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

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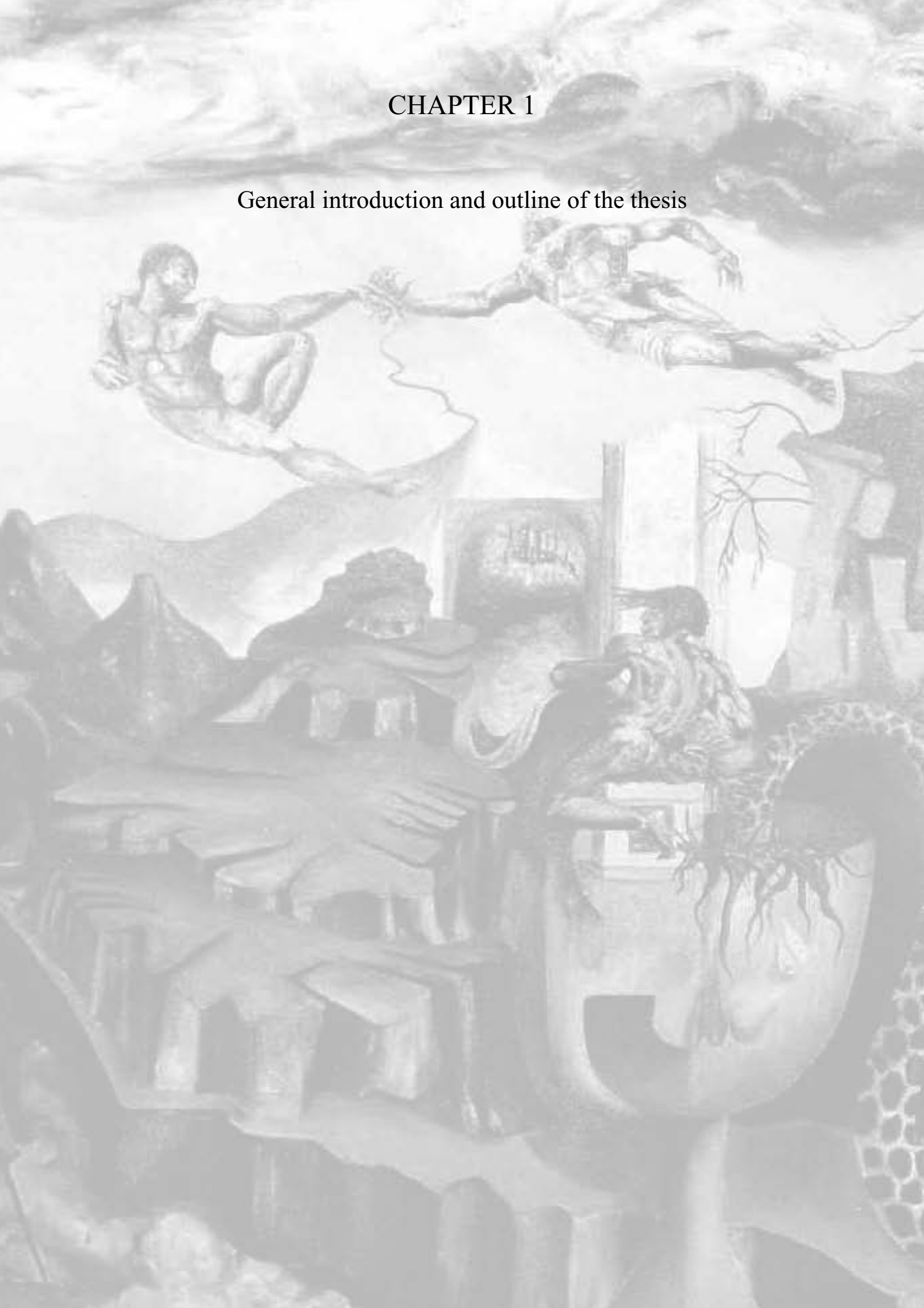
CHAPTER 1

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).

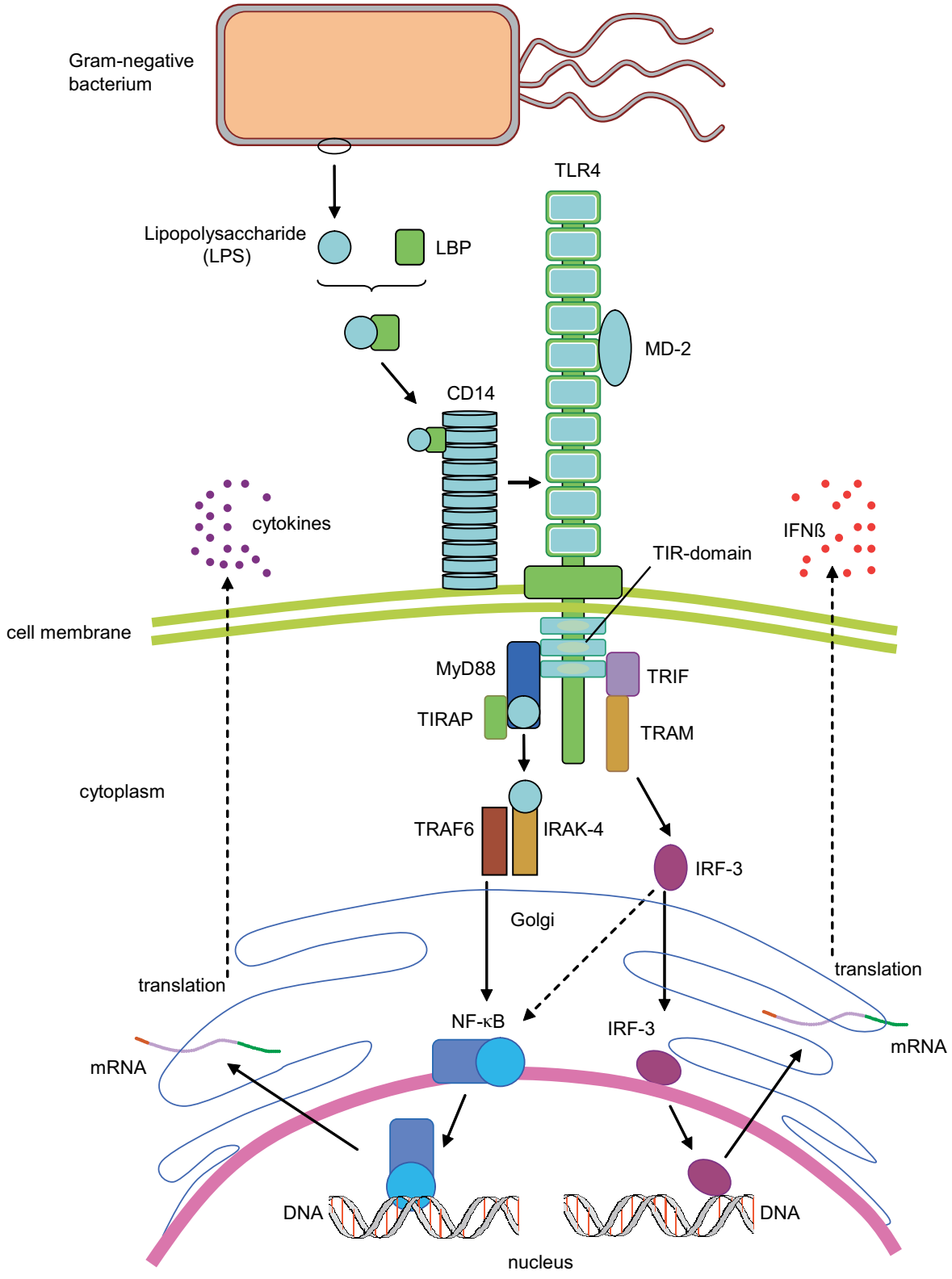


Figure 1: TLR4 signaling of LPS. LPS=lipopolysaccharide, LBP= lipopolysaccharide binding protein, TLR4=toll-like receptor-4, MD-2=myeloid differential protein, TIR-domain=Toll-Interleukin-1 resistance domain, MyD88=myeloid differentiation factor 88, TIRAP= TIR domain-containing adapter protein, IRAK-4=interleukin-1 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).

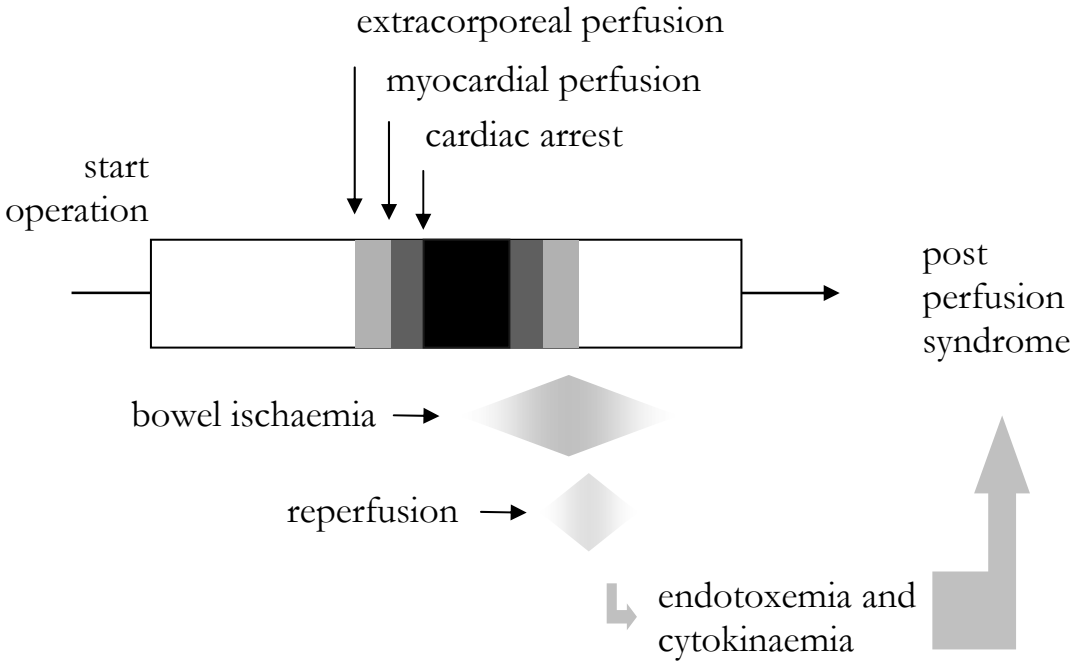


Figure 2. Schematic representation (time-line) of the *in vivo* model. In patients undergoing cardiac surgery, extracorporal perfusion is applied. In the first phase of the procedure, the heart is perfused and stops beating, circulation and oxygenation is maintained by an extracorporal system('Heart-Lung 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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