making direct extrapolation of our findings possible.

In summary, based on our results and the findings in the literature, we propose that it is likely that the IL-10

promoter polymorphism at position −1082 base pairs relative to the transcriptional start site plays a functional role in LPS mediated IL-10 production capacity in vitro and in vivo. The common TLR4 polymorphisms do not interact with factors determining IL-10 production capacity in vivo and ex vivo. Although part of the large inter-individual variation found in the in vivo and ex vivo responses to LPS can be explained by known polymorphisms in the IL-10 promoter region, overall their effects are limited and can only explain about 5−10% of the variation. Further studies are needed to determine to what extent the remaining inter-individual variation found in the IL-10 response to LPS is determined by genetic or random, environmental factors.

4. Materials and methods

4.1. Patients

The study was performed at the Leiden University Medical Center, an 800-bed secondary and tertiary referral hospital. To be eligible for enrolment, the patients had to be aged 18 years or older and being scheduled for elective cardiac surgery with cardiopulmonary bypass between July 1, 1998, and December 30, 1999. We obtained institutional approval from the local medical ethics committee (protocol #P168/96). Each patient gave a written, informed consent. The patients studied were 159 consecutive patients undergoing elective cardiac surgery with cardiopulmonary bypass. Of these, 122 had been included in a previously published study on the effect of selective gut decontamination (SGD) on endotoxemia and cytokine activation [37]. Of these 122 patients 24 received preoperative SGD consisting of polymyxin B and neomycin. In the previously mentioned study, perioperative endotoxemia and subsequent cytokine activation were found not to be influenced by the SGD regimen. Also, the results were not different when these patients were excluded from the analysis. Patients were followed-up throughout their stay in the hospital. Demographic characteristics were systematically collected for all the patients entering the study.

4.2. Study design

On the day of surgery blood samples for ex vivo whole blood LPS stimulation, using pyrogen-free tubes (Kabi-Vitrum, Amsterdam, The Netherlands), and DNA extraction were drawn before anesthesia. Blood samples for the determination of in vivo endotoxin and cytokine levels were collected from each patient before anesthetic induction, on aorta declamping, 30 min into body reperfusion (i.e. 30 min after stopping the extra corporal perfusion), and at the ICU admission (approximately 2 h after surgery). In a previous pilot study, we found that endotoxemia occurs most likely at these time-points [37].

4.3. Whole blood LPS stimulation

Cytokine production was determined in whole blood samples ex vivo as previously described [38]. Briefly, whole blood samples were mixed 1:1 with RPMI 1640 (Gibco, Germany) and lipopolysaccharide (*E. coli* 0111:B4, Difco, Detroit, MI) was added to final concentrations of 10, 100 and 1000 ng/mL and cells were stimulated for 24 h at 37 °C under 5% CO_2 atmosphere. Interleukin-10 concentrations were measured in the supernatant as described below.

4.4. Endotoxin measurements

Blood for endotoxin determination was collected in pyrogen-free tubes (Kabi-Vitrum, Amsterdam, The Netherlands) and the platelet rich plasma was prepared by centrifugation. Endotoxin was determined by a quantitative photometric assay with end-point measurement as described elsewhere [39]. The assay's lower detection limit for endotoxin was about 3.0 pg/mL [39].

4.5. IL-10 measurements

Blood for determination of IL-10 was collected in pyrogen-free ethylenediaminetetraacetic acid (EDTA) tubes (Chromogenics, Amsterdam, Netherlands) and immersed in ice. Plasma was prepared by centrifugation at 3000*g* for 5−10 min at 4 °C and stored at −70 °C. Interleukin-10 concentrations in the supernatants of the ex vivo whole blood assay and the samples from the in vivo IL-10 production were analysed at completion of the study. Interleukin-10 concentrations were determined with a standard ELISA technique (Central Laboratories for Bloodtransfusion, Amsterdam, The Netherlands; Medgenix diagnostics, Floury, Belgium); the lower detection limit for IL-10 was 4.0 pg/mL.

4.6. DNA isolation

DNA was isolated from citrate blood from each patient, red blood cells were lysed with three volumes of lysis buffer (1.55 M NH_4Cl, 0.1 M $KHCO_3$, 0.01 M EDTA, pH 7.4); the remaining cells were incubated for 17 h at 37 °C with SDS and proteinase K. The DNA was extracted using phenol and chloroform as described by Maniatis et al. [40].

Table 5
Sequence and specificity of biotin labeled sequence-specific oligonucleotides and critical washing temperature (CWT) in °C

Polymorphism	Primer sequence (5′ → 3′)	CWT	Specificity
−1330	ACCTAGGTCAATGTTCCTCC	52	G
	GGAGGAACACTGACCTAGGT	54	A
−1082	TTCTTTGGGAGGGGGAAG	51	G
	ACTTCCCCTTCCCAAAGAA	52	A
−819	CAGGTGATGTAACATCTCTGTGC	62	C
	GCACAGAGATATTACATCACCTGT	63	T
−592	CCGCCTGTCCTGTAGGAA	50	C
	TTCCTACAGTACAGGCGGG	52	A

4.7. Typing of the proximal IL-10 promoter polymorphism

The polymorphic regions at positions −1330, −1082, −819 and −592 were evaluated by PCR amplification using the following primers: 5′-CCCTGACTATA-GAGTGGCAG-3′ (forward) and 5′-GTGCTGAGC-TGTGCATGCCT-3′ (reverse). The single nucleotide polymorphisms were determined using Allele Specific Hybridization (ASH-blot) as previously described [10]. Briefly, the PCR products were dot blotted on the membranes (Hybond−N^+, Amersham, Buckinghamshire, UK) and hybridized overnight at 42 °C with the specific biotin labeled oligoprimer. After hybridization the blot was washed with ASH-buffer at the critical temperature. Sequences of the sequence-specific oligoprimers and their respective critical washing temperatures are given in Table 5.

4.8. Typing of distal IL-10 promoter polymorphisms

Typing for the IL-10 single nucleotide polymorphisms at positions −3575, −2849 and −2763 was performed by PCR amplification. Forward and reverse primers used are given in Table 6 [28]. For the positions −2849 and −2763 a second, nested-PCR was performed using P2BR as reverse primer (Table 6). Primers were purchased from Biosource International (Foster City, CA). PCR amplifications were performed using the Thermolyne Amplitron II Thermal Cycler (Barnstead/Thermolyne, Dubuque, IA). PCR-amplified products were electrophoresed on a 10% polyacrylamide gel to detect the size differences of the fragments between the polymorphisms, following digestion with a specific restriction enzyme (Table 6).

4.9. TLR4 mutation analysis

The presence of the TLR4 Asp299Gly and Thr399Ile mutations was determined as previously described [17].

4.10. Calculation of ex vivo LPS/IL-10 dose−response

For each patient two characteristics (i.e. EC_{50} and E_{max}) of the LPS-induced IL-10 release were calculated by non-linear regression with the dose−response model according to the Hill equation (Eq. (1)).

$$E_N = E_{N,max} \times C/(EC_{50} + C) \quad (1)$$

where E_N is the observed IL-10 production at a given LPS concentration C, EC_{50} is the estimated LPS concentration at which 50% of the maximal IL-10 response is reached, and $E_{N,max}$ is the estimated maximal IL-10 production. In a previous pilot study we found that measuring the IL-10 production at the LPS concentration we used (i.e. 10, 100 and 1000 ng/mL) was sufficient to calculate the dose−response characteristics EC_{50} and $E_{N,max}$ (E.S., unpublished observation, report in preparation).

4.11. Statistical analysis

IL-10 concentrations in vivo and ex vivo in different haplotype groups and genotypes were not distributed

Table 6
Primers sequences and restriction enzymes used for detection of the polymorphisms in the IL-10 gene promoter and size of the PCR products and restriction fragments

Polymorphism	Primer sequence (5′ → 3′)	PCR product (bps)	Enzyme	Size	Allele
−3575	GGTTTTCCTTCATTTGCAGC	231	*Bam*HI	121 + 110	TT
	ACACTGTGAGCTTCTTGAGG			231	AA
−2849	CTGTAATCTCAGCACTCTGG	193	*Alw*I	153 + 40	GG
	ACCAAGTCTGGCCCTTGGAA/GTTCAAGCCATTCTCCTGCC			193	AA
−2763	CTGTAATCTCAGCACTCTGG	193	*Dde*I	114 + 71 + 8	CC
	ACCAAGTCTGGCCCTTGGAA/GTTCAAGCCATTCTCCTGCC			185 + 8	AA

normally, and so non-parametric testing has been used throughout. IL-10 concentrations are quoted as the median with interquartile ranges between parentheses. For statistical analysis blood samples with IL-10 and endotoxin levels below the limit of detection were entered as half of the value of the lower detection limit (2.0 and 1.5 pg/mL, respectively). To determine whether polymorphisms in the IL-10 promoter were associated with levels of in vivo IL-10 production, repeated measurements models (SPPS 11.0, mixed) were used to determine whether the patterns of IL-10 production over time were different between polymorphisms. For this analysis log-transformed IL-10 and endotoxin levels were used. The ex vivo and in vivo IL-10 production characteristics between the various haplotypes and genotypes were compared using Kruskal–Wallis test, individual groups were compared by the Mann–Whitney *U* test. Correlations were assessed non-parametrically using Spearman correlation test. Statistical significance was tested two-tailed, with the α set to 0.05.

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CHAPTER 7

Cytokine response to endotoxin in individuals heterozygous for the Delta32 mutation of chemokine receptor CCR5

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Cytokine response to endotoxin in individuals heterozygous for the Δ32 mutation of chemokine receptor CCR5

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Abstract

Studies of mice with a targeted disruption of the CCR5 gene suggest that the CC chemokine receptor 5 (CCR5) is a determinant of the cytokine response to endotoxin. In humans, a naturally occurring mutation of the CCR5 gene is a 32-basepair (bp) deletion which precludes the translation of the gene into a functional transmembrane protein. To evaluate the cytokine phenotype of heterozygosity for the 32 deletion, we studied the endotoxin-stimulated release of tumor necrosis factor-α, Interleukin (IL)-6, IL-8, IL-10, and IL-12 in whole blood ex-vivo of healthy volunteers and patients undergoing elective cardiac bypass surgery. This operation represents a major surgical trauma associated with ischemia-reperfusion-injury and triggers a profound inflammatory response. In these patients, cytokine plasma concentrations were measured during and after cardiac surgery. No difference was found between the frequencies of the observed and expected CCR5 genotypes in the groups of individuals studied. Furthermore, no significant difference in ex-vivo or peri- and postoperative cytokine plasma concentrations was detected between CCR5 wild-type homozygotes and individuals carrying one defective CCR5 allele. Our results indicate that heterozygosity for the 32 bp deletion of CCR5 is not associated with an altered cytokine response to endotoxin or to a major surgical trauma when compared with individuals homozygous for the wild-type allele.

Keywords: CC chemokine receptor 5; Cytokines; Endotoxin; Genetic polymorphism

1. Introduction

The seven-transmembrane spanning G protein-coupled receptor CCR5 binds the CC chemokines macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and RANTES [1]. It is expressed on monocytes as well as CD45RO+ (memory) T cells [1,2]. Particular interest in this molecule comes from its role as an HIV co-receptor required for the infection of CD4 positive cells with macrophage-tropic HIV isolates [3]. A genetic variant of this molecule exists consisting of a 32-nucleotide deletion (Δ32) in the region encoding the second external loop [4]. This deletion causes a frame shift and the introduction of a premature stop codon. The protein encoded by this deleted allele is not translated [4]. Homozygosity for this defect was reported as a mechanism of resistance to HIV infection in multiply exposed individuals [4,5]. Heterozygosity for the Δ32 mutation is associated with a slower progression to AIDS and a longer disease-free survival compared with wild-type HIV infected individuals [5,6]. The frequency of the mutated allele is 10% in Caucasians [7]. Carriers of the Δ32 allele are healthy and, so far, under normal conditions no phenotype could be attributed to the heterozygous or homozygous genotype. This suggests that CCR5 function may be dispensable under normal conditions.

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Studies in mice with a targeted disruption of this molecule presented evidence for a role of CCR5 in host defense against infection [8]. After intraperitoneal challenge with different concentrations of endotoxin CCR5 deficient mice had a higher survival rate than CCR5 wild types. Also, the LD_{50} value of endotoxin was significantly lower in wild-type compared with the knock-out mice (18.15 vs. 34.05 mg endotoxin/kg animal body weight). Moreover, peritoneal macrophages of CCR5 knock-out animals differed from their wild-type littermates with regard to the cytokine production. Macrophages of mice lacking CCR5 display reduced endotoxin-stimulated production of Interleukin (IL)-1β and IL-6. No difference in the release of tumor necrosis factor (TNF)-α was found between knock-out and wild-type mice [8]. Since these results of animal studies relate CCR5 expression to cytokine release, we hypothesized that heterozygosity for the Δ32 allele might be associated with differences in the cytokine response to endotoxin in humans as well. We studied healthy human volunteers and patients scheduled for elective cardiac surgery and evaluated the ex-vivo cytokine release after incubation of whole blood with endotoxin. In addition, peri- and postoperative cytokine plasma concentrations were determined in the patients undergoing cardiac surgery.

2. Results

Ninety blood donors and 122 cardiac surgery patients were genotyped for the CCR5 polymorphism. In both groups the frequencies of the wild-type (CCR5) allele and of the mutated Δ32 allele were found to be 93 and 7%, respectively. Thus, the genotype frequencies were 87% for CCR5/CCR5 and 13% for the heterozygous genotype (CCR5/Δ32). Statistical analysis did not reveal a difference between the frequencies of the observed genotypes and those of the expected genotypes, indicating that both study groups were in Hardy–Weinberg equilibrium.

The concentrations of the cytokines TNF-α, IL-1β, IL-6, and IL-8 obtained after stimulating whole blood of blood donors with 100 ng/ml endotoxin for 4 h are given in Table 1. Overall, no significant difference was found in the cytokine release of individuals homozygous for the CCR5 allele or CCR5/Δ32 heterozygotes.

Tables 2 and 3 summarize the data obtained from patients scheduled for elective cardiac surgery. In the whole blood samples taken before surgery and stimulated ex-vivo with various concentrations of endotoxin, a dose-dependent increase in TNF-α, IL-10, and IL-12 release was observed that did not differ between patients carrying either one of the CCR5 genotypes (Table 2). Furthermore, the concentrations of TNF-α, IL-10, and IL-6 in the blood of these patients during and after cardiac surgery (Table 3) did not reveal a significant difference between the CCR5/CCR5 and CCR5/Δ32 genotype, neither on release of the aortic cross-clamp nor on arrival at the intensive care unit (ICU) 2 h after completion of surgery.

Table 1
Whole blood cytokine response of healthy volunteers to endotoxin according to CCR5 genotypes

	CCR5/CCR5 ($n = 78$)	CCR5/Δ32 ($n = 12$)	p Values
TNF-α	2198 (1684–3709)	3278 (2237–3872)	0.345
IL-1β	381 (247–737)	280 (175–725)	0.544
IL-6	2876 (2329–4634)	3586 (2944–4368)	0.717
IL-8	1452 (833–2186)	1025 (582–2670)	0.430

Cytokine concentrations are ng/ml. Values are given as median with interquartile ranges (25th–75th percentile) in parentheses. Whole blood was stimulated with 100 ng/ml endotoxin (*E. coli* O5 : B55) for 4 h at 37 °C. Cytokine plasma concentrations were determined by measuring immunoreactivity by means of chemiluminescence.

3. Discussion

The main finding of the present study is that in humans the endotoxin-stimulated cytokine release did not differ between individuals homozygous for the wild-type CCR5 allele and persons heterozygous for CCR5 Δ32. This conclusion is based on two observations. Firstly, using an established and validated whole blood system to assess the release of TNF-α, IL-1β, IL-6, IL-8, and IL-12 after 4 h incubation with endotoxin, and IL-10 after 24 h stimulation, the cytokine release was not affected by CCR5 genotype. This assay avoids confounding factors including adherence-induced expression of cytokines that can be associated with isolation of certain cell populations such as monocytes. In addition, whole blood probably represents the most physiological environment for cytokine-producing cells. In a second approach, the cytokine release during and after surgery was determined in patients undergoing cardiac surgery. Open heart surgery with cardiopulmonary bypass triggers the release of pro- and anti-inflammatory cytokines into the systemic circulation [9,10]. Also in the peri- and postoperative blood samples, the cytokine concentrations did not differ according to the CCR5 genotype of the patients.

As a response to cardiac surgery plasma concentrations of TNF-α and IL-6 increased whereas IL-10 levels decreased. This finding is in agreement with previous reports [11–13]. The sources of plasma TNF-α are monocytes/macrophages and the myocardium [14]. IL-6 is produced by a variety of cells including monocytes, fibroblasts, and hepatocytes [15]. Wan et al. [16] identified the liver as the primary source of IL-10 in the perioperative phase of cardiac surgery.

In previous approaches to relate a phenotype to the Δ32 mutation, the concentrations of the natural ligands

Table 2
Ex-vivo cytokine release after endotoxin-stimulation of whole blood of patients before elective cardiac bypass surgery

10 ng/ml endotoxin		100 ng/ml endotoxin		1000 ng/ml endotoxin	
CCR5/CCR5	CCR5/Δ32	CCR5/CCR5	CCR5/Δ32	CCR5/CCR5	CCR5/Δ32
TNF-α 9323 (6930–12277)	8380 (7031–15203)	12589 (9034–17009)	13559 (8617–22320)	16975 (13408–22127)	18097 (12848–29217)
IL-10 798 (517–1128)	699 (409–1198)	1492 (1006–1962)	1397 (816–1985)	3475 (2543–4510)	3269 (2041–5070)
IL-12 1.0 (0.0–5.0)	3.5 (1.5–8.8)	2.0 (0.0–7.8)	5.5 (1.3–11.3)	7.0 (2.0–18.0)	12.0 (5.0–28.0)

Cytokine concentrations are in ng/ml. Values represent median with interquartile range (25th–75th percentile) in parentheses.
All *p*-values for differences between homozygous and heterozygous genotype > 0.40 (TNF-α $n = 106$ and 16; IL-10 $n = 101$ and 16; IL-12 $n = 102$ and 16).
Whole blood was incubated with endotoxin (*E. coli* 0111:B4) for 4 h (TNF-α, IL-12) or 24 h (IL-10) at 37 °C. Cytokine plasma concentrations were determined by ELISA.

of this receptor, the chemokines MIP-1α, MIP-1β, and RANTES were studied. Higher concentrations of these chemokines were reported in two individuals homozygous for the Δ32 mutation compared with wild-type homozygotes [17]. Yang et al. [18] studied chemokine concentrations in individuals homozygous for the wild-type and in Δ32 heterozygotes. This group was unable to find a difference in the chemokine synthesis after endotoxin stimulation.

In contrast, animal studies related the lack of the CCR5 molecule to the cytokine production. Mice with a targeted disruption of CCR5 showed a 50% reduction in the levels of IL-1β and IL-6 after endotoxin stimulation of peritoneal macrophages whereas no difference was observed for TNF-α [8]. We therefore hypothesized that the cytokine response may characterize the CCR5/Δ32 genotype. The Δ32 allele is a mutation introducing a premature stop codon and the deleted allele is not translated [4]. Carriers of the genotype CCR5/Δ32 thus lack one functional allele. One would expect that individuals carrying one Δ32 allele which does not code for an intact protein have about half the number of CCR5 molecules on the cell surface. However, the amount of CCR5 surface expression showed a 20-fold variation among individuals with two intact CCR5 alleles, using an MIP-1β binding assay or anti-CCR5 monoclonal antibodies [19,20]. The reasons for this high interindividual variability in wild-type homozygotes remain unclear. It cannot be excluded that genetic factors other than the Δ32 mutation determine the cellular density of CCR5. This high variance may by far outnumber the role of the defective CCR5 allele.

Among the reasons for the lack of association between cytokine release and CCR5 genotype, genetic polymorphisms of the cytokine genes have to be mentioned. Such genetic variations have been identified for all cytokines determined in our study [21–24]. These polymorphisms were shown to influence the synthesis of the respective cytokine. For instance, the TNF-α gene has a polymorphism at position −308 of the promoter region which regulates gene transcription [21]. The promoter region of the IL-1β gene bears two polymorphic sites, −31 and −511 [22]. The −31 polymorphism affects DNA binding and is in a linkage disequilibrium with the −511 site. Similarly, the promoter of the IL-6 gene was also found to carry a polymorphism consisting of a G/C substitution at position −174 [23]. In a reporter gene assay, the −174C oligonucleotide had a lower expression than the −174G construct suggesting a higher IL-6 synthesis by the G allele. Polymorphisms were also found in the IL-10 gene and associations with gene transcription were reported [24]. The study by Zhou et al. [8] suggested that in mice the concentrations of IL-1β and IL-6 are under the influence of the CCR5 polymorphism. It is not known

Table 3
Perioperative and postoperative plasma concentrations of various cytokines in patients undergoing elective cardiac bypass surgery

	Upon release of aorta cross-clamp		Upon arrival at ICU	
	CCR5/CCR5	CCR5/Δ32	CCR5/CCR5	CCR5/Δ32
TNF-α	2.4 (0.6–4.3)	2.7 (0.2–5.9)	4.3 (2.3–7.5)	5.1 (3.1–7.5)
IL-10	32 (11–81)	39 (6–207)	9 (4–26)	5 (0–17)
IL-6	39 (20–64)	35 (13–67)	300 (103–750)	146 (103–198)

Cytokine concentrations are in ng/ml. Values represent median with interquartile range (25th–75th percentile) in parentheses.
All *p*-values for differences between homozygous and heterozygous genotype > 0.40, except for IL-6 values upon arrival at ICU, $p = 0.068$.
Data on homozygous and heterozygous genotype available for TNF-α $n = 86$ and 16; IL-10 $n = 110$ and 21; IL-6 $n = 57$ and 9. Plasma cytokine levels were determined by ELISA.

whether the above-mentioned IL-1β and IL-6 polymorphisms which were described in humans also exist in mice. In humans, these cytokine genetic variations might outweigh the effect of a defective CCR5 molecule.

In conclusion, our study suggests that heterozygosity for the Δ32 mutation of CCR5 is not associated with a significant phenotype affecting the cytokine response to an infectious or inflammatory stimulus when compared with that in individuals homozygous for the wild-type allele.

4. Materials and methods

4.1. Subjects and cytokine measurements

Written informed consent from each individual and approval of the local medical ethics committee were obtained. Two groups of subjects were studied. The first group consisted of Caucasian healthy blood donors (aged 18–65 years). Twenty milliliters of heparin-anticoagulated blood was drawn and mixed 1 : 1 with RPMI 1640 (Gibco BRL, Karlsruhe, Germany) and stimulated with 100 ng/ml endotoxin (*Escherichia coli* O5 : B55, Sigma, St. Louis, MO) for 4 h at 37 °C. The plasma concentrations of TNF-α, IL-1β, IL-6, and IL-8 were determined by measuring immunoreactivity by means of chemiluminescence (Immulite, DPC Biermann, Bad Nauheim, Germany).

The second group were Caucasian patients undergoing elective cardiac surgery with cardiopulmonary bypass. On the morning just before surgery blood samples for the determination of ex vivo cytokine production were collected using heparin-anticoagulated tubes. Cytokine production was determined in whole-blood samples ex vivo as previously described [25]. Lipopolysaccharide (*E. coli* 0111 : B4, Difco, Detroit, MI) was added to final concentrations of 10, 100, and 1000 ng/ml and cells were stimulated for 4 or 24 h at 37 °C. The operative procedures including premedication and anesthesia are described elsewhere [9]. During operation, blood samples were taken immediately upon release of the aorta clamp and upon arrival at the ICU 2 h after completion of surgery. Blood samples were collected in pyrogen-free ethylenediaminetetraacetic acid (EDTA) tubes and immersed in ice. Plasma was prepared by centrifugation at $3000 \times g$ for 5–10 min at 4 °C and stored at −70 °C. TNF-α, IL-10, IL-12, and IL-6 concentrations were determined with an ELISA technique (Central Laboratories for Bloodtransfusion, Amsterdam, The Netherlands and Medgenix diagnostics, Floury, Belgium) [26].

4.2. Genotyping for the CCR5 Δ32 polymorphism

Genomic DNA was isolated from peripheral blood cells using the QIAmp Blood kit (QIAgen, Hilden, Germany). Twenty nanograms of DNA was used in a 50 μl polymerase chain reaction (PCR) containing 1.5 mM $MgCl_2$ and 20 pmol of the forward primer 5′-ATCTCAAAAAGAAGGTCTTCATTAC-3′ and the reverse primer 5′-GAGTAGCAGATGACCATGACAA-3′, as described previously [5]. PCR conditions were as follows: 94 °C for 5 min followed by 35 cycles with 94 °C for 20 s, 55 °C for 30 s, 72 °C for 20 s, and a final elongation step (72 °C for 5 min). The PCR product was visualized on a 2% agarose gel. Based on the band size an allele was classified as wild-type (187 bp) or Δ32 (155 bp).

4.3. Statistical analysis

Data analysis was performed with the SPSS for Windows Release 10.0.7 program. The 2 × 2 table chi-square test was used to test for the Hardy–Weinberg equilibrium by comparing the expected with the observed genotype frequencies. Cytokine concentrations are given as median and interquartile range (25th–75th percentile). The Wilcox Mann–Whitney-test served for the comparison of cytokine data between the two CCR5 genotypes. Statistical significance was set at *p*-levels <0.05.

Acknowledgements

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CHAPTER 8

Polymorphisms in the TNF-α and IL-10 promoter region, TLR-4 and the in vivo and in vitro response to LPS: relevance on the outcome of patients undergoing elective cardiac surgery

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Submitted

Abstract

Background: Cardiopulmonary bypass surgery (CPB) may serve as a model for studying the host inflammatory response to bacterial products that appear in the systematic circulation as a result of ischaemia-reperfusion damage. In a minority of patients the inflammatory response eventually progresses into organ dysfunction and death.

Objective: To relate perioperative endotoxemia, cytokine release and polymorphisms in the IL-10 and TNF-α promoter and the coding regions of TLR4 to clinical outcome parameters in patients undergoing elective cardiac surgery involving CPB.

Design: Prospective, observational, clinical study, with systematic collection of postoperative data.

Setting: Tertiary-care university teaching hospital.

Patients: One hundred fifty-nine patients (66 % males [n= 105], median age 65 and 67 years for males and females, respectively).

Measurement and Main Results: High perioperative endotoxin and IL-10 concentrations are independently associated with postoperative hemodynamic instability and pulmonary dysfunction. Interleukin-10 promoter polymorphism genotypes associated with high IL-10 production (the presence of the AGCC allele and/or the absence of the GATA allele), helped explain some of the differences in clinical outcome parameters. Of note, perioperative TNF-α concentrations, TNF-α and TLR4 polymorphisms did not contribute to postoperative outcome.

Conclusion: High postoperative endotoxin and IL-10 concentrations, independent of TNF-α, are associated with hemodynamic instability and pulmonary dysfunction. These findings suggest that IL-10 may cause cardio-pulmonary dysfunction, largely independent of the ongoing pro-inflammatory response.

Introduction

Cardiac surgery with cardiopulmonary bypass (CPB) induces a variable systemic inflammatory response that may progress from a relatively mild to a severe and a potentially life threatening situation. The response is accompanied by complement activation, release of cytokines, leukocyte activation along with expression of adhesion molecules, and the production of substances such as oxygen-free radicals, arachidonic acid metabolites, platelet-activating factor (PAF), nitric oxide (NO), and endothelins. In severe cases the inflammatory response contributes to increased cardiac output in the presence of reduced systemic vascular resistance (SVR) and high oxygen consumption, generally referred to as 'hyperdynamic instability' or postperfusion (or "post-pump") syndrome, and requires fluid replacement and treatment with vasoactive agents (1-4). Ultimately, the derangement of hemodynamic variables may be complicated by lactic acidosis, impaired organ perfusion, and pulmonary dysfunction, which cause an extended intensive care unit stay and even fatal outcome.

Among other factors (e.g., exposure of blood to artificial surfaces, complement activation, surgical trauma) bacterial endotoxin, derived from the gut, is regarded as an important precipitating factor of the inflammatory response (5-13). Splanchnic ischemia and gut reperfusion injury occurs frequently during and after cardiopulmonary bypass with consequent disturbance of gut barrier function marked by translocation of endotoxin to the systemic circulation (14-18). In a previous study we showed that lowering the pool of aerobic Gram-negative bacteria in the gut was not associated with a reduction of the postoperative endotoxemia and inflammatory response (10). However, an association was found between the occurrence of perioperative endotoxemia and postoperative inflammatory response, indicating endotoxin as etiologic factor in the cytokine release in patients undergoing CPB (8-10). Since the inflammatory response following CPB is accompanied by changes in many regulatory systems, the culprit factor remains unknown. In general, a lot of focus existed on the proinflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8). Recently, it has been shown that in an artificial endotoxemia model, rhIL-10 blunts the human inflammatory response to lipopolysaccharide, without affecting the cardiovascular response (19). This could imply that endotoxin exerts its negative effects on the cardiovascular system independently of the inflammatory process, or that the deleterious effect is exerted directly by IL-10, independently of the proinflammatory response.

Furthermore, the highly variable nature of the syndrome in its occurrence and severity, make it crucial to identify factors that help explain this variation, including possible genetic determinants that play a role in the inflammatory response in CPB (20). Studies in patients with infectious diseases have shown that inter-individual variation in cytokine

production capacity, as well as severity and outcome of infectious diseases, can be explained partly by differences in the genetic background of the subjects. Since cytokine release is most likely transcriptionally regulated, polymorphisms in gene promoters of the cytokines involved (i.e., TNF-α and IL-10), are significant targets for this research. Many single nucleotide polymorphisms (SNPs) in the promoter region of the TNF-α and IL-10 gene have been described. Associations between these SNPs and (auto)inflammatory and infectious diseases have been described (21-32). More recently a polymorphism in the coding region of the toll-like receptor-4 (TLR4) has been found, however it's clinical significance remains unclear (33-39). Since the inflammatory response to CPB has strong similarities with sepsis, the condition of natural occurring endotoxemia may serve as an elegant model for the host response in sepsis. Currently, it is unknown to what extend the known SNPs contribute to the observed large inter-individual variation found in the inflammatory response following CPB and the development of the post-pump syndrome. The recognition of genetic polymorphisms that are associated with an increased risk of development of severe inflammatory response following CPB, should enable clinicians to develop preventive strategies targeted at patients that are at increased risk for poor clinical outcome associated with this response.

The aim of the present, prospective study was to investigate in patients undergoing elective cardiac surgery with CPB, the role of perioperative endotoxemia, cytokine activation and known SNPs in the promoter region of IL-10, the coding region of TLR4 on postoperative response leading to hemodynamic stability, morbidity, complications and outcome.

Materials and Methods

The study was performed at the Leiden University Medical Center, an 800-bed secondary and tertiary referral hospital. To be eligible for enrolment, the patients had to be aged 18 years or older and being scheduled for elective cardiac surgery with cardiopulmonary bypass between July 1, 1998, and December 30, 1999. We obtained institutional approval from the local medical ethics committee (protocol #P168/96). Each patient gave a written, informed consent.

The patients studied were 159 consecutive patients undergoing elective cardiac surgery with CPB. One hundred and twenty-two patients had been included in a previously published study on the effect of selective gut decontamination (SGD) on endotoxemia and cytokine activation (10). Of these 122 patients, 24 received preoperative SGD consisting of polymyxin B and neomycin. In that study, SGD was not associated with a reduction of perioperative endotoxemia and subsequent cytokine activation. The anaesthesia protocol and procedures have been described previously (10). Briefly, anaesthesia was induced and

maintained by administration of sufentanil and midazolam. Corticosteroids were not routinely administered during the anaesthesia induction phase. Patients were heparinised at induction, at the end of the procedure the heparin action was antagonized by protamine. CPB was established using a membrane oxygenator with nonpulsatile flow rates of about 2.4 L/min/m^2. Cardioplegia was induced with cold crystalloids; the core temperature was maintained at 28 ^{0}C.

Data collection.

Demographic characteristics, co-morbid conditions, details on the surgical procedure and the logistic 'European System for Cardiac Operative Risk Evaluation' score (EuroSCORE) were systematically collected for all patients entering the study (40). During their stay at the Intensive Care Unit (ICU) and throughout their stay in the hospital, patients were closely monitored. The ICU uses a computerized patient data management system (PDMS), wherein all consecutive (clinical) data were entered by the attending nurse. Data on body temperature, heart rate, hemodynamic parameters (central venous pressure, mean arterial blood pressure and cardiac output), artificial ventilation parameters (inspired fraction of oxygen [FiO_2], tidal volume, ventilation frequency, level of end-expiratory pressure [PEEP]) and the administration of vasoactive agents (dopamine, dobutamine and enoximon) were recorded every hour. During the study, vasopressive agents (epinephrine and norepinephrine) were not administered. For the analysis we calculated, for each patient, the mean value for each of these parameters over 6 hour intervals. Data on intravenously administered intravenous fluids (colloids, crystalloids, blood products) and urinary output, were routinely recorded as total volumes for consecutive 24-hour postoperative interval. Blood gas analysis (i.e. $PaCO_2$ and PaO_2, oxygen saturation) was entered directly following measurement. Oxygenation index (OI) was calculated by dividing the PaO_2 by the FiO_2. Finally, clinical outcome parameters (i.e. duration of artificial ventilation, length of stay [LOS] in the ICU, LOS in the hospital and outcome [fatal or non-fatal]) were recorded. All data were collected and entered in a database without previous knowledge of the results of the endotoxin, cytokine and genetic measurements. Only at the end of the study period, all data were combined in a single database, together with data on perioperative endotoxin, TNF-α and IL-10 concentrations, as well as the results of the genetic determinants described previously(8-10;41). Briefly, for this analysis data on the promoter polymorphisms of IL-10 (positions -592, -819, -1082, -1330, -2763, -2849 and -3575) and the common TLR4 SNPs (Asp299Gly and Thr399Ile) were available.

Analysis of Data.

Correlations were assessed non-parametrically using Spearman correlation test, comparisons between groups were made using Kruskal-Wallis test, individual groups were

compared by the Mann-Whitney *U* test, unless indicated otherwise. Comparison of distribution between categorical variables was performed using χ^2 test. Time-trends were assessed using log-rank test, censored for fatality. Statistical significance was tested two-tailed, with the α set to 0.05.

Results

We studied 159 consecutive patients undergoing elective cardio-thoracic surgery with cardiopulmonary bypass. The patient characteristics have been described previously(10). Briefly, there was a predominance of male patients (66 %), the median age for males and females was 65 and 67, respectively. Active smoking occurred in 35 patients (22 %) whereas 20 patients (12 %) had diabetes mellitus. Surgical procedures were extensive; 20 patients (12 %) underwent coronary artery bypass surgery (CABG) combined with valve replacement, 49 patients (30 %) underwent valve replacement only, whereas 89 patients (54 %) underwent CABG only. Eight patients underwent other surgical procedures, mainly aortic surgery. Patients aged 65 or older, had significantly longer median duration of artificial ventilation (1.25 versus 1.00 days), ICU admission (3.0 versus 2.0) and hospital stay (12.0 versus 10.0 days).

Fourteen patients out of the 159 patients (8.8 %) died in the hospital, mortality rates were higher in patients aged 65 and older (13.1 versus 4.0%, $p = 0.043$), in patients with diabetes (20.0 versus 6.6 %, $p = 0.042$) and females (17.3 versus 4.7, $p = 0.008$). Multiple organ dysfunction (MOF) occurred more often in patients with diabetes as compared to patients without diabetes (15.0 versus 2.2%, $p = 0.005$). EuroSCORE was higher in patients with fatal outcome (6.1 versus 3.6, $p = 0.014$), and correlated significantly with the duration of artificial ventilation ($r = 0.235$, $p = 0.005$), duration of ICU stay ($r = 0.231$, $p = 0.004$) and hospital stay ($r = 0.347$, $p < 0.00001$).

Correlation between perioperative endotoxemia, cytokine release and clinical parameters.
The relationship between perioperative endotoxemia and cytokine release has been described previously (8;9). Perioperative endotoxemia was positively correlated with TNF-α and IL-10 release. In the current analysis we assessed correlations between perioperative endotoxin concentrations and clinical outcome. We found a consistent positive correlation between the endotoxin concentration at aorta declamping (time-point 2) and the amount of colloids administered to the patient during the first 24, the first 48 hours, and the 24-48 hour interval at the ICU ($r = 0.186$; $p = 0.040$, $r = 0.247$; $p = 0.006$, $r = 0.225$; $p = 0.012$, respectively). Not only but also, the same trend was observed in the correlation between the endotoxin concentration 30 mins into reperfusion and upon arrival at the ICU (time-points 3 and 4, respectively) and the amount of colloids administered

during the same postoperative intervals (i.e. the first 24-hours, the first 48-hours and between 24-48 hours) however these correlations were less strong and only marginal statistically significant (i.e., $r = 0.169$; $p = 0.060$, $r = 0.198$; $p = 0.027$, $r = 0.167$; $p = 0.064$ and $r = 0.124$; $p = 0.163$, $r = 0.169$; $p = 0.055$, $r = 0.194$; $p = 0.028$, respectively). After dichotomization, the endotoxemia positive (defined as endotoxin level $\geq$ 5 pg/mL) received significantly larger amounts of colloids during the first 24, 48 and 72 hours of their postoperative stay at the ICU (Figure 1, Mann-Whitney *U* test) as compared to the endotoxemia negative patients (defined as endotoxin level < 5 pg/mL). Also, the endotoxemia positive patients received significantly larger amounts of crystalloids during the first 72 postoperative hours at the ICU ($p = 0.042$). Furthermore, a positive correlation was found between the endotoxin concentrations at all post-operative time-points (time-point 2, 3 and 4) and the amount of dopamine (µg/kg/min) administered in the early postoperative phase (i.e. the first three 12-hour intervals of admission to the ICU, *r* ranging from 0.163 to 0.253, *p* ranging from 0.065 to 0.003). A trend of larger amounts of dopamine administered to the endotoxemia positive patients during the first 48-hours of their ICU stay was observed, however this did not reach the level of statistical significance ($p = 0.148$, $p = 0.076$, $p = 0.037$, $p = 0.090$ for the first four consecutive 12-hour intervals, respectively). Furthermore, patients having significant endotoxemia (defined as endotoxin level $\geq$ 5 pg/mL) upon arrival at the ICU (time-point 4) had significantly lower oxygen saturation, arterial oxygen pressure (PaO_2) and oxygenation index (OI, i.e. arterial PaO_2 divided by inspired fraction of oxygen [FiO_2]) during the first 24-hours at the ICU (Table 1).

Table 1. Postoperative pulmonary function parameters during the first 24-hours in patients with or without endotoxemia upon arrival at ICU.

	OI (PaO_2/FiO_2)			O_2 saturation			PaO_2		
	endotoxin level			endotoxin level			endotoxin level		
	≤5	>5	*p*	≤5	>5	*p*	≤5	>5	*p*
0-6 hours	39.0 (29.1-51.9)	34.5 (28.8-45.4)	0.249	98.7 (97.6-99.0)	97.7 (96.9-99.0)	0.312	17.8 (14.2-21.0)	16.2 (12.0-21.9)	0.013
6-12 hours	41.8 (31.8-47.0)	34.9 (26.1-42.4)	0.034	98.5 (98.0-99.0)	98.0 (96.5-99.0)	0.041	17.0 (14.0-20.0)	15.1 (11.3-19.1)	0.006
12-18 hours	38.5 (29.5-47.4)	31.5 (24.6-40.2)	0.018	98.0 (97.0-99.0)	97.7 (96.5-99.0)	0.041	15.6 (13.3-17.9)	14.0 (11.5-17.0)	0.027
18-24 hours	37.7 (33.0-46.2)	30.1 (24.3-36.3)	0.005	98.0 (97.0-99.0)	97.7 (96.0-98.5)	0.005	14.7 (13.2-17.6)	13.0 (11.3-15.3)	0.012

OI = oxygenation index (kPa); PaO_2 = arterial oxygen pressure; FiO_2 = inspired fraction of oxygen. Endotoxin concentrations in pg/mL. Median values and interquartile ranges between parentheses. Mann-Whitney *U* test.

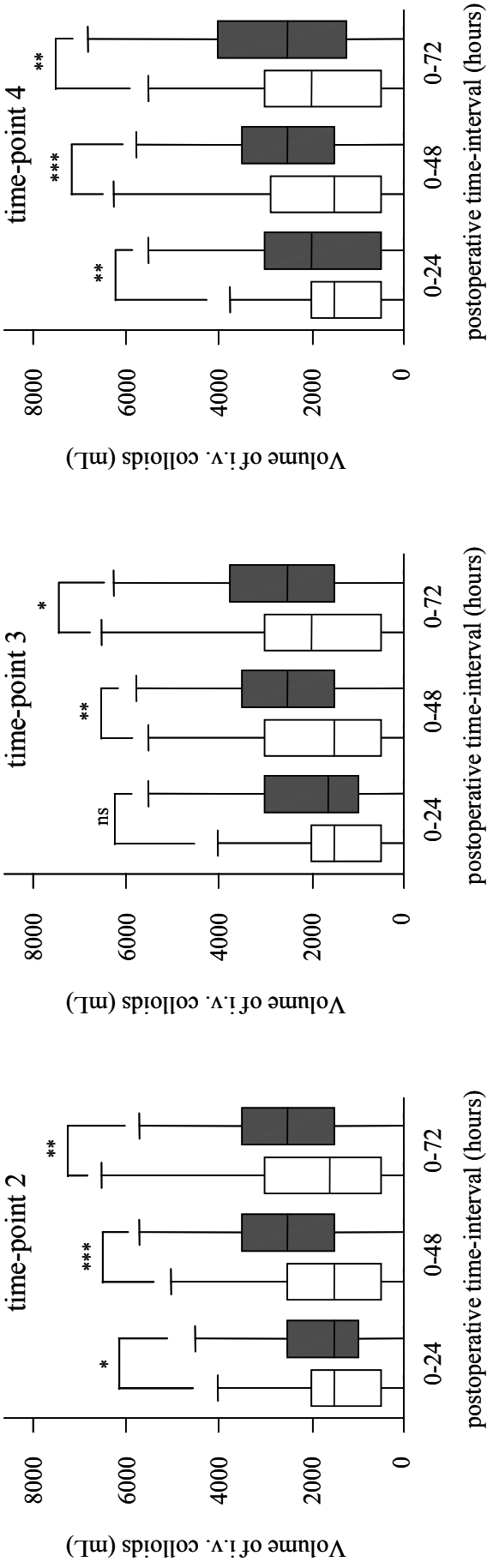

Figure 1. Volume of colloids administered according to the presence (≥ 5 pg/mL, dark bars [right]) or the absence of endotoxemia (< 5 pg/mL, light bars [left]) at time-point 2, 3 and 4 respectively. *p < 0.05, **p < 0.03, ***p < 0.01, ns = not significant. Mann-Whitney *U* test.

We found no associations between the intensity of endotoxemia and differences in central venous pressure, cardiac output, cardiac index, mean arterial pressure, heart rate and mechanical ventilator parameters (i.e., among others tidal volume, PEEP level). Since these major indices of the hemodynamic status, in general, are targets of medical interventions and therapeutic adjustments and by medical intervention are thus kept between desired ranges, these interventions are better markers of the clinical status of the patient than these parameters.

Secondly, the correlations between the perioperative cytokine release and clinical parameters were assessed. We observed a negative correlation between IL-10 concentrations at aorta declamping, 30 mins into reperfusion and upon arrival at the ICU and the amounts of colloids administered during the first 24, and the first 48 hours during ICU admission (r = -0.218; p = 0.017, r = -0.221; p = 0.017, r = -0.185; p = 0.038 and r = -0.200; p = 0.029, r = -0.226; p = 0.013, r = -0.118; p = 0.187, respectively). Interleukin-10 concentrations at aorta declamping were positively correlated with the amount of postoperative administered dobutamine (μg/kg/min) during the first three 24-hour intervals (r = 0.210; p = 0.018, r = 0.239; p = 0.007, r = 0.192; p = 0.032, respectively). The same trend was observed in the correlation between the IL-10 concentration 30 mins into reperfusion and upon arrival at the ICU and the amount of dobutamine (μg/kg/min) administered during the same postoperative intervals (i.e. the first three 24-hours intervals) however these correlations were less strong and less statistically significant (r = 0.129; p = 0.153, r = 0.162; p = 0.072, r = 0.141; p = 0.120 and r = 0.148; p = 0.092, r = 0.174; p = 0.047, r = 0.220; p = 0.012, respectively). For dopamine and enoximon a positive correlation was found at all perioperative IL-10 concentrations, however this was not statistically significant (data not shown). The ratio's of TNF-α and IL-10 concentrations, at aorta declamping, were negatively correlated with the amount of dobutamine during the first three 24-hour intervals (r = -0.195; p = 0.047, r = -0.256; p = 0.008, r = -0.264; p = 0.006, respectively). Positive correlations were observed between IL-10 concentrations at aorta declamping and central venous pressure during the first eight 6-hour intervals (r = 0.243; p = 0.012, r = 0.265; p = 0.006, r = 0.193; p = 0.047 and r = 0.229; p = 0.019, r = 0.248; p = 0.019, r = 0.293; p = 0.007, r = 0.319; p = 0.003 and r = 0.281; p = 0.016, respectively). The same trend, but to a lesser extend, was observed at 30 mins into reperfusion (r ranging from 0.168 to 0.306, p ranging from 0.085 to 0.005). A negative correlation was found between IL-10 concentrations at aorta declamping and cardiac output during the first eight 6-hour intervals (r = -0.204; p = 0.109, r = -0.312; p = 0.014, r = -0.418; p = 0.001 and r = -0.308; p = 0.016, r = -0.467; p < 0.001, r = -0.429; p = 0.002, r = -0.375; p = 0.007 and r = -0.407; p = 0.005, respectively). Again, the same trend was

observed at 30 mins into reperfusion and upon arrival at the ICU however these correlations were less statistically significant (r ranging from -0.104 to -0.342, p ranging from 0.415 to 0.009 and r ranging from -0.238 to -0.427, p ranging from 0.052 to 0.001, respectively). No consistent correlations were found between perioperative IL-10 concentrations and the other clinical parameters. Finally, no consistent correlations between the perioperative TNF-α release and the clinical parameters were observed. However, we observed a positive correlation between IL-6 concentrations at both of the measured time-points (i.e. at aorta declamping and upon arrival at the ICU) with the amount of dopamine administered during the first three 24-hour intervals (r = 0.317; p = 0.010, r = 0.300; p = 0.014, r = 0.254; p = 0.039 and r = 0.240; p = 0.054, r = 0.276; p = 0.026, r = 0.268; p = 0.031, respectively). For dobutamine, enoximon and the other clinical parameters no significant correlations with IL-6 were observed.

Correlation between genetic factors and clinical outcome parameters.

Next we investigated whether a correlation could be found between the genetic polymorphisms and the aforementioned clinical parameters. In none of the clinical parameters a differences was found between carriers of the common polymorphisms in TLR4 (Asp299Gly or Thr399Ile carriers as compared to wild-type homozygotes). The proportion of patients initiating therapy with dopamine, dobutamine or enoximon directly following arrival at the ICU, were significantly different according to the proximal IL-10 promoter haplotypes. Patients carrying the GATA allele, initiated dopamine therapy significantly more frequently as compared to the patients not carrying these alleles (73.2 versus 52.3 %, p = 0.013, Table 2), whereas patients carrying the AGCC allele initiated therapy with dopamine less frequently (53.8 versus 72.5 %, p = 0.023, Table 2).

Table 2. Proportion of patients initiating treatment with vasoactive agents according to proximal IL-10 promoter haplotype

	GATA			AGCC			GACC		
	absent	present	*p*	absent	present	*p*	absent	present	*p*
Dobutamine									
yes	42	24	0.407	19	47	0.146	27	39	0.281
no	42	32		30	44		37	37	
Dopamine									
yes	44	41	0.013	36	49	0.023	43	42	0.150
no	40	15		13	42		21	34	
Enoximon									
yes	21	18	0.356	14	25	0.890	16	23	0.489
no	63	38		35	66		48	53	

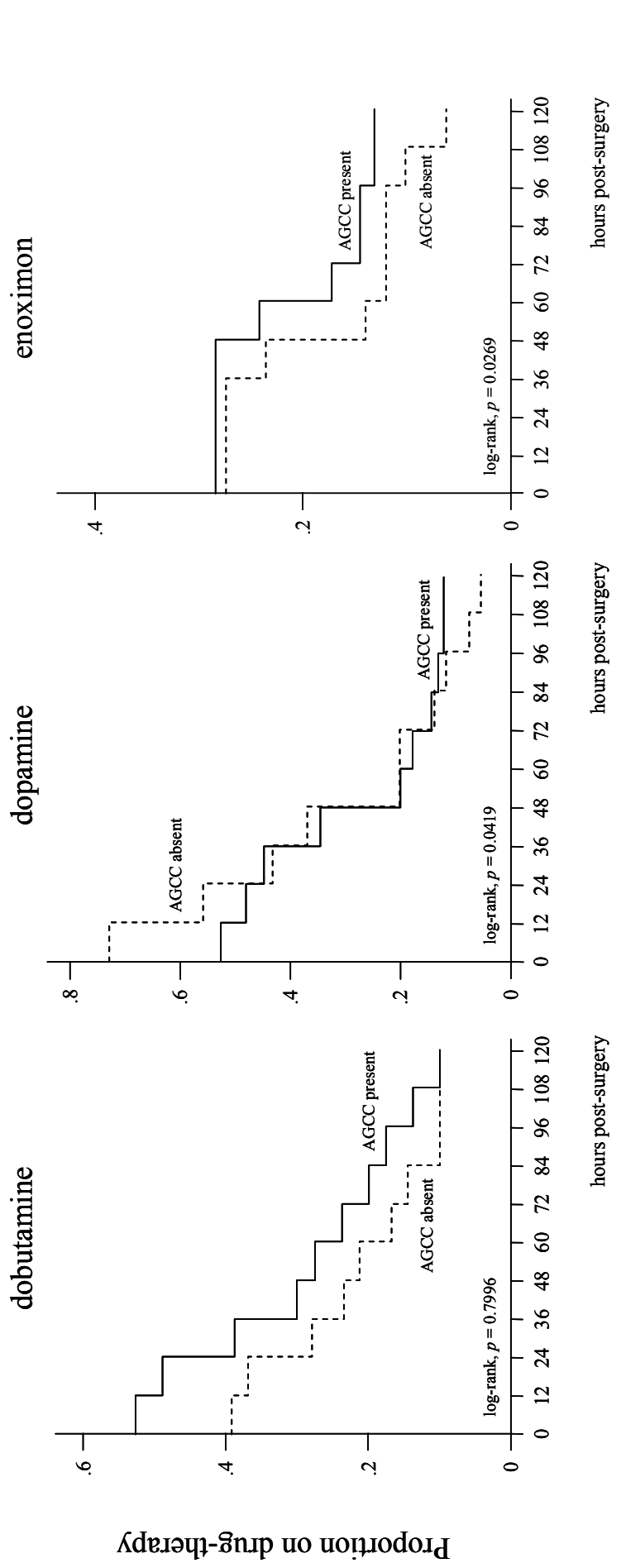

Figure 2. Proportion of patients remaining on drug-treatment after direct post-CPB initiation of treatment with dobutamine (left), dopamine (middle) and enoximon (right), according to the presence or absence of the IL-10 promoter AGCC allele. Log-rank analysis performed on curves after setting the proportion of patients in each group starting on agent to 1.0. Curves censored for fatal outcomes.

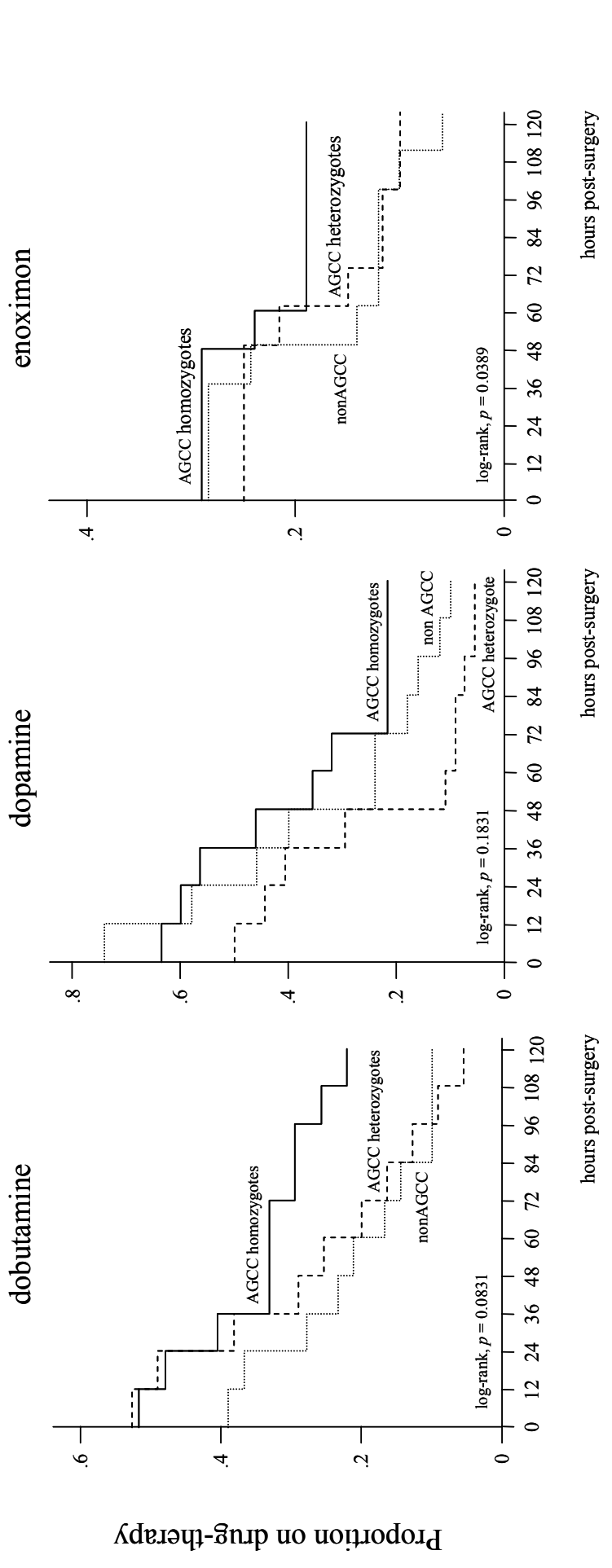

Figure 3. Proportion of patients remaining on drug-treatment after direct post-CPB initiation of treatment with dobutamine (left), dopamine (middle) and enoximon (right), according to the 3 possible AGCC allele genotypes (i.e., homozygous AGCC/AGCC, heterozygous AGCC or complete absence of AGCC allele. Log-rank analysis performed on curves after setting the proportion of patients in each group starting on agent to 1.0. Curves censored for fatal outcomes.

In the patients initiating therapy with either dopamine or enoximon the use of these agents was significantly longer in patients carrying the AGCC allele as compared to patients not carrying this allele (p = 0.0419 and 0.0269, respectively, Figure 2). Moreover, homozygous carriers of the AGCC allele used dobutamine and enoximon longer than the heterozygous carriers, whereas patients not carrying the AGCC allele had the lowest use of these agents (p = 0.0831 and 0.0389, respectively, Figure 3). For dopamine use this was not observed (Figure 3).

Discussion

The main finding of the present study is that perioperative endotoxemia and high IL-10 concentrations are associated with postoperative hemodynamic instability and pulmonary dysfunction in elective cardiac surgery patients undergoing CPB. Patients with significant endotoxemia upon aorta declamping received higher amounts of colloids during the first 72 hours of their postoperative ICU stay, moreover perioperative endotoxin concentrations were positively correlated with the amount of postoperative administered dopamine. Furthermore; during the first 24-hour post CPB the oxygenation index (OI) was lower in patients with significant perioperative endotoxemia, indicating impaired oxygenation. Also, high IL-10 concentrations at aorta declamping and 30 minutes into reperfusion were associated with a postoperative clinical profile characterised by increased central venous pressure (CVP) and use of dobutamine, together with a decrease in the volume of administered colloids, decreased cardiac output, and decreased urinary output.

With respect to this study and methodology, several issues need to be considered. We repeatedly sampled patients undergoing elective CPB, and performed in these samples paired measurements of endotoxin and cytokine concentrations in each patient over time. This enabled us to study the inflammatory response to surgery and endotoxemia, and relate these to clinical outcome: e.g., hemodynamic status, pulmonary dysfunction, days on artificial ventilation and length of intensive care and hospital stay. Since in the ICU hemodynamic and ventilatory indices are targets of medical intervention and therapeutic adjustments (e.g. an undesired trend in hemodynamic parameters is quickly countered by adjustments of intravenous fluids and vasoactive drugs), we also quantitatively analysed trends in these medical interventions. The role of the nonpulsatile flow CPB, routinely used in our study, on the magnitude of endotoxemia is controversial, but might have led to a higher proportion of patients experiencing endotoxemia, as compared to other CPB modalities (42;43). In a minority of patients, the postoperative response leads to ultimately fatal multiple organ dysfunction (MODS). Little is know about the determinants of the broad clinical spectrum of responses observed in patients after CPB. Sofar, no study has

convincingly shown association between perioperative endotoxin concentrations and patient outcome. Of note, although we studied a reasonably large cohort of patients, this study had insufficient power to detect differences for genetic polymorphisms that occur only in small minority (less than 5-10%) of patients.

The occurrence of endotoxemia was associated with an increased need for postoperative circulatory support, indicated by the administration of larger volumes of colloids, crystalloids and amounts of dopamine. At the same time, higher perioperative endotoxin concentrations were associated with higher urinary output during the first 72 hours after ICU admission, indicating swift normalisation of the hemodynamic status after volume loading, and increased excretion of excessive extracellular fluids later. Of note, these observations were not accompanied by changes in indices of the hemodynamic status of patients i.e., CVP, cardiac output, cardiac index and ABP. However, keeping these parameters constant was the target of the interventions above. In the same way the oxygenation index, is a more suitable marker of pulmonary dysfunction as compared to inspiratory oxygen fraction or arterial oxygen pressure alone. Patients with endotoxemia needed more respiratory support, as indicated by lower OI, as compared to patients without endotoxemia. Pulmonary dysfunction following CPB occurred frequently, and led to delayed tracheal extubation. The aetiology of pulmonary dysfunction is multifactorial, occurring as a result of the combined effects of anaesthesia, CPB, and surgical trauma. It has been suggested that the inflammatory response that results in increased pulmonary capillary permeability is involved in the pathogenesis of the pulmonary dysfunction as well. One study in elective cardiac surgery described an association between circulating endotoxin and high postoperative oxygen consumption (13). In that study patients with higher perioperative endotoxin levels had high postoperative oxygen consumption. Our data support this finding; to our knowledge no other studies in CPB patients have addressed this question.

In patients with high perioperative IL-10 concentrations a hemodynamic profile occurred indicative of cardiac depression. Typically these patients are characterised by a good venous filling state (high CVP, low volumes of administered colloids) and a tendency towards forward failure (low cardiac output and a need for dobutamine). Similarly, one other study in CPB patients described an association between high circulating IL-10 levels and risk of organ dysfunction in the early postoperative phase (44). Most studies, however focused on proinflammatory cytokines (i.e., TNF-α, IL-1, IL-6), and therefore the adverse effect of high circulating IL-10 may have been overlooked. In patients with infection high IL-10 appears deleterious, likely by inhibiting bacterial clearance (45;46). However a reduced bacterial clearance does not apply to the present situation. In a recent study conducted by Kumar et al., healthy subjects were artificially challenged with a intravenous

bolus of *Escherichia coli* lipopolysaccharide (19). This infection was preceded by a single shot of rhIL-10, at various dosages and time-intervals. The interesting finding of the study was that, although the rhIL-10 diminished the proinflammatory response to the LPS administration (e.g., reduced the TNF-α, IL-6 and IL-1Ra levels), it did not attenuate the hemodynamic changes observed after the administration of LPS. Subjects receiving rhIL-10 two hours before administration of LPS had lower mean arterial pressure (MAP) five hours after LPS administration, as compared to subjects receiving placebo instead of rhIL-10 (19). This suggests that a high level of IL-10, present during LPS administration, augments the deleterious hemodynamic effects of LPS, despite down regulation of proinflammatory cytokine release (IL-6, IL-1Ra and IL-1β). The observation limits the use of rhIL-10 as a immunomodulating agent in sepsis, but also highlights the role of IL-10 in the pathophysiology of endotoxin-related hemodynamic deterioration resulting in organ dysfunction.

As to explore the genetic basis of the high IL-10 concentrations we found that patients carrying the AGCC haplotype, generally regarded as the high IL-10 producing allele, initiated treatment with dopamine less frequently as compared to the patients not carrying the AGCC haplotype. This is consistent with the findings described above. However, once initiated on dopamine or enoximon, these patients used these agents for a longer period of time as compared to patients not carrying this allele. This observation seems conflicting since it suggests that the IL-10 promoter haplotype associated with high IL-10 production to endotoxin is not associated with the clinical profile associated with high perioperative circulating IL-10 levels that we described in the previous paragraph. We are not sure as to how to explain this observation. In one earlier study, high levels of circulating IL-10 were, like in our study, associated with the development of organ dysfunction in the early postoperative period (44).

Of note, perioperative TNF-α concentrations did not predict clinical outcome parameters. This suggests that the adverse effects of IL-10 occur largely independent of an ongoing proinflammatory reaction.

In summary, endotoxemia in CPB triggers an inflammatory response as reflected by the release of TNF-α and IL-10. Perioperative TNF-α concentrations did not predict postoperative outcome. However, postoperative hemodynamic and respiratory function is negatively affected by high IL-10, largely independent of the concentration of TNF-α. Furthermore, these IL-10 associated hemodynamic changes occurred according to the proximal IL-10 promoter genotype associated with high and low IL-10 production capacity in vitro and in vivo.

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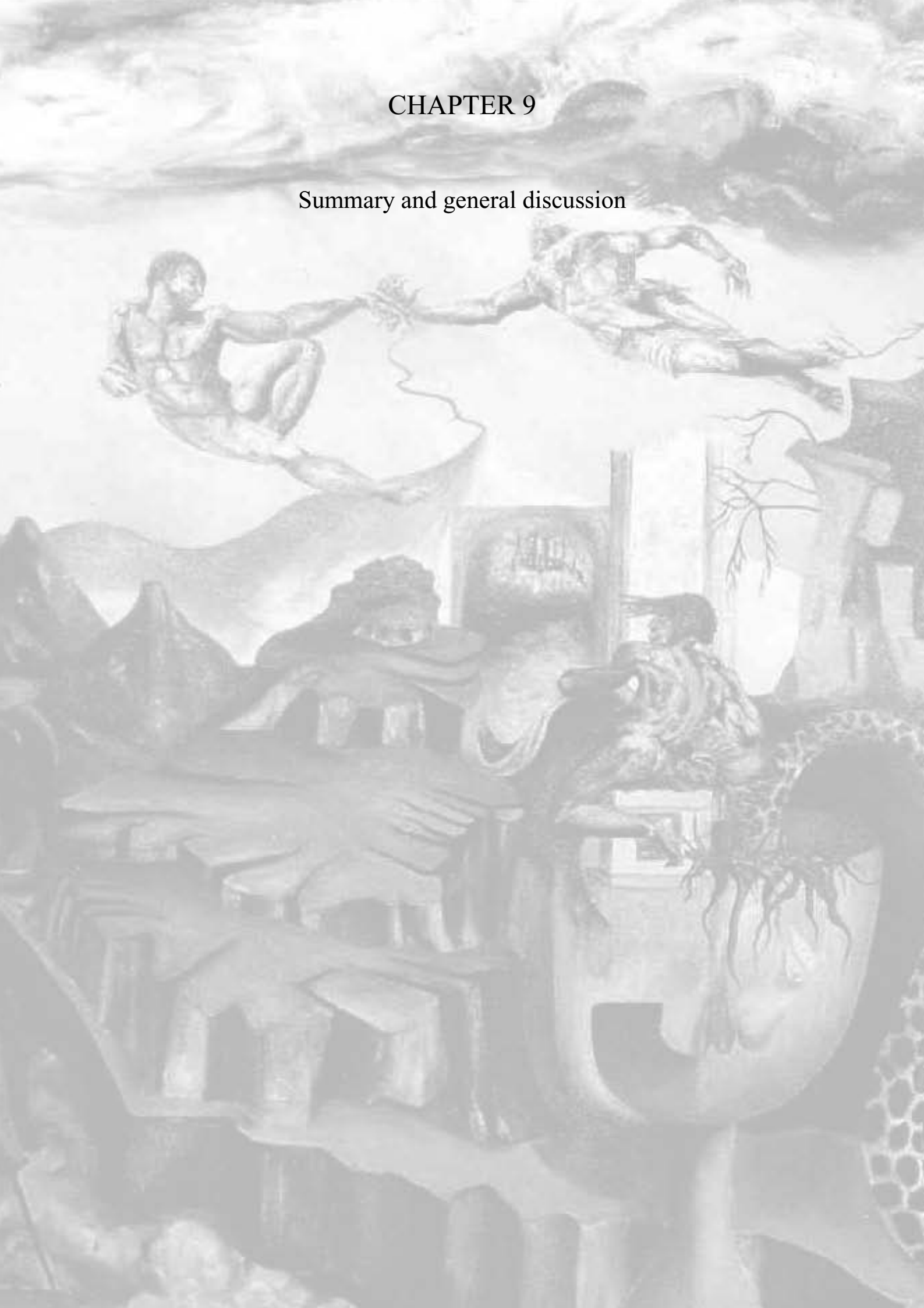
CHAPTER 9

Summary and general discussion



The results of the studies described in Chapters 2 through 8 will be briefly summarized and discussed in the following sections.

Effect of preoperative selective gut decontamination and the role of perioperative endotoxemia on postoperative cytokine activation and clinical outcome in elective cardiac bypass surgery.

In the study described in **Chapter 2 and 3**, we found no effect of preoperative selective gut decontamination (SGD) on perioperative endotoxemia and cytokine activation in cardiac surgery patients undergoing elective cardio-pulmonary bypass (CPB). This is notwithstanding the positive relationship between occurrence of endotoxemia and intensity of the perioperative cytokine response. This observation that bowel decontamination did not reduce the incidence of endotoxemia is in contrast with two previous studies, one performed in rats and one in human subjects. In our study, over half of the patients experienced translocation of endotoxin from the gut into the systemic circulation following the surgical procedure. Although SGD was highly effective in eliminating the aerobic Gram-negative bacteria from the feces, it did not reduce the percentage of patients with endotoxemia or the level of endotoxemia. This indicates that during SGD the pool of endotoxin in the bowel lumen is not a key limiting factor in the pathophysiological mechanism that controls translocation of endotoxin from the gut into the bloodstream. In addition, it should be realised that aerobic Gram-negative microorganisms constitute only about 0.5-1% of the Gram-negative bowel flora, and the remaining anaerobic Gram-negative bacteria might well be responsible for a large part of the intraluminal endotoxin potentially available for translocation.

Although we were unable to register an alternative - causative - mechanism associated with the occurrence of endotoxemia, several possible factors might come into play. First, there is the compromised circulatory state during and directly following the procedure and this is generally believed to be the most critical mechanism leading to translocation of endotoxin from the gut. The intestine is amongst the metabolically most active organs and therefore sensitive to hypoperfusion, any compromise rapidly leading to tissue hypoxia. In animal studies, experimental shock inflicted by various means (i.e., hemorrhagic shock, trauma, thermal injury), led to similar occurrences of endotoxemia. In humans, the systemic blood pressure might be a poor indicator of gut tissue perfusion during non-pulsatile flow. As a physiologic mechanism, splanchnic blood flow is down-regulated during hypotension by regional vasoconstriction. In patients with vascular occlusive disease of the intestine, not uncommon in patients undergoing coronary artery bypass grafting for atherosclerosis narrowing of the arteries, these factors might have amplified the effect of hypoperfusion of the gut, in about half the patients leading to critical

ischemia-reperfusion injury and subsequent translocation of endotoxin, despite a seemingly adequate systemic blood pressure during the surgery.

Several studies found that in Gram-negative infection the mortality of patients is substantially higher in those experiencing endotoxemia as well. Likely, endotoxemia is associated with the level of bacteremia, i.e., the actual number of microorganisms per mL blood, and not all patients with Gram-negative bacteremia develop endotoxemia. Moreover, endotoxemia does not occur exclusively in Gram-negative bacteraemia. In patients with shock and culture proven Gram-positive infection, endotoxemia exists in a significant proportion of patients. This observation suggests that the role of endotoxin in septic shock goes beyond Gram-negative infection. Furthermore, in various diseases not related to infection, any state of shock (hemorrhagic, cardiac, burns) was associated with endotoxemia in a substantial proportion of the patients. Taken together, these observations imply that it is not Gram-negative infection per se that causes endotoxemia and a poor outcome, but rather suggest that endotoxemia should be considered both as indicator of poor tissue perfusion allowing translocation of endotoxin from the gut, as well as inflammatory mediator triggering an inflammatory response that leads to a deterioration of a compromised hemodynamic situation. Such a hypothesis would explain why endotoxemia is an indicator of poor outcome in infections caused by microorganisms that do not carry endotoxin, e.g., the gram-positive bacteria, and in inflammatory states due to causes other than infection. In future studies it would be of great interest the look in severe infections in more detail to other markers of insufficient gut perfusion, and relate these to the incidence of endotoxemia and outcome.

In the studies described in **Chapters 5 and 6**, we described the correlation between the level of endotoxemia during the reperfusion phase of CPB and the perioperative release of TNF-α and IL-10. The positive, proportional relationship between these mediators indicates that endotoxin is responsible for at least part of the cytokine activation in these patients. Although the correlation was statistically significant, the extent of its scope seemed limited (i.e., the correlation was relatively weak) and this implicates other factors triggering the cytokine response, such as the surgical insult, cardiac stunning, activation of cells by tubing, etc. plat a role. In accordance we found, as was shown by others as well, that during CPB cytokine activation can occur in the absence of endotoxemia. Despite the presence of additional factors causing cytokine release, the finding indicated that the surgical procedure accompanied by CPB may serve as a model to study the *in vivo* response to endotoxemia.

In **Chapter 8** we investigated clinical parameters and outcome in relation to the occurrence and intensity of perioperative endotoxemia and subsequent cytokine release into the systemic circulation. In this respect, the occurrence of pathophysiological changes (e.g. hemodynamic status, pulmonary dysfunction) and clinical outcome (i.e. days on

artificial ventilation, length of intensive care and hospital stay) of the patient in the period following CPB were studied. Since in the ICU hemodynamic and ventilatory indices are targets of routine medical intervention and therapeutic adjustments (e.g., undesired trends in hemodynamic parameters let the attending physician adjust the amount of intravenous fluids and vasoactive drugs), we also quantitatively analyzed trends in these medical and drug interventions. It was reasoned that the amount of circulatory support needed to keep hemodynamic parameters (i.e., central venous pressure [CVP], blood pressure, cardiac output and urinary output) within certain preset levels, these would serve as an adequate surrogate marker for the underlying hemodynamic status of a patient. In the same way the generally used indicator of respiratory support, i.e., the oxygenation index (OI calculated by dividing the PaO_2 by the FiO_2), is a better marker of pulmonary dysfunction as compared to inspiratory oxygen fraction or arterial oxygen pressure alone.
We found that the occurrence of endotoxemia was associated with hemodynamic instability, and this was generally reversed within 48 hours. Of note, patients with endotoxemia needed a longer period of respiratory support, as compared to patient without endotoxemia.

Another finding of our study described in **Chapter 8** was that in patients with high perioperative IL-10 concentrations cardiac depression occurred. Typically these patients are characterised by an adequate venous filling state (i.e., high CVP in combination with low volume of colloids being administered) and a tendency towards forward failure as evidenced by a low cardiac output and high amounts of dobutamine administered. Little is known about adverse effects of high circulating concentrations of IL-10, partly because most studies have focused on pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6. Nevertheless, some studies found deleterious effects of high circulating concentrations of IL-10 in patients with acute infectious disease, most likely, but not exclusively, as a result of reduced bacterial clearance by the anti-inflammatory effect of IL-10. However, in our study patients did not suffer from infection as bacterial products translocated to the circulation as a result of ischemia-reperfusion damage and so stimulated cytokine producing cells. Therefore, the argument of a reduced bacterial clearance does not apply. In healthy subjects, administration of IL-10 shortly before an intravenous bolus of LPS, diminished the pro-inflammatory response (e.g., resulted in a lower TNF-α, IL-6 and IL-1Ra levels as compared to untreated controls), but - of interest here - did not attenuate the deleterious hemodynamic effects of LPS. Thus, subjects receiving IL-10 two hours before administration of LPS had lower mean arterial pressure (MAP) five hours after LPS administration, as compared to subjects receiving placebo (in stead of rhIL-10). This suggests that high circulating levels of IL-10, shortly before LPS administration, potentates the deleterious hemodynamic effects of LPS, despite down regulation of pro-inflammatory cytokine release (IL-6, IL-1Ra and IL-1β). Obviously, the observation limits the potential

use of rhIL-10 as an immunomodulating agent in sepsis. Also, it suggests a potential role for IL-10 in the pathophysiology of endotoxin induced hemodynamic changes leading to organ dysfunction.

We did not find a correlation between perioperative TNF-α concentrations and clinical outcome parameters. This was somewhat unexpected because generally TNF-α is regarded as the most potent cytokine leading to pro-inflammatory response and is held responsible for the development of harmful effects of a progressing systemic inflammatory response, such as capillary leak, hypotension, acute respiratory distress syndrome (ARDS), and multiple organ system failure. We did not observe such a trend in this model of natural occurring endotoxemia.

An individual's *ex vivo* cytokine response to LPS and the *in vivo* response to endotoxin: which *ex vivo* parameter foresees best the *in vivo* response?

The main finding of the study described in **Chapter 4** was that the dose-response characteristics of TNF-α and IL-10 release by human peripheral whole blood, upon stimulation with a wide range of LPS concentrations, can be described adequately by a receptor-ligand interaction model. This model is fully characterized by two parameters, i.e., EC_{50}, the estimated LPS concentration at which half of the cytokine concentration is reached and the E_{max}, the estimated maximal concentration of cytokine released. We found that these two parameters were highly constant for individuals, yet differed between individuals. For instance, we detected significant differences between two subjects with respect to the cytokine release at the lowest LPS concentration and, more importantly, the dose-response parameters based on the whole range of LPS concentrations remained significantly different between the subjects. Such differences in dose-response characteristics would have been discarded, however, if only one or two LPS concentrations had been used to test the cytokine response of these individuals. Thus, relevant information on cytokine release is lost when the commonly applied approach is taken, i.e. testing the TNF-α and or IL-10 release after stimulation with a single, and often high, LPS concentration. Such an approach yields a single value that doesn't represent a physiological model of release and often shows significant variation over time. By contrast, we estimated two parameters that fully characterize an underlying model and are intrinsic parameters that are much less sensitive to day-to-day variation.

In **Chapter 5** we describe that the *in vivo* release of TNF-α was correlated with the maximal TNF-α release (the TNF-α_{max}) *ex vivo* upon stimulation with LPS. This indicates that the maximal TNF-α production capacity measured *ex vivo* is at present the best predictor of *in vivo* TNF-α levels in the perioperative stage of cardiac surgery. Similarly, in **Chapter 6** we found that the *in vivo* release of IL-10 during reperfusion (i.e., at aorta

declamping and 30 min into reperfusion) in patients experiencing endotoxemia was predicted by the IL-10 production *ex vivo* upon stimulation with 1000 ng LPS/mL and the estimated maximal IL-10 production capacity (the $IL\text{-}10_{max}$). Thus, the *ex vivo* LPS stimulation assay is a predictor of the *in vivo* release of IL-10 during endotoxemia after cardiac surgery. Of note, the correlation was best in the patients experiencing endotoxemia, indicating the relevance of endotoxin as stimulus in the *in vivo* TNF-α and IL-10 production.

TNF-α and IL-10 promoter polymorphisms and the *in vivo* and *ex vivo* response to LPS.

Since several studies indicated a role of genetic determinants in the cytokine responses in innate immunity, we investigated if and to what extend known polymorphism in the TNF-α and IL-10 promoter are to be held responsible for differences in the release of the cytokines *in vivo* and *ex vivo* upon stimulation with LPS.

In **Chapter 5** we described that *in vivo* endotoxin-stimulated release of TNF-α did not differ between patients according to their TNF-α promoter polymorphisms, including the -308 G/A substitution. Although earlier studies described an increased susceptibility and/or severe outcome of sepsis or septic shock in carriers of a common TNF-α promoter polymorphism (i.e. -308 G/A), in none of the studies was the *in vivo* release of TNF-α positively correlated with this increased risk. The findings suggest that this TNF-α promoter polymorphism does not exert its effect on sepsis susceptibility and/or outcome by causing a differential gene expression and/or release of TNF-α. Overall, the effects on TNF-α release by promoter polymorphism appeared rather limited, both in vivo in patients experiencing endotoxemia, and ex vivo upon stimulation of peripheral blood cells by LPS. Given its proximity to many other innate immune genes located on chromosome 6 and to the HLA system, it cannot be excluded that the TNF-α promoter polymorphism acts as marker of another gene variation.

In **Chapter 6** we described that patients carrying the AGCC allele of the IL-10 promoter, had slightly higher post-operative IL-10 levels as compared to carriers of all other haplotypes combined. Homozygous carriers of the GATA allele had lower postoperative IL-10 levels as compared to all other patients. Furthermore, AGCC allele carriers had higher LPS sensitivity *ex vivo*, whereas carriers of the GATA allele showed lower LPS sensitivity. Furthermore, homozygous GATA carriers also had lower IL-10 production *in vivo*. This emphasizes the importance of the SNP at position -1082 in the transcriptional activity of the proximal promoter. The phenotype of increased LPS sensitivity was confirmed *ex vivo* in AGCC haplotype carriers, and is further supported by the significantly higher circulating IL-10 levels following cardiopulmonary bypass in the

AGCC haplotype carriers as compared to carriers of the other haplotypes combined.

Although part of the large inter-individual variation found in the *in vivo* and *ex vivo* responses to LPS can be explained by known polymorphisms in the IL-10 promoter region, overall their influence is limited and can only explain about 5-10% of the variation in IL-10 concentrations. Similar reasoning applies to the release of TNF-α. This raises the question whether or not the observed differences, although small in the light of large inter-individual differences, are relevant to the individual. This question appears the more fascinating since the human population shows a preserved heterogeneity in some specific alleles. When assuming that these polymorphisms each arise from one single spontaneous mutation in one of our ancestors, their conservation in evolution must have bared some advantage, even though that may not exist at present time. In this respect it should be realized, nevertheless, that in a complex, highly organized biological system, a sustained and repeated 5% difference in the direction of a particular response might add up to a highly relevant overall difference. In such systems, a beneficial outcome or catastrophe may follow directed, repeated small events. To fully appreciate this issue, an analogy can be made to the analysis of the dynamic behavior of a macrophage lining the lung surface reaching for a bacterium delivered into the alveolus. In the presence of such a target for phagocytosis, chemotaxis is an important component of the immune response, the success of which depends on the time to ingestion of the bacterium relative to rate of replication of the microorganism (i.e., producing two, four, eight and so on separate targets). To reach an effective encounter time to control bacterial multiplication, some directed motion of the phagocyte is necessary. Analyses of dynamic models of this process have demonstrated that the biggest reduction in average encounter time results in very small changes in probability of moving in the direction of the target rather than exhibiting random movement, i.e., when only 5 to 15 percent is directed movement. The complex immune system, somewhat artificially divided up into a pro- and anti-inflammatory pillar, similarly depends on the interplay of multiple, repetitive signals and is susceptible to disturbances of this delicate balance. An exaggerated pro-inflammatory response may lead to an undesired outcome due to ‘collateral’ damage, whereas an inhibited, slow reaction may lead to an unacceptable lag in response. Clearly, further studies should elucidate what balance is optimal in what situation, and to extent to which the inter-individual variation in cytokine responses are determined by preprogrammed, genetic or random, environmental factors.

Polymorphisms in LPS signaling molecules (i.e., Nod2, TLR4, CCR5) and the *in vivo* and *ex vivo* response to LPS.

In the study described in **Chapter 5**, we did not detect an influence of the 3020insC mutation in the Nod2 gene on the *in vivo* and *ex vivo* TNF-α production capacity upon

endotoxin. In view of the conflicting data in the literature, the finding indicates that in studies exploring presumed ligand-receptor relations, great care must be taken to use highly purified ligands rather than crude materials that often contain contaminants that act as ligands for the same or other receptors.

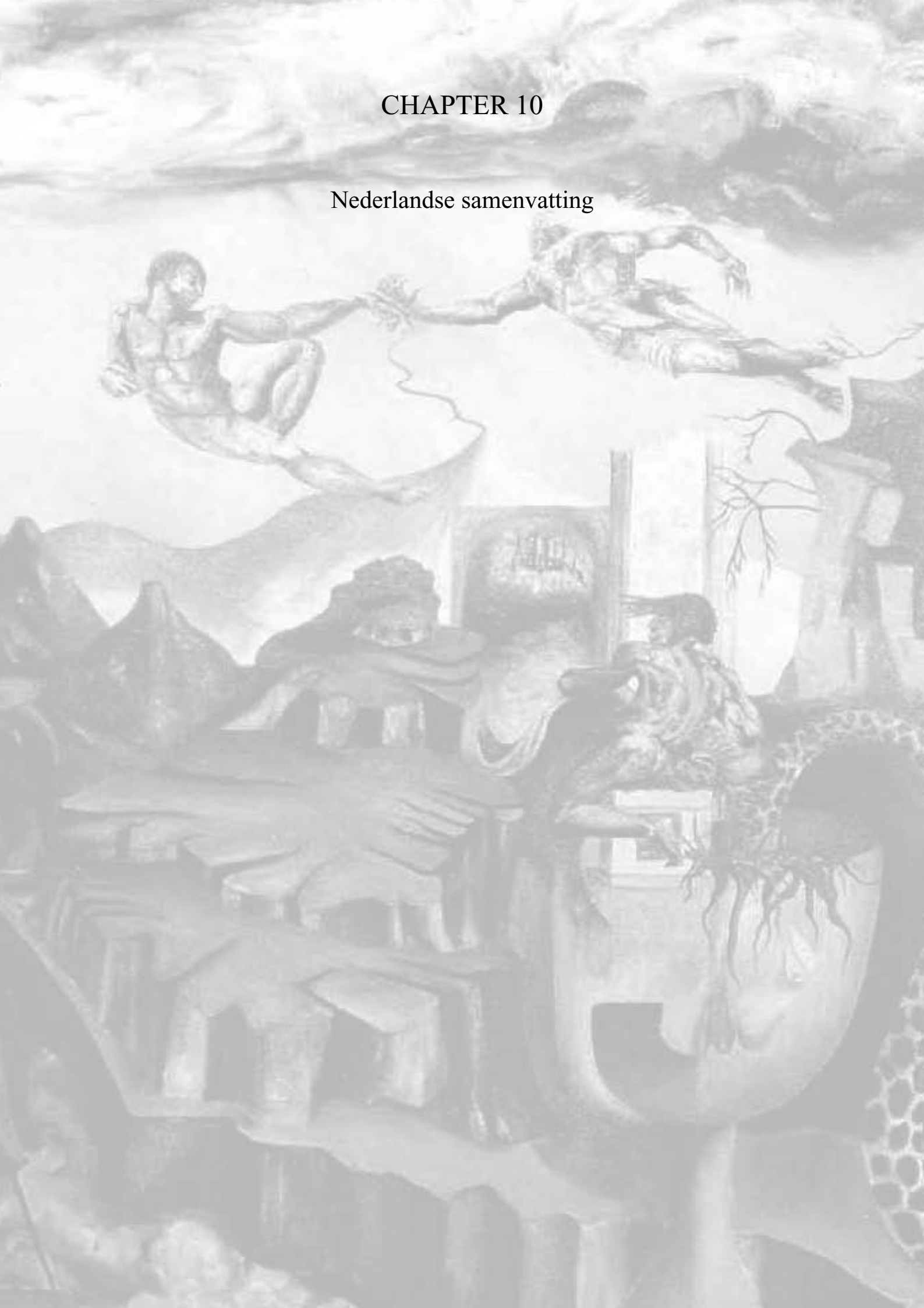
In the study described in **Chapter 5** and **6**, we found no correlation between the level of perioperative and *ex vivo* TNF-α and IL-10 production, and the common TLR4 polymorphisms (Asp299Gly and Thr399Ile). Therefore their role, if present, appears rather limited, and cannot explain the large inter-individual differences found in TNF-α and IL-10 production capacity. In the study described in **Chapter 6**, we found that the common TLR4 polymorphisms were associated with slightly higher IL-10 production capacity *ex vivo*; however, this did not reach the level of statistical significance. At present it remains unclear which and to what extent additional polymorphisms in molecules involved in LPS triggering (e.g. sCD14, MD-2, LBP) add to the large inter-individual variation found in *in vivo* and *ex vivo* responses to LPS.

In **Chapter 7**, we found that the endotoxin-stimulated cytokine release *ex vivo* and *in vivo* did not differ between the individuals homozygous for the wild-type CCR5 allele and persons heterozygous for CCR5 Δ32. In mice, a disruption of CCR5 showed a large reduction in the production of some cytokines upon stimulation with LPS. In humans, however, polymorphisms in competing genes involved in the cytokine production pathway outweigh the effect of a defective CCR5 molecule

In conclusion, our studies in patients undergoing elective cardiac surgery and CPB indicate that naturally occurring endotoxemia can be a stimulus for the release of cytokines, even in the absence of overt infection. Because endotoxemia cannot be predicted before the surgery and occurs in only about half of the patients, i.e., the others may serve as control, studying the elective surgery presents an elegant model of the effect of a relatively frequent yet naturally occurring endotoxemia and cytokine release. The pattern of cytokine responses to endotoxemia *in vivo* appeared much more complex than the relatively simple to describe endotoxin dose-dependent increase in cytokine release *ex vivo*. The cytokine responses to endotoxin appear to be influenced by many factors, most of which are at present ill-defined. Of these factors the genetic background of the individual plays only a minor yet - through its consistent direction - not to be discarded role.

CHAPTER 10

Nederlandse samenvatting



Al sinds mensenheugenis is het mensen opgevallen dat infectieziekten bij de ene persoon veel ernstiger verlopen dan bij de andere. Terwijl de één nauwelijks ziekteverschijnselen vertoont wordt de ander levensbedreigend ziek. Vaak is er wel een duidelijk aanwijsbare oorzaak voor deze verschillen. Zo zijn mensen met al aanwezige ziekten, zoals ziekten van de bloedvaten of kanker meer kwetsbaar. Soms ligt de oorzaak in het feit dat iemand een bijzonder agressieve bacterie heeft opgelopen, die in korte tijd ogenschijnlijk kerngezonde mensen doodziek kan maken (vleesetende bacterie, nekkramp bacterie). Het komt ook voor dat iemand door een infectie zodanig wordt verrast dat hij of zijn arts de ziekte onderschat en er te laat een behandeling wordt ingesteld. Vaak is er echter geen voor de hand liggende verklaring voor de grote verschillen tussen de ziekteverschijnselen van dezelfde ziekte bij verschillende personen.

Om beter te begrijpen hoe de verschillen verklaard kunnen worden, is het belangrijk te kijken naar de werking van het afweersysteem van de mens. Ons afweersysteem is vrij ingewikkeld en bestaat uit allerlei verschillende onderdelen die voor een deel met elkaar zijn verweven. Tijdens ons leven ontwikkelen wij een afweersysteem dat specifiek gericht is tegen bepaalde ziekteverwekkers (verworven immuniteit, acquired immunity). Dit systeem wordt levenslang getraind en zal bij ieder contact met een nieuwe ziekteverwekker een afweerreactie tot stand brengen. Omdat het afweersysteem daar enige tijd voor nodig heeft zijn we dan wel een tijdje (meestal een kleine week) ziek. Als er vervolgens geen complicaties optreden herstellen we volledig en zijn we voor de rest van ons leven immuun tegen deze ziekteverwekker. Van deze laatste eigenschap van het verworven afweersysteem wordt dankbaar gebruik gemaakt door mensen te vaccineren. Door het inbrengen van een entstof wordt de infectie nagebootst en zijn we, vaak levenslang, beschermt tegen deze ziekte. Hoewel dit afweersysteem prachtig werkt, heeft het ook een groot nadeel. Het heeft vrij veel tijd nodig om met een reactie te komen. Waarschijnlijk is de mens daarom nog met een ander soort afweersysteem uitgerust, dat dit nadeel niet heeft. Dit onderdeel van het afweersysteem is al direct bij de geboorte aanwezig en wordt om die reden het aangeboren afweersysteem (innate immunity) genoemd. Dit afweersysteem is in staat om heel snel na het binnendringen van een ziekteverwekker te reageren, en vormt in feite de eerste verdedigingslinie. Ziekteverwekkers verraden hun aanwezigheid door het al dan niet opzettelijk afgeven van stoffen aan hun omgeving. Deze stoffen worden herkend door een groot arsenaal aan antennes (receptoren) die vóórkomen op cellen van het afweersysteem. Bij het gelijktijdig prikkelen van verschillende antennes ontstaat een patroon dat het afweersysteem in staat stelt een gerichte reactie te maken tegen de binnengedrongen ziekteverwekker. Lipopolysaccharide (LPS) is zo'n stof en is afkomstig uit de celwand (het omhulsel) van sommige bacteriën. LPS wordt herkend door cellen van het aangeboren afweersysteem (vooral door de zogenaamde monocyten) en deze cellen

worden er vervolgens door geactiveerd. Ze gaan activerende stoffen produceren waarvan de belangrijkste Tumor Necrosis Factor(TNF)-α wordt genoemd. Deze stof zweept het gehele afweersysteem op tot een verhoogde activiteit. Tegelijkertijd worden er ook remmende stoffen geproduceerd, de belangrijkste daarvan is Interleukine(IL)-10. De balans tussen deze twee stoffen is van groot belang. Als de balans in één van beide richtingen doorslaat ontstaat er een probleem. Zowel een te hoge als een te lage activiteit van het afweersysteem kan ertoe leiden dat er schadelijke effecten voor het lichaam ontstaan, hetzij door de afweerreactie zelf of door de binnendringende ziekteverwekker. Bloedvergiftiging (sepsis) is een ernstige ziekte die kan optreden zodra een infectie uit de hand loopt. In Nederland sterven per jaar naar schatting 5000 mensen aan bloedvergiftiging. De meest voorkomende ziekten die bloedvergiftiging kunnen veroorzaken zijn longontsteking, buikvliesontsteking en blaasontsteking.

Als we ervan uitgaan dat de balans tussen stimulatie en remming van het afweersysteem van belang is voor het verdere verloop van een infectie, dan ligt het voor de hand om aan te nemen dat erfelijke factoren hierbij een rol spelen. In genen van verschillenden personen blijken soms heel kleine verschillen te zitten, die polymorphismen worden genoemd. Vaak gaat het om een verschil in slechts 1 schakeltje (base-paar, Single Nucleotide Polymorphism of SNP) in een lange DNA keten dat we een gen noemen. Uit eerder onderzoek is gebleken dat deze kleine verschillen verantwoordelijk kunnen zijn voor verschillen in het verloop van, onder andere, infectieziekten. Het onderzoek beschreven in dit proefschrift was erop gericht meer inzicht te krijgen in de rol van bekende, genetische verschillen in genen die betrokken zijn bij het tot stand komen van een afweerreactie op LPS.

Zoals hiervoor beschreven herkent het afweersysteem ziekteverwekkers door de aanwezigheid van stoffen die door ziekteverwekkers worden afgegeven aan hun omgeving. LPS bindt aan een oppervlaktestructuur (antenne) die toll-like receptor-4 (TLR4) wordt genoemd (zie Figuur 1 in hoofdstuk 1). Na binding aan TLR4 wordt een cascade in beweging gezet (domino effect) die uiteindelijk leidt tot activatie van genen in de kern van de desbetreffende cel. De genen die worden geactiveerd bevatten de genetische code voor eiwitten, cytokines genaamd (zoals TNF-α en IL-10) die vervolgens door de cel worden uitgestort in hun omgeving. In de genen van TLR4, TNF-α en IL-10 zijn in de afgelopen jaren diverse ‘single nucleotide polymorphisms’ (SNPs) ontdekt. In het onderzoek beschreven in dit proefschrift hebben wij onderzocht in hoeverre deze SNPs verantwoordelijk zijn voor verschillen in de reactie op LPS. Om dit bij mensen te onderzoeken zijn we op zoek gegaan naar een omstandigheid waaronder patiënten met LPS worden geconfronteerd. We zijn terecht gekomen bij patiënten die een operatie aan hun hart moeten ondergaan. Tijdens deze operatie worden patiënten aangesloten op een hart-

longmachine. Het hart kan dan tijdelijk worden stilgezet, zodat de chirurg de operatie rustig kan uitvoeren. De hart-longmachine houdt de bloedsomloop kunstmatig in stand. Hoewel dit op zich goed lukt, is de bloedsomloop toch minder goed dan onder normale omstandigheden. Vooral de darmen worden onder deze omstandigheid minder goed van zuurstof voorzien. Daardoor raken de darmen een beetje doorlaatbaar (lek), en komen stoffen die zich in de darminhoud bevinden in het bloed terecht. Omdat het gaat om heel kleine hoeveelheden, is dat voor de meeste stoffen geen probleem. Een belangrijke uitzondering is het LPS. Zelfs heel kleine hoeveelheden van deze stof kunnen een afweerreactie tot stand brengen. In de praktijk blijkt dat ongeveer de helft van de patiënten die een dergelijke operatie ondergaat een zekere mate van afweerreactie op LPS vertoond. Om deze reden hebben wij deze situatie gebruikt om ons onderzoek uit te voeren. Uiteindelijk hebben we bijna 200 patiënten onderzocht. Kort voor de operatie hebben we van deze mensen bloed afgenomen waaraan we in het laboratorium LPS hebben toegevoegd en vervolgens ook de cytokines hebben gemeten. Tijdens en kort na de operatie hebben we bloed afgenomen dat werd onderzocht op de aanwezigheid van LPS en meerdere cytokines (zoals TNF-α en IL-10). Daarnaast hebben we van deze patiënten de genen onderzocht en gekeken of er bepaalde SNPs aan- of afwezig waren. Vervolgens hebben we de verschillende metingen met elkaar vergeleken om te onderzoeken welke factoren de verschillen in productie van cytokines verklaarden.

In het onderzoek beschreven in hoofdstuk 2 en 3 hebben we onderzocht of het toedienen van antibiotica in de week voor de operatie kan voorkómen dat er tijdens en na de operatie LPS in de bloedsomloop terecht komt. Hoewel het aantal bacteriën in de darm duidelijk lager werd, bleek er geen vermindering op te treden in de hoeveelheid LPS die in de bloedsomloop terecht kwam. Verder was ook de mate van afweerreactie niet verschillend tussen de patiënten die antibiotica hadden gebruikt en zij die dat niet hadden toegediend gekregen. Uit verder onderzoek zal moeten blijken wat de verklaring is voor deze waarneming.

In het onderzoek beschreven in hoofdstuk 5 en 6 hebben we onderzocht wat de samenhang was tussen de mate van LPS dat tijdens de operatie - en kort daarna - in de bloedsomloop kwam en de hoeveelheid vrijgekomen cytokines (TNF-α en IL-10). We hebben gevonden dat er een duidelijke samenhang is. Hoe meer LPS er tijdens de operatie in het bloed komt hoe groter de hoeveelheid aan cytokines die wordt geproduceerd. We hebben echter ook waargenomen dat er cytokines vrijkomen bij patiënten die helemaal geen LPS in hun bloed hadden. Dit betekent dat er nog andere factoren betrokken zijn bij het vrijkomen van cytokines. Uit vervolgonderzoek zal moeten blijken welke factoren dat zijn.

In hoofdstuk 8 hebben we onderzocht in hoeverre het verschijnen van LPS in de bloedsomloop en de productie van cytokines invloed had op de toestand van de patiënt na

de operatie. Wat we gevonden hebben is dat patiënten die meer LPS in hun bloed hadden grotere problemen kregen met het functioneren van hun bloedsomloop. Verder hadden patiënten met een hoge concentratie van IL-10 in het bloed na de operatie een slechter functionerend hart.

In hoofdstuk 4, 5 en 6 hebben we onderzocht of het mogelijk is om met een bloedonderzoek te voorspellen hoe mensen op LPS in hun bloed zullen reageren. We hebben dat onderzocht door bloed, afgenomen voor de operatie, in het laboratorium bloot te stellen aan LPS. Vervolgens hebben we de hoeveel cytokines die vrijkwamen gemeten. Tijdens de operatie hebben we in het bloed van deze mensen soortgelijke metingen gedaan. Door de metingen van iedere patiënt naast elkaar te leggen, konden we kijken of er bepaalde patronen optraden die een zekere mate van voorspelling gaven. Het bleek dat de hoeveelheid TNF-α die in het laboratorium vrijkwam na stimulatie met een hoge concentratie LPS in grote mate voorspellend was voor de hoeveelheid TNF-α die tijdens de operatie vrij kwam. Voor IL-10 hebben we hetzelfde gevonden.

In hoofdstuk 5 hebben we onderzocht in hoeverre SNPs in het TNF-α gen effect hebben op het vermogen om TNF-α te produceren, zowel tijdens de operatie als in het laboratorium. We hebben hiervoor geen bewijs kunnen vinden.

In hoofdstuk 6 hebben we hetzelfde onderzocht als in hoofdstuk 5, maar dan voor het IL-10 gen. We hebben van een aantal SNPs in het IL-10 gen kunnen aantonen dat dit effect heeft op de hoogte van de IL-10 productie. Inmiddels zijn er ook onderzoeken door anderen verricht die soortgelijke resultaten hebben opgeleverd. We hebben hier dus een belangrijke aanwijzing gevonden dat deze SNPs voor een deel verantwoordelijk zijn voor de verschillen die worden gevonden in de wijze van het ontwikkelen van een afweerreactie.

In de hoofdstukken 5, 6 en 7 hebben we onderzocht in hoeverre genetische verschillen in de receptoren van LPS (TLR4, nod2) en het CCR5 gen effect hadden op het vermogen om te reageren op LPS. We hebben van de verschillende genetische polymorphismen geen effect kunnen aantonen.

De belangrijkste conclusie van ons onderzoek is dat het bestuderen van patiënten die een hartoperatie ondergaan een goede manier is om onderzoek te doen naar de afweerreactie op LPS. Het vrijkomen van LPS is bij deze patiënten een belangrijke factor bij het ontstaan van een afweerreactie. We moeten ons echter wel realiseren dat er nog vele andere factoren een rol spelen bij het ontstaan van deze afweerreactie. Deze factoren zijn nog grotendeels onbekend en toekomstig onderzoek zal hierin opheldering moeten brengen.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 21 mei 1965 te Utrecht. In 1981 behaalde hij het MAVO diploma aan de Willibrord MAVO in Noordwijk. Datzelfde jaar werd begonnen met de opleiding tot analist aan de Middelbare Laboratorium Opleiding van de Laboratorium School Rijnland te Leiderdorp. Nog datzelfde jaar ging hij over naar het Voorbereidend Jaar Hogere Laboratorium Opleiding waarna in 1982 werd begonnen met de Hogere Laboratorium Opleiding (richting medische microbiologie en hematologie). In het kader van deze opleiding werden stages gelopen bij de afdeling Stralengenetica en Mutagenese (hoofd: Prof.dr. F.H. Sobels) van het Sylvius Laboratorium te Leiden en het Laboratorium Medische Microbiologie (hoofd: Dr. G.L. Smit) van het Diaconessenhuis te Leiden. Na het behalen van het analisten diploma was hij gedurende een jaar werkzaam als medisch microbiologisch analist op het laatst genoemde laboratorium. In 1986 werd begonnen met de studie geneeskunde aan de Rijksuniversiteit Leiden. In 1990 werd het doctoraal examen gehaald. Na het artsexamen in 1992, werkte hij achtereenvolgens bij de afdeling Chirurgie van het Elisabeth Ziekenhuis te Leiderdorp en de afdeling Cardiologie van het Academisch Ziekenhuis te Leiden. De opleiding tot internist vond plaats tussen 1993 en 1999, de eerste twee jaar in het Groene Hart Ziekenhuis te Gouda (opleider Dr. K.J. Heering) en van 1995 tot 1999 in het Academisch Ziekenhuis te Leiden (opleider Prof. Dr. A.E. Meinders). In juli 1999 werd hij ingeschreven in het specialistenregister Inwendige Geneeskunde. Aansluitend werd hij opgeleid in het aandachtsgebied Infectieziekten (opleider Prof. Dr. J.T. van Dissel), resulterend in de registratie als infectioloog in 2001. Tussen september 2001 en medio 2004 werd het in dit beschreven onderzoek verricht. Vanaf 1 juli 2001 is hij werkzaam als staflid op de afdeling Infectieziekten van het Leids Universitair Medisch Centrum.

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