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TOLL-LIKE RECEPTOR-INDUCED CYTOKINE SECRETION BY HUMAN MONOCYTES IN HEALTHY DONORS AND SEPTIC PATIENTS ASSESSED AT THE SINGLE CELL LEVEL

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ABSTRACT

Following the ligation of Toll-like receptors (TLRs) by bacterial-derived signature molecules, innate immune cells such as monocytes, macrophages and neutrophils initiate the inflammatory process by secreting chemokines and cytokines with a wide range of immunomodulatory effects. Under normal conditions, these mediators work to compartmentalize the inflammatory process and eradicate the intruder, while simultaneously recruiting more immune cells to the site of infection. In sepsis, however, the virtues of this containment are lost as the response against a disseminated bacterial infection becomes systemic with an excessive production of cytokines that can lead to tissue injury, organ dysfunction and ultimately death. Here we have investigated TLR-induced cytokine secretion *in vitro* by monocytes and granulocytes from healthy donors and septic patients using the ELISpot and/or FluoroSpot assays. By looking at the secretion of cytokines directly *ex vivo*, the ELISpot assay offered the potential of being able to better define the immunological status of septic patients. We investigated the lipopolysaccharide (LPS)-induced cytokine secretion by peripheral blood mononuclear cells (PBMC) and granulocytes from healthy donors using the ELISpot assay. Cytokines (IL-1β, IL-6, IL-10, IL-12p40, TNF-α, GM-CSF) and chemokines (IL-8, MIP-1β) important in inflammatory processes were assessed. Granulocytes were found to selectively secrete IL-8 and MIP-1β. Also TNF-α was secreted but by considerably fewer cells. In contrast, PBMCs secreted all evaluated cytokines with $CD14^+$ monocytes being the main source of production. Next, we analyzed the cytokine secretion by enriched monocytes from healthy donors in response to LPS and lipoteichoic acid (LTA). The FluoroSpot technique allowed for the simultaneous analysis of two cytokines from the same population of isolated cells. With this approach, a recurring pattern of cytokine co-secretion was observed, identifying several distinct cytokine-secreting profiles for the TNF-α-, GM-CSF-, IL-10-, IL-12p40-secreting monocytes and those secreting IL-6 or IL-1β. Finally, the spontaneous and LPS-induced cytokine secretion by total leukocytes isolated from septic patients (n=32) and healthy controls (n=30) was evaluated using the ELISpot assay. Surprisingly, we found no increase in the number of constitutively cytokine-secreting cells from any of the septic patients despite significantly increased levels of cytokines (IL-6, IL-1β, TNF-α, GM-CSF, IL-10, IL-12p40) in their plasma. Simultaneously, the LPS-induced *in vitro* capacity revealed a maintained (IL-6, TNF-α) as well as reduced (IL-1β, GM-CSF, IL-10, IL-12p40) number of cytokine-secreting monocytes in the patients compared to normal donors. This selective reduction for some of the cytokines could be correlated with disease severity. In conclusion, our data indicate that circulating monocytes are not the major source of increased cytokine levels in patients with sepsis.

LIST OF PUBLICATIONS

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CONTENTS

1 INTRODUCTION 1
2 BACKGROUND 2
2.1 Immunology 2
2.1.1 The innate immune system 2
2.1.2 The adaptive immune system 4
2.1.3 Cytokines and chemokines 6
2.2 Innate immunity sensors: a call to arms 8
2.2.1 Signaling pattern-recognition receptors (PRRs) 8
2.2.2 The family of TLRs 10
2.2.3 Intracellular signaling following TLR-activation 11
2.2.4 Negative TLR-regulators 13
2.3 Monocytes, macroph., neutro. and the inflammatory response 14
2.3.1 Initiation of the inflammatory response 14
2.3.2 Activation of the coagulation system 17
2.3.3 Macrophage differentiation 17
2.3.4 Macrophage plasticity and the resolution of inflammation 19
2.4 Monocyte heterogeneity 21
2.4.1 Three monocyte subsets and their production of cytokines 21
2.4.2 Methodological considerations 24
2.5 The pathophysiology of sepsis 25
2.5.1 Role of monocytes in sepsis 26
2.5.2 The anti-inflammatory phase of sepsis 27
3 AIMS OF THESIS 29
4 MATERIALS AND METHODS 30
4.1 Isolation of PBMC (paper I-II) 30
4.2 Isolation of granulocytes (paper I) 30
4.3 Depletion of CD14 positive cells (paper I) 30
4.4 Isolation of monocytes (paper II) 31
4.5 Isolation of $CD16^{+}$ monocytes (paper II) 31
4.6 Isolation of leukocytes using dextran (paper III) 31
4.7 ELISpot assay (paper I-III) 32
4.8 ELISA assay (paper I) 32
4.9 FluoroSpot assay (paper II) 32
4.10 Flow cytometry (paper II) 33
4.11 Luminex assay (paper III) 33
4.12 Injection of endotoxin into healthy volunteers (paper III) 33
4.13 Patients (paper III) 34
4.14 Statistical analysis (paper I-III) 34
5 RESULTS AND DISCUSSION 35
5.1 Paper I - ELISpot analysis of LPS-stimulated leukocytes: Human granulocytes selectively secrete IL-8, MIP-1beta and TNF-alpha 35

5.2 Paper II- FluoroSpot analysis of TLR-activated monocytes reveals several distinct cytokine-secreting subpopulations 39
5.3 Paper III- Circulating monocytes are not the major source of plasma cytokines in patients with sepsis 44
6 CONCLUSIONS 50
7 POPULÄRVETENSKAPLIG SAMMANFATTNING 51
8 ACKNOWLEDGEMENTS 54
9 REFERENCES 57

LIST OF ABBREVIATIONS

AMP	Anti-microbial peptides
APC	Antigen presenting cell
CARS	Compensatory anti-inflammatory response syndrome
CLR	C-type lectin receptor
DAMP	Danger associated molecular pattern
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot assay
ERK	Extracellular-signal-regulated-kinase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBP	Heparin binding protein
HMGB1	High mobility group box protein 1
HNP	Human neutrophil peptide
ICAM	Intercellular adhesion molecule
ICS	Intracellular cytokine staining
IKK	IκB kinase
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
IFN	Interferon
IRAK	IL-1 receptor-associated kinase
IRF3	Interferon-regulatory factor 3
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinases
MBL	Mannan-binding lectin
MCP-1	Monocyte-chemoattractant protein 1
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MIP-1β	Macrophage inflammatory protein 1 beta
MyD88	Myeloid differentiation primary response gene 88
MyD88s	Myeloid differentiation primary response gene 88 short
NF-κB	Nuclear factor κB
NK	Natural-killer
NLR	Nod-like receptor
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PRR	Patter recognition receptor
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RLR	RIG-like receptor
SARM	Sterile-α and Armadillo motif containing proteins

SIKE	Suppressor of IκBε
SIRS	Systemic inflammatory response syndrome
SOCS-1	Suppressor of cytokine signaling 1
SOFA	Sequential Organ Failure Assessment score
TAK1	Transforming-growth-factor-β-activated kinase 1
TF	Tissue-factor
TGF-β	Transforming growth factor-β
Th	T-helper
TIR	Toll/IL-1 receptor
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOLLIP	Toll-interacting protein
TRAF6	TNF-receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor inducing IFN-β

1 INTRODUCTION

The role of microbes in infectious diseases is today common knowledge. However, the first schools of medicine were understandably challenged in explaining how these lethal illnesses could originate and be transmitted. Physicians in the middle ages spoke of an airborne poison as a reasonable explanation due to the similarities in symptoms of some infectious diseases and those seen after intoxication. The theory named this poison “miasma”, which was believed to originate as a noxious gas following the decomposition of organic material present within dying patients or in slow-moving waters such as swamps. An alternative viewpoint was that a poisonous material, termed “contagion” caused lethal illnesses and that this dangerous material originated in rotten fish or meat. Different from the theory of miasma, the contagion did not spread through the air, only by direct physical contact [1].

Although the two models of miasma and contagion failed to explain the true nature of infectious diseases, it was not until the 19th century that a causative connection was made to that of living microorganisms. At that time, Luis Pasteur demonstrated in a series of experiments that bacteria were responsible for the decay of organic material. Furthermore, he recognized that bacteria were not only capable of causing infectious disease but they were actually the single requirement for an infection to be initiated. Robert Koch went on to show how specific microorganisms gave rise to certain infectious diseases and laid out the principles for how to determine the causal relationship between the two [1]. Nevertheless, once science transitioned from a pre-microbial to a post-microbial era, a new set of questions began to emerge [2]: How does the body recognize the bacteria? What kind of bacterial products elicits the bodily reactions associated with disease?

Thankfully, due to great discoveries in innate immunity during the last 15 years, those questions have finally been answered at a molecular level [3]. We today understand how bacterial infections are perceived by the host and what kind of bacterial products are recognized, yet much work remains to be done in defining the nature of the response once it has been initiated and propagated throughout the complexity of a living organism. This need is perhaps best exemplified by the continued difficulties of finding good therapeutic options for patients overreacting to bacterial infections [4]. Understanding and balancing the adverse systemic inflammatory response in sepsis, severe sepsis and septic shock represents a major challenge that will require an even deeper knowledge of cytokines and how the innate immune system can be regulated. This thesis is an attempt to simply provide additional sentences to this unfinished story, focusing on the cellular immune response of monocytes in healthy donors and septic patients at the single cell level.

2 BACKGROUND

2.1 IMMUNOLOGY

In jawed vertebrates, including animals like fish, amphibians, reptiles, mammals and birds, the defense system against foreign invaders is divided into two branches of immunity. One part, the innate immune system, is considered a first line of defense and is solely dependent on the encoded behavior passed down through our germ-line, i.e. its response is immediate, genetically pre-determined and present from the moment of birth. The second part, the specific or adaptive immune system, comprising T and B lymphocytes is, in comparison, much more specialized and ensues first after innate immunity has attempted to eliminate an infectious organism. Thus, the adaptive immune response takes longer to fully develop but is antigen-specific, meaning that it is focused in its effector functions against discrete parts of a microbe [5, 6]. I will in the following sections briefly outline the organization of these two systems primarily from an anti-microbial perspective.

2.1.1 The innate immune system

Innate immunity is divided into a cellular and a soluble arm, the former being comprised of specialized cells and the latter of serum factors such as complement and anti-microbial peptides. In addition, elements of innate immunity also include the natural barriers of our body such as the skin and the epithelium. These surfaces are in constant contact with the outside environment and therefore a primary route of infection for the pathogens that we encounter. Here, mucus membranes, lowered pH and secreted enzymes help protect the host against numerous attacks even before they are able to induce an immunological reaction [5, 6].

Monocytes/macrophages and **granulocytes** primarily combat invasive microorganisms through a process called phagocytosis in which the foreign invader is engulfed, killed and digested inside phagolysosomal granules containing toxic radicals and antimicrobial molecules [5]. Phagocytosis is enhanced by several classes of so called pattern-recognition receptors (PRRs) which help to facilitate the identification and uptake of invading bacteria. These receptors, categorized into either endocytic or signaling PRRs [7], identify key structural components that are either inherent to the pathogen itself or have been deposited there by other factors of the immune system. PRRs include, among others, scavenger receptors, mannose receptors and Toll-like receptors (TLRs). Upon activation, phagocytes also release soluble substances including cytokines, which not only work as effector molecules but also enable communication with other cells. Some of these are referred to as chemokines as they possess chemotactic activity and, when released, serve to recruit and guide other cells to sites of infection [5, 6]. Other soluble substances that are released by phagocytes are

included in the so-called respiratory burst of reactive oxygen species (ROS) and comprise superoxide anion, hydrogen peroxide and hypochlorous acid, all with potent microbicidal activity [6, 8]. Moreover, superoxide anion can also react with endogenously produced nitric oxide, giving rise to reactive nitrogen species (RNS) that complement the oxidative attack by ROS [9]. Macrophages and neutrophils are essential for withstanding infections and, due to their widespread location in tissues and swift recruitment, they are often the first to make contact with a foreign intruder [5].

Dendritic cells (DCs), named for their characteristic cell membrane with protruding dendrites, are specialized in antigen uptake and have a very important function as antigen-presenting cells (APC) [10]. Antigen presentation is a key factor in T-cell stimulation, as T cells do not "see" antigens in their native form (e.g. an intact bacterium or a protein) but as short peptides bound to MHC (major histocompatibility complex) molecules on DCs or other cells with antigen-presenting capacity such as macrophages and B cells [11, 12]. Dendritic cells hereby fulfill an important link between innate and adaptive immunity, and being the only cells [10] that can properly activate naïve T cells they are often referred to as professional APCs. To accomplish their role, DCs effectively bind to, internalize and degrade a pathogen. Degradation takes place within the phagolysosome and results in the formation of short antigenic peptides which, in turn, bind to MHC molecules and become translocated to the cell surface. Here they are recognized by antigen-specific T cells through their receptors, which can recognize and bind foreign peptides only in the context of a peptide-MHC complex [13]. Like other members of the innate immune system, dendritic cells express numerous PRRs which facilitate the identification of pathogens and mediate cell activation. Thus, upon bacterial encounter and ligation of specialized PRRs, dendritic cells undergo a process of maturation that leads to increased antigen uptake, migration into the draining lymph node, upregulation of activating cell surface molecules and production of various cytokines, all essential for the peptide presentation and activation of T cells with the appropriate antigen specificity [14, 15].

Natural-Killer (NK) cells are categorized as innate lymphoid cells (ILCs) of the innate immune system and constitute approximately 1-6% of circulating leukocytes in the peripheral blood [16, 17]. NK cells are especially critical in the early host response against viruses but are also important in immunity against certain classes of intracellular bacteria. NK cells target and destroy host cells which display altered features of "self", notably down-regulated MHC class I molecules often induced by the pathological processes of viral infection and tumorigenesis. Killing is achieved not by phagocytosis but by release of cytotoxic proteins and proteases which permeate the target cell and induce osmotic cell lysis or programmed cell death (apoptosis) [5, 6].

The **complement system** is central in the soluble arm of innate immunity and consists of a multitude of circulating proteins that can mediate the clearance of bacteria through three types of complement cascades: the classical, the alternative and the mannose-binding lectin (MBL) pathways [18]. Initiation of these different pathways relies on the recognition of key structures found on surfaces of bacteria, either directly or through an

element which has been located there by other parts of the immune system. For example, the classical pathway is primarily activated by the recognition of antigen-antibody complexes whereas the alternative and MBL pathways are initiated by direct complement binding to the bacterial membrane. All pathways eventually end in the formation of the membrane attack complex (MAC) which weakens the integrity of the cell membrane and induces bacterial lysis [19]. In addition, complement fragments deposited on the surface of bacteria also act as opsonins for receptors on neutrophils and macrophages facilitating the process of phagocytosis. Moreover, a number of cleaved fragments are referred to as anaphylatoxins and can mediate systemic effects by binding to specific receptors, influencing the blood flow controlled by localized smooth muscle and the release of inflammatory proteins from nearby mast cells [5]. Together, these factors help to intensify the inflammatory response by increasing the accumulation of fluids and cells recruited to the site of infection.

All species resist bacterial invasion through the production of **anti-microbial peptides (AMPs)** [20]. These are short, soluble, amphipathic molecules with a broad range of anti-microbial effects including direct disruption of bacterial cell membranes, modulation of the inflammatory response and recruitment of phagocytes. AMPs are generally divided into defensins and cathelicidins and are produced by several different types of immune cells, including epithelial cells, macrophages and neutrophils [21]. Upon microbial infection, they are either quickly synthesized and released or cleaved from precursors stored in cellular granules [20].

2.1.2 The adaptive immune system

The adaptive immune system can be divided into a cellular and a humoral arm, mediated by T and B lymphocytes, respectively. Different from the innate immune system, T and B cells recognize pathogens in a much more specific manner and can through the generation of memory cells remember past insults by a previously defeated pathogen. More specifically, individual T cells and B cells exhibit specificity against a particular antigen by virtue of their individual T- and B-cell receptors, both of which are generated through a randomized form of gene rearrangement and unique for each single lymphocyte. Due to this process and the process of clonal expansion that is a result of antigen activation of naïve T or B cells, adaptive immune responses against a pathogen take longer to initiate and develop [5, 6].

B cells and their secreted products, antibodies, constitute the humoral part of adaptive immunity. IgM, IgG, IgA and IgE antibodies are the secreted entities of the membrane expressed B-cell receptor and recognize a specific and defined part of an antigen (i.e. an epitope). Upon binding to its antigen, antibodies help to direct and focus the immune response; neutralizing the invader by direct blockage and/or promoting increased opsonization for elimination by other mechanisms of the immune system (e.g. complement activation, enhanced phagocytosis and antibody-dependent cellular cytotoxicity, ADCC). Although active against a variety of pathogens, humoral

immunity is believed to be particularly important in the protection against extracellular microbes [5].

The effector arm referred to as cell-mediated immunity is comprised of several subsets of **T cells** with specialized functions. T cells are especially effective against intracellular pathogens such as viruses and certain classes of bacteria [5]. As mentioned above, the initial contact between antigen and an individual T cell is dependent on the presentation of the antigen on MHC class I and II molecules performed by APCs such as macrophages and dendritic cells. These cells patrol tissues armed to encounter pathogens, which then will be taken up and brought to secondary lymphoid organs where they will be presented to the T cells. The process of presentation divides T cells into two main classes depending on which MHC molecule on the APC that is engaged; the $CD4^+$ T cells recognize peptides presented by MHC class II molecules, whereas $CD8^+$ T cells recognize peptides presented by MHC class I [5]. Once activated, $CD8^+$ T cells differentiate into cytotoxic T cells that kill infected target cells in a cognate, cell-to-cell interaction [22]. Conversely, antigen primed $CD4^+$ T cells expand and differentiate into various subsets of T-helper (Th) cells with a broad array of immunoregulatory functions. These subsets are defined primarily on the basis of the cytokines they produce and currently at least five distinct subsets have been defined (Table 1) [23]. The initial contact between an APC and the naïve $CD4^+$ T cell is here supposed to play a crucial role in directing the T cell toward a specific Th-phenotype. Several APC and environmental signals, such as the nature of the antigen presented, cytokines secreted and co-stimulatory molecules engaged are integrated by the T cell and decide the final outcome of Th-development [24-26]. In a microbial setting, antigen presentation typically results in Th1 differentiation and has been shown to be dependent on the production of Interleukin (IL)-12 by the presenting APC [27]. Th1 cells, in turn, induce the propagation of inflammation suitable for eliminating bacteria by regulating macrophage activation and the production of special subclasses of antibodies by the B cells. In contrast, Th2 cells and the cytokines these cells produce (e.g. IL-4 and IL-13) promote B cells to secrete IgE and activate macrophages for an effective immune response against various types of extracellular parasites [5].

Table 1: Cytokine secreting profiles of T helper (Th) subsets

Th1	Th2	Th9	Th17	Th22
IFN-γ	IL-4 IL-13	IL-9 IL-10	IL-17A IL-21	IL-22

IFN: Interferon, IL: Interleukin

2.1.3 Cytokines and chemokines

As evident from above, the engagement and co-operation of both innate and adaptive immunity is often necessary in order to mount a response against a pathogen. The cellular communication that signifies these processes is to a large extent dictated by a group of proteins called **cytokines**. These chemical messengers, usually having a molecular weight between 10 and 30 kDa, regulate the initiation, duration and course of the immune response by binding to specific receptors on the surface membrane of cells. Their secretion is tightly controlled and they are typically present in very low concentrations (10^{-10} to 10^{-15} M). Once a particular cytokine has been secreted, it can either bind to receptors located on the secreting cell itself (i.e. autocrine production), receptors on nearby cells (paracrine production) or to receptors on cells located in distal parts of the body (endocrine production). Different cytokines generate different types of responses based on the modifications in gene expression induced by the cytokine receptors activated. Thus, a receptor-bound cytokine is able to influence the behavior of a target cell by activating or inhibiting basic functions such as activation, differentiation and/or proliferation [5, 6].

The effects of cytokines are generally pleiotropic and may exhibit additive, synergistic or antagonistic features when secreted together. A level of redundancy exists in that many cytokines appear to fulfill very similar functions. Clearly, these features make it difficult to distinguish a precise role of a cytokine in a given situation but categorizations have still been made based on their structure, receptor specificity and function [6]. The latter usually encompasses a generalized division into pro-inflammatory or anti-inflammatory [28]. Here, classical examples of pro-inflammatory cytokines include IFN-γ, TNF-α, IL-1β, IL-12p70 and IL-23, whereas IL-10, TGF-β and IL-1ra are considered as anti-inflammatory [29]. There are also cytokines such as IL-6 which have been demonstrated to play a role in both sides of this spectrum [30].

The **chemokines** are a special subfamily of cytokines that regulate chemotaxis [6]. They control the migration of several leukocyte populations by binding to G-protein-coupled receptors on the surface membrane of nearby target cells. The recruitment is guided by a concentration gradient that attracts cells to move in the direction of the chemokine-secreting source [31]. This is especially highlighted in situations of an encountered pathogen, where typically macrophages and/or neutrophils secrete chemokines in order to guide and recruit other immune cells to the site of infection. The chemokines are small with a molecular weight of only 7-12 kDa and are categorized into four families based on the position of conserved cysteine residues important in forming their 3-dimensional structure [32]. Some chemokines are considered pro-inflammatory as they are primarily released in situations of infectious stimulation, while others are housekeeping chemokines, being constitutively produced and important in regulating migratory homeostasis and development [31]. Well-described chemokines especially related to this thesis include IL-8 (also known as CXCL8), MIP-1β (CCL4) and MCP-1 (CCL2).

Research studying the properties of the different cytokines and chemokines has been greatly facilitated by the development of immunoassays using cytokine-specific monoclonal antibodies [33]. Methods such as the enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot assay (ELISpot) and intracellular cytokine staining (ICS) in combination with flow cytometry has allowed investigators to gain insight into when and where different cytokines are produced and secreted. As implied by its name, ICS detects cytokines inside of cells, whereas both ELISA and ELISpot detect the cytokine after it has been secreted. This distinction is important in situations where production and secretion may be separately regulated by the activated cell. Moreover, while ELISA measures the amount of secreted cytokine in solution, ELISpot detects the frequency of cytokine-secreting cells within a population of cells [6].

2.2 INNATE IMMUNITY SENSORS: A CALL TO ARMS

While different with regard to their effector functions, innate and adaptive immunity are interlinked through an intricate and sophisticated network of co-operation. Here, the ancient building blocks of innate immunity have formed the support structures necessary for the evolution of the more advanced adaptive immune system. Thus, the inflammatory response, originating from within the innate immune system and used for shaping the most appropriate antigen-specific response, has in retrospect been catered to guide and influence the pathways taken by the adaptive immune system [34]. How innate immunity in this way "sense" its environment and mounts a rapid response based on cytokine release, conveying the nature of the pathogen encountered, was for many years unknown to immunologists [35].

A theory of inherited receptors as a way of communicating this information was described in 1989 by Charles Janeway [36]. With brilliant insight, Janeway argued that, due to the heterogeneity and randomness of receptors generated in adaptive immunity, B and T cells would be unsuitable for the initial recognition of self versus non-self. Instead, he proposed that cells within the innate immune system, expressing receptors with a much broader type of pattern recognition would be better suited for this initial contact. Once triggered, they could serve not only as a first line of defense but also as instructors to the adaptive immune system. The first hints of the existence and nature of these special types of receptors were provided in 1996, when Jules Hoffman discovered how the gene "Toll" controlled the antifungal immune response in fruit-flies [37]. The year afterwards, Janeway himself identified a human homologue of Toll which controlled the activation of several cytokines and expression of co-stimulatory molecules in the monocytic cell line THP-1 [38]. Finally, in 1998, Bruce Beutler fully unlocked the relationship between a Toll receptor and its specific ligand when he demonstrated how mice unresponsive to lipopolysaccharide (LPS) had a point mutation in the Toll gene *tlr*4 rendering its function defective [3]. Consequently, mice were in the possession of a germ-line encoded receptor, which allowed them to recognize and respond to a major component derived from the cell wall of gram-negative bacteria. Without its proper functioning, mutant mice were overly susceptible to live infections using gram-negative bacteria but at the same time unscathed by a normally lethal injection of LPS [39]. These results highlighted the very important role in anti-microbial immunity of this receptor which today is referred to as TLR4 [2]. Together, these discoveries have provided fundamental insights into the biology of innate immunity and how this is interlinked with the adaptive immune system.

2.2.1 Signaling pattern-recognition receptors (PRRs)

To date, several families of these "innate immunity sensors" or signaling PRRs have been identified. Apart from the Toll-like receptors (TLRs), they include C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and the RIG-like receptors (RLRs) [40-

42]. The PRRs can be expressed either at the cell membrane or intracellularly, ensuring detection of both intra- and extra-cellular pathogens (Figure 1).

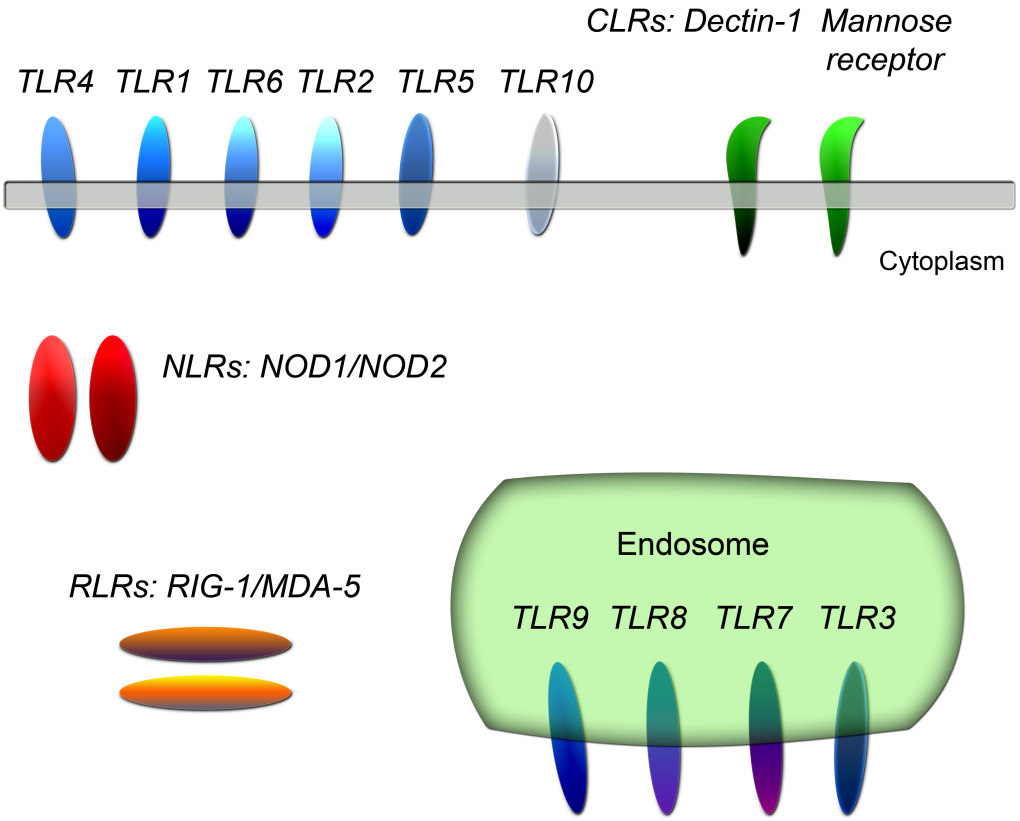

Figure 1.
Simplified illustration describing the cellular localization of the different families of PRRs (TLRs, CLRs, NLRs and RLRs). Modified from [43].

Typically, PRRs recognize and bind to structures on the pathogens, which have been conserved throughout evolution. Collectively, these motifs are called pathogen-associated molecular patterns (PAMPs) and include diverse molecules such as lipids, carbohydrates and nucleic acids [40-42]. For example, RLRs and NLRs are both located in the cytoplasm were they recognize viral nucleic acids and bacterial peptidoglycans, respectively [44, 45], while CLRs are transmembrane receptors interacting with microbial carbohydrates [46]. Not only do PRRs bind and respond to PAMPs but a number of them have also been shown to recognize so called danger-associated molecular patterns (DAMPs). These are endogenously produced molecules released by injured or necrotic cells [47]. Notably, degraded components of the extracellular matrix, certain heat-shock proteins and the nuclear factor high-mobility group box 1 protein (HMGB1) have all been classified as DAMPs capable of interacting with TLR4 [42]. Thus, in addition to its function as a receptor for LPS, TLR4 gives cells the ability to sense and react to an ongoing destruction of own host cells.

The activation of PRRs leads to the triggering of a series of downstream intracellular signaling pathways which, in turn, control the transcription of inflammatory mediators,

including cytokines, modulators of PRR signaling as well as many uncharacterized proteins [40-42]. While activation of different PRRs can lead to the production of the same or a similar set of cytokines, the level of expression can be quite different even within the same family of PRRs [48]. In addition, variants of a particular ligand binding the same PRR may result in different levels of cytokine production, despite activating the same type of signaling pathway [49].

2.2.2 The family of TLRs

As detailed above, TLRs were the first family of PRRs discovered and subsequent studies have identified ten functional human receptors (TLR 1-10) in this family, many of which are expressed by monocytes and neutrophils [50, 51]. TLRs can be grouped depending on cellular location; TLR1, 2, 4, 5 and 6 are found on the surface of cells and primarily detect a variety of microbial membrane products such as peptidoglycan (PGN), lipoteichoic acid (LTA), LPS and flagellin (FLG). In contrast, TLR3, 7, 8 and 9 are found inside of cells, more specifically in the membrane of endosomes where they primarily recognize viral and bacterial nucleic acids. TLR10 is expressed at the cell surface but its function is currently unknown [41]. All TLRs share a conserved structure involving an intracellular Toll/IL-1 receptor (TIR) signaling domain necessary for communicating TLR-activation to the nucleus and an extracellular, leucine rich repeats (LRRs) domain that gives the TLR its ability to bind specific ligands [40-42]. Upon PAMP ligation, TLRs are stabilized as dimers, forming a specific "m-shape" architecture that allows for the intracellular TIR domains to be properly aligned [41]. The dimerizing property of TLRs can in certain cases generate new binding abilities depending on the combination of TLR-receptors. For example, TLR2 can form heterodimers with either TLR1 or TLR6 to expand its repertoire of PAMP recognition to include not only peptidoglycan but also common bacterial derivatives such as lipoprotein (TLR1/TLR2) and LTA (TLR2/TLR6) [40-42]. In contrast, TLR4 exclusively forms homodimers (TLR4/TLR4). These, however, associate with myeloid differentiation factor 2 (MD-2) and the LPS co-receptor CD14 [52] to form an LPS-binding complex. In addition, a serum component, LBP (LPS-binding protein) facilitates the transfer of LPS molecules to the receptor complex and is thus an important component in LPS-signaling [53]. For LTA recognition, both CD14 and CD36 have been described as co-receptors in the activation of TLR2/TLR6 [54]. A summary of the current knowledge of human TLRs, their co-receptors and examples of their respective ligands is shown in Table 2.

Table 2. TLRs, their co-receptors and examples of specific ligands.

Receptor	*Co-receptor (s)*	**Location**	**TLR-ligand**
TLR1/2		Cell surface	Triacyl lipopeptides
TLR2	*CLR Dectin-1*	Cell surface	Peptidoglycan
TLR3		Endosome	dsRNA virus
TLR4	*MD-2, CD14, LBP*	Cell surface	LPS
TLR5		Cell surface	Flagellin
TLR2/6	*CD36, CD14*	Cell surface	LTA
TLR7		Endosome	ssRNA
TLR8		Endosome	ssRNA
TLR9		Endosome	CpG DNA
TLR10		Cell surface	Unknown

Note: Adapated from [41]

2.2.3 Intracellular signaling following TLR-activation

The activation of TLRs triggers specific types of signaling pathways and distinct cytokine responses [42]. These differences in activation are primarily explained by the recruitment of different adaptor molecules, i.e. myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor inducing IFN-β (TRIF), TIR domain-containing adaptor protein (TIRAP) and TRIF-related adaptor molecule (TRAM), to the aligned TIR domains of each dimerized receptor. All TLRs, except TLR3, recruit and initiate MyD88 for further downstream signaling, and consequently this pathway is referred to as the MyD88-dependent pathway [42]. Intracellular signaling further relies on the recruitment of several IL-1 receptor-associated kinases (IRAKs) called IRAK4, IRAK1 and IRAK2 (Figure 2).

Sequential activation of these proteins leads to the downstream interaction with TNF-receptor-associated factor 6 (TRAF6), which in turn activates transforming-growth-factor-β-activated kinase 1 (TAK1). TAK1 phosphorylates a complex composed of IκB kinase (IKK) α, IKKβ and nuclear factor κB essential modulator (NEMO). This complex releases the key transcription factor nuclear factor κB (NF-κB) by phosphorylating the inhibitory IκB proteins that limit its accessibility to the nucleus. In parallel, TAK1 also activates the mitogen-activated protein kinases (MAPKs), including extracellular-signal-regulated-kinase (ERK) 1, ERK2, p38 and c-jun N-terminal kinase (JNK). Further downstream, MAPKs modulate translation and regulate

transcription factors such as activator protein (AP)-1, which together with the liberated NF-κB, lead to the up-regulation of pro-inflammatory cytokines and other TLR-inducible genes [40-42].

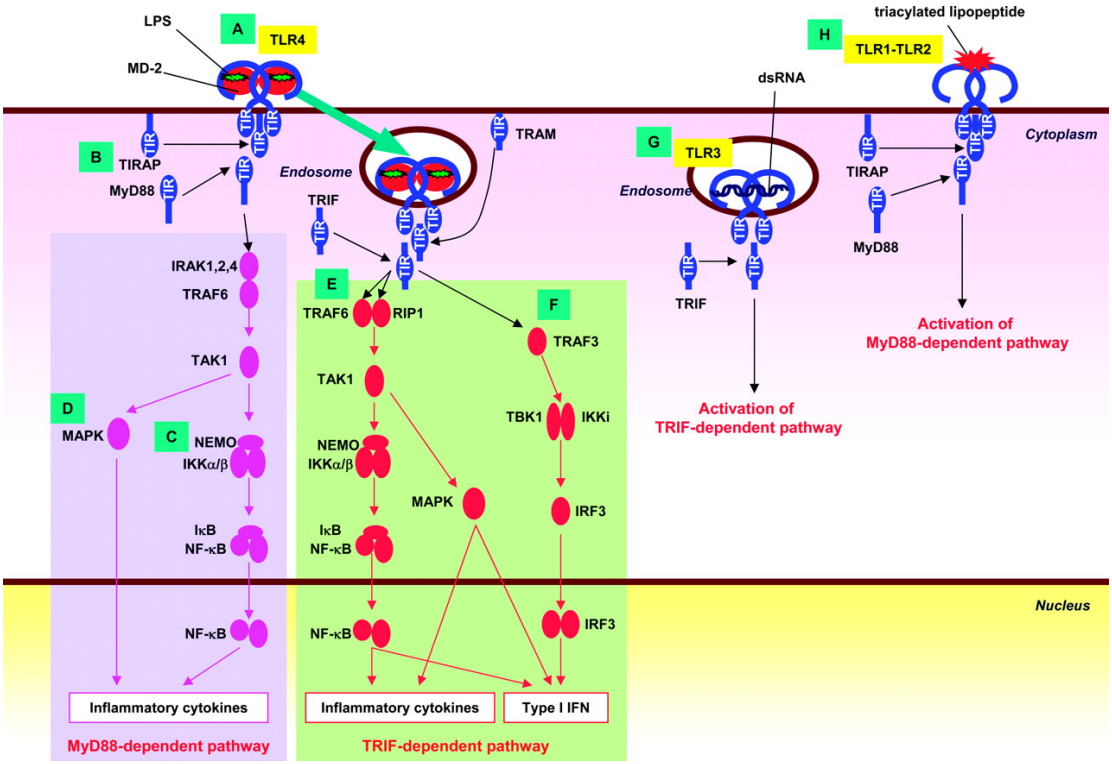

Figure 2.

TLR-activation elicits the production of inflammatory cytokines and/or type I interferons through the Myd88- and/or TRIF-dependent pathways. Reprinted with permission from Oxford University Press [55].

In contrast to the MyD88-dependent pathway, TLR3 relies on the adaptor molecule TRIF for activation of the transcription factors NF-κB and IRF3. As a result, this activation pathway has been named the TRIF-dependent pathway. Here, the induction of cytokines and type I interferons has been shown to be especially important in anti-viral immunity. Similar to the signaling pathway of MyD88, TRIF also recruits and activates TRAF6 and TAK1 for downstream activation of NF-κB. However, IRF3 is translocated into the nucleus following its phosphorylation by another set of proteins, involving the recruited signaling complex TANK-binding kinase 1 (TBK1) plus IKKε (also known as IKKi) together with TRAF3. TLR4 is unique in this setting as it activates both the MyD88- and TRIF-dependent pathways in a sequential order, first through the adaptor molecule TIRAP for MyD88-dependent activation, and then secondly, after the TLR4 receptor has been internalized into endosomes, the TRIF-dependent pathway by a complex-formation with TRAM [40-42] (Figure 2).

2.2.4 Negative TLR-regulators

While activation of TLRs is essential for the establishment of inflammation in response to invading microorganisms, the signaling of these receptors must also be tightly controlled in order to avoid chronic inflammation and excessive immune responses to pathogens [42]. Researchers have identified several negative regulators that affect signaling at multiple levels of the TLR-signal transduction pathways. These include, among others, Toll-interacting protein (TOLLIP), MyD88 short (MyD88s), IRAK-M, suppressor of cytokine signaling (SOCS)-1, A20, SHIP-1, sterile-α and Armadillo motif containing proteins (SARM) and suppressor of IκBε (SIKE) [56-58]. These proteins exert their inhibitory effects in different ways. For example, TOLLIP inhibits TLR2 and TLR4 by sequestering IRAK1, thereby resulting in impairment of NF-κB activation. MyD88s works by forming heterodimers with the full length MyD88, impeding the natural phosphorylation of IRAK1 by IRAK4. Similarly, the alternative kinase IRAK-M associates with IRAK4 and has been reported to disrupt the assembly of MyD88-IRAK4-IRAK1 complexes, thereby preventing the downstream activation of TRAF6. SOCS1 is member of a family of proteins with important roles in suppressing cytokine signaling. In relation to TLR4, mouse macrophages lacking SOCS1 show increased production of pro-inflammatory cytokines and nitric oxide in response to LPS. This lack of inhibition is suggested to stem from the ability of SOCS1, an E3-ubiquitin ligase, to target TIRAP for protein degradation. A20 lowers LPS-activation by negatively influencing the activation of TRAF6, whereas SHIP-1 interferes with the normal activity of TBK1. SARM, an alternative adaptor molecule, is specifically involved in down-regulating TRIF-dependent TLR-signaling. Likewise, SIKE has been found to impede IRF3 activation [56-58]. Together, these proteins obviously exert a complex influence on the final outcome of TLR-induced gene expression. This is especially highlighted by the well-studied phenomenon called endotoxin tolerance whereby monocytes subjected to an initial dose of LPS become refractory and fail to produce pro-inflammatory cytokines in response to a secondary challenge with LPS [59]. Here, the downturn in production of cytokines has in part been explained by the late up-regulation of several negative TLR-regulators following the first LPS challenge [60]. Thus, the induced gene expression will change and be modified as the TLR4 response progresses from an initial phase dominated by pro-inflammatory mediators to a secondary, LPS-tolerant phase characterized by anti-inflammatory cytokines and proteins such as TOLLIP, IRAK-M, SHIP-1, SOCS1, SARM and SIKE [56-58]. Finally, endotoxin tolerance has also been linked to the induction of so-called microRNAs [61]. These are small RNA molecules, induced e.g. after LPS-activation, that downregulate gene-expression at the post-transcriptional level by binding to the transcribed mRNA of certain genes. Over 700 microRNAs have been identified within the human genome, and of these, seven have been linked to the regulation of the TLR4 signaling pathway [62]. Here, the most well studied example is the microRNA called miR-146a that downregulates TNF-α production by binding to the mRNA of TRAF6 and IRAK-1. By transfecting miR-146a into unstimulated THP-1 cells, a phenomenon resembling LPS tolerance is artificially induced [63].

2.3 MONOCYTES, MACROPHAGES, NEUTROPHILS AND THE INFLAMMATORY RESPONSE

Inflammation arises through a complex cascade of serological and cellular events following injury or infection. During this evolutionary well-conserved process, the immune system is mobilized, the harmful stimuli eliminated and processes initiated that result in the repair of damaged tissues [64]. Inflammation can in many cases be recognized due to the tell-tale signs in afflicted sites of the body including swelling, redness, heat and pain, all manifestations of the immune system's attempt at combating the injurious stimuli and bringing back the tissue to its original homoeostatic state [6]. Inflammation is often described as a double-edged sword; beneficial and protective, but also the cause of tissue damage as an unavoidable part of its engagement. Vital to the initiation, propagation and final outcome of this process are monocytes/macrophages and the PRR-inducible cytokines that they command [65, 66].

Monocytes originate from lineage-committed hematopoietic stem cells in the bone marrow and constitute around 4-10% of leukocytes in human blood [11]. After approximately 3 days in circulation as non-proliferating cells, monocytes migrate across the endothelium and differentiate not only into tissue-specific macrophages or monocyte-derived dendritic cells but, depending on the environmental signals, also to other cell types including microglia cells and osteoclasts [67]. Thus, monocytes can be looked upon as transitional cells serving to replenish these tissue cell populations when required. Upon injury or infection, the recruitment of monocytes from bone marrow is increased with a large number of these cells migrating toward the inflamed tissue [68].

Monocytes and macrophages are ubiquitous throughout the animal kingdom being found in mammals, amphibians and fish. In fact, macrophage-like cells are also present in insects and simple multicellular organisms [11]. Apart from their fundamental role as surveillance cells, safeguarding us from infection, monocytes and macrophages also help the body to regulate various homeostatic functions like the clearance of apoptotic cells, removal of toxic compounds and tissue remodeling. In addition, through their role as APCs, monocytes/macrophages constitute accessory cells for the induction of antigen-specific immune responses [11]. Together, macrophages, dendritic cells, monocytes and their progenitors in the bone marrow form a network of cells called the mononuclear phagocyte system (MPS) [69, 70]. This network is represented in virtually all tissues of our bodies and is an important source of TLR-induced cytokines in the development of the inflammatory response [65].

2.3.1 Initiation of the inflammatory response

As described in the background section, tissue-residing macrophages are often the cells making the first contact with an invading pathogen [64]. This encounter will normally result in the phagocytosis of the pathogen and the activation of TLRs through binding of PAMPS like LPS or LTA. This, in turn, leads to the induction and secretion of pro-

inflammatory cytokines and chemokines like IL-6, IL-8, MIP-1β, TNF-α and IL-1β [71] which induce a wide range of secondary effects on nearby cells and tissues including up-regulation by the endothelium of adhesion molecules such as selectins and the intercellular adhesion molecule 1 (ICAM-1). Furthermore, the cytokines also increase vascular permeability leading to the typical swelling and redness associated with inflammation and, if they reach the blood, production of acute-phase proteins by the liver. Examples of these include mannan-binding lectin (MBL) and C-reactive protein (CRP) both of which can bind bacterial structures and form complexes that trigger complement activation. In addition, IL-6, TNF-α and IL-1β are all known as endogenous pyrogens as they induce fever by affecting the hypothalamus in the brain [5, 6].

In response to the initial "call to arms", circulating neutrophils and monocytes extravasate from the bloodstream guided by the concentration of chemotactic signals and adhere to the vessel wall in areas of activated endothelial cells [6]. The essential steps of this process are similar for neutrophils and monocytes involving rolling, activation, endothelial arrest and transendothelial migration (Figure 3) [72].

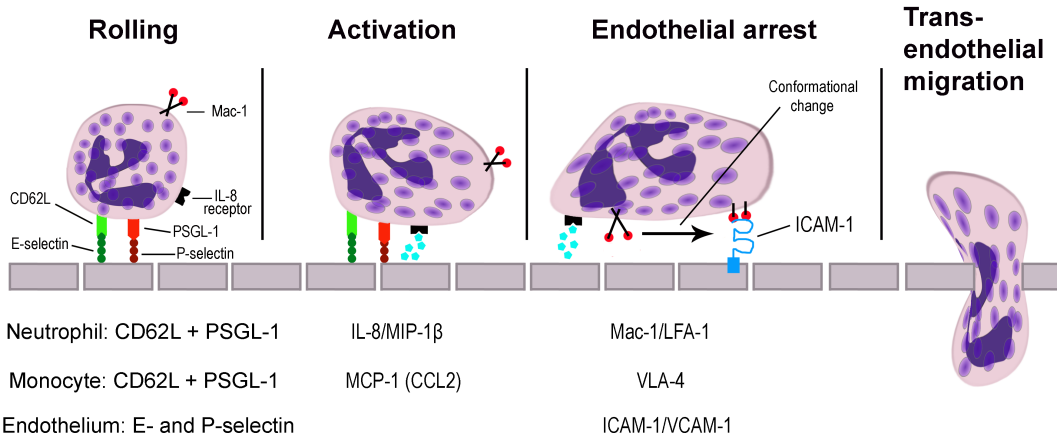

Figure 3.
Illustration of neutrophil extravasation showing the main receptor-ligand interactions between leukocyte and endothelium that allow for transendothelial migration into tissues. The essential steps of rolling, activation, endothelial arrest and transendothelial migration is similar for monocytes but involves different combinations of receptors and chemotactic factors as indicated. Adapted from [6].

Neutrophils begin rolling by binding to E- and P-selectins on the endothelium through their expression of L-selectin (CD62L) and P-selectin glycoprotein ligand-1 (PSGL-1) [73]. These receptor-ligand interactions slow down cellular movement along the area of inflamed endothelium and permit chemokines such as IL-8 and MIP-1β to bind to receptors on the neutrophils and induce conformational changes in their integrin molecules LFA-1 (CD11a/CD18 or Lymphocyte function-associated antigen 1) and MAC-1 (CD11b/CD18 or Macrophage-1 antigen) [74]. These changes strengthen the neutrophil affinity for ICAM-1 and lead to complete endothelial arrest. Finally, transendothelial migration allows neutrophils to migrate into tissues. Here,

chemoattractants such as bacteria-derived peptides and components of the complement cascade serve as additional signals to IL-8 and MIP-1β for the movement of neutrophils towards the site of infection [6]. Monocytes rely on a very similar sequence of events using CD62L to start rolling. However, their integrin molecules also include very late antigen 4 (VLA-4) that binds to vascular cell adhesion molecule 1 (VCAM-1) on the activated endothelial cells [73]. Moreover, integrin activation in monocytes occurs primarily through the binding of MCP-1 (CCL2) [6].

When neutrophils arrive at the site of infection, they instantly attack the intruder through the process of phagocytosis, the production of ROS/RNS and the release of granules containing an array of antimicrobial substances and proteolytic enzymes [75]. Neutrophil granules are divided into primary, secondary and tertiary granules which release their content in hierarchal order beginning already during the process of transendothelial migration [76]. Here, the tertiary granules, containing extracellular matrix degrading enzymes, help the neutrophil to extravasate. Then, at the site of infection, the contents of the secondary and primary granules are released, either extracellularly or inside the neutrophil through the membrane of internalized phagocytic vacuoles. The secondary and primary granules are "loaded" with acid hydrolases and antimicrobial substances, including the human neutrophil peptides 1-3 (HNP 1-3), heparin binding protein (HBP), myeloperoxidase (MPO), LL-37, and members of the serine protease family like cathepsin and elastase [76, 77]. Apart from the direct role of these granule proteins in eradicating bacteria, a growing body of research has demonstrated that many of these components also possess immunoregulatory capacity. For example, the antimicrobial peptides LL-37, HBP and HNP1/2 have all been shown to have chemotactic effects and can induce cytokine production in a range of other immune cells, including monocytes and dendritic cells [78-80].

Neutrophils reportedly express most members of the TLR family, except for TLR3 and TLR7 [51]. Consequently, many bacteria-derived PAMPs, will be able to stimulate neutrophils and induce the secretion of cytokines and chemokines [81]. Apart from a well-established production of IL-8, MIP-1β and IL-1ra following LPS-stimulation *in vitro*, several studies have reported on neutrophils secreting a wide range of both inflammatory (IL-6, TNF-α, IL-1β, IL-12p40) and anti-inflammatory (IL-10) cytokines [82-85]. However, other investigators have questioned this and have attributed the detection of several of these cytokines to the presence of contaminating monocytes [86, 87]. Nevertheless and irrespective of their true cytokine profile, the proven production of IL-8 and MIP-1β, demonstrates that neutrophils not only display microbicidal activity but that they can also communicate with other immune cells via chemotactic factors and recruit these to the site of infection [88].

2.3.2 Activation of the coagulation system

Inflammation in response to infection also affects the coagulation system. Bacterial-derived LPS and cytokines produced by monocytes/macrophages promote the intravascular deposition of fibrin in the immediate vicinity of inflamed tissues and prevent spreading of pathogens from the site of infection by forming a physical barrier or clot [89]. This process relies upon the interaction between a molecule called tissue-factor (TF) and the coagulation factor VIIa in plasma. TF is a membrane-expressed protein found constitutively in subcutaneous tissues but is also up-regulated on the cell surface of monocytes and endothelial cells in response to cytokines such as IL-6 [90]. The formation of an activated complex between tissue-factor and factor VIIa (TF-FVIIa) catalyzes the conversion of the plasma enzymes factor IX and X, which trough a sequence of activation cascades, leads to the generation of thrombin and the subsequent conversion of circulating fibrinogen into fibrin [89, 91]. Activated platelets increase the efficiency of this process by bringing certain components of the coagulation cascade in close proximity at their phospholipid-containing surfaces. In addition, plasma and platelet derived factor XIIIa, cross-links the strands of converted fibrin thereby stabilizing the clot generated [92].

2.3.3 Macrophage differentiation

Once monocytes have migrated into the infected tissue, they differentiate into macrophages or monocyte-derived dendritic cells [93]. Here, cues in the microenvironment, including PAMPs, DAMPs, cytokines and cognate interactions are believed to influence and dictate their differentiation into various macrophage phenotypes suited for the particular type of pathogen and tissue [94]. In humans, two well established macrophage phenotypes have been described; the classically activated macrophages, designated as M1, and the alternatively activated macrophages designated as M2 [95]. In the case of an acute bacterial infection, the classical M1 phenotype is favored over M2 due to the differentiating signals of NK-cell derived IFN-γ and microbial PAMPs. These two stimuli are critical for M1 polarization and give rise to macrophages that are specially adapted to kill microbes by virtue of their enhanced production of pro-inflammatory cytokines/chemokines, increased antigen-presenting capacity (upregulation of MHC class II, CD80, CD86) and heightened production of ROS/RNS [96, 97]. In addition, these inflammatory macrophages can also produce IL-12p70 and IL-23 which promote the differentiation of Th1 and Th17 responses, respectively [98]. In contrast, the alternatively activated macrophages are better equipped to fight extracellular parasites and to promote wound healing and are considered as being mainly anti-inflammatory in their nature. M2 macrophages have been further subdivided into M2a, M2b and M2c depending on differences in their phenotype and the signature mediators which elicit their development *in vitro* (Figure 4) [71, 99]. For example, the M2a macrophages are induced in response to IL-4 or IL-13 and secrete many immunoregulatory cytokines such as IL-10, IL-1ra and chemokines (CCL17/18/22/24). In addition, they express characteristic receptors such

as the mannose and scavenger receptors. M2b macrophages develop in response to a combination of immune complexes and TLR-agonists, forming IL-10 producing macrophages with concomitant secretion of pro-inflammatory cytokines and expression of co-stimulatory molecules (CD80, CD86). Finally, the M2c phenotype is found after exposure to IL-10, TGF-β or glucocorticoid hormones. Similar to M2a, they express the mannose and scavenger receptors and, in addition to IL-10 and IL-1ra, they also produce TGF-β [71]. While these phenotypes have been used as a framework by which the heterogeneity of macrophages has been outlined, it is also clear that they only partially represent the full complexity of these cells both *in vitro* and *in vivo* [94]. Exceptions to the different polarization states are frequently found and in mice a more flexible classification has been suggested where the different macrophage functions (inflammatory, wound healing and regulatory) have been described as a continuum of blended phenotypes within a color wheel [98]. For simplistic reasons, I will in the following parts of this thesis refer to the various phenotypes of M2a, M2b and M2c as the alternatively or M2 activated macrophages.

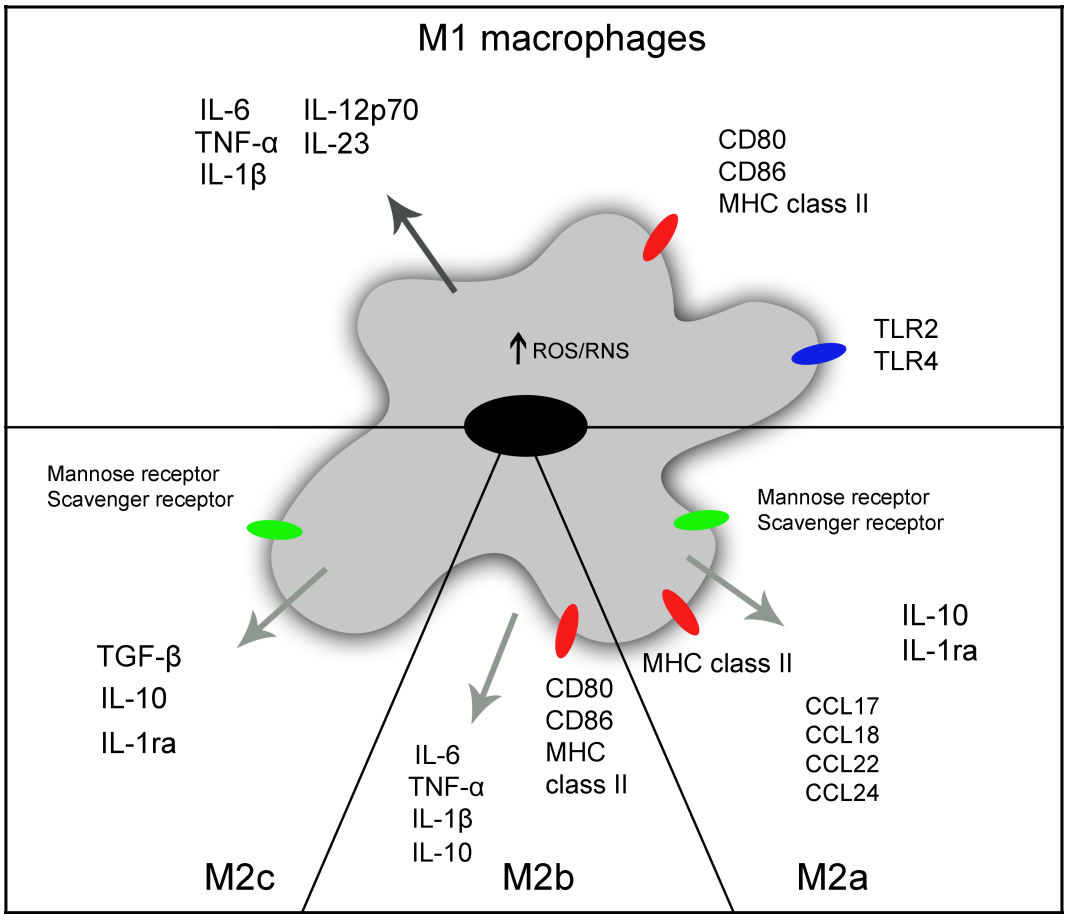

Figure 4.

The phenotype and cytokine producing capabilities of macrophages polarized into M1, M2a, M2b and M2c. Modified from [71].

2.3.4 Macrophage plasticity and the resolution of inflammation

In contrast to T cells, which give rise to several subsets through somewhat stable epigenetic changes in gene expression [100], macrophages are known to display a high degree of plasticity being able to switch back and forth between opposing phenotypes given the proper set of differentiation signals [98, 101]. For example, M2 macrophages can be pushed towards an M1 phenotype through exposure to recombinant IFN-γ [102, 103] and identically differentiated macrophages may respond differently to the same stimuli when placed in new or alternative environmental settings [94]. This form of cellular "adjustability" is also apparent in response to PRR-activation, where the genes expressed early after LPS stimulation differ significantly from those expressed at a later stage [104]. In addition, macrophages commonly revert back to a basal phenotype when left unstimulated after a period of cytokine exposure. For example, *in vitro* differentiation of macrophages into immature dendritic cells using the cytokines IL-4 and GM-CSF can essentially be reversed following the continued culturing of these cells for a few days in the absence of any cytokines [105]. In line with this, macrophage phenotype and function will change as the inflammatory response progresses, giving rise to a population of cells that from an outside perspective is confusingly heterogeneous; the emergence of pro-inflammatory M1 macrophages adapted to killing microbes is followed, through a transition, by various subsets of M2 cells suited to bring down inflammation and reestablish tissue homeostasis [94, 106, 107].

The exact mechanisms mediating this phenotypic shift are currently incompletely understood [98, 107, 108]. However, in the context of invading microorganisms, the cues in the microenvironment will certainly change as the clash between cellular immunity and the offending pathogen progresses and finally resolves. A natural decline in microbial products coupled with an accumulation of late warfare material like apoptotic neutrophils, anti-inflammatory cytokines and metabolites are believed to be critical in the conversion of M1 macrophages into M2. For example, following complete bacterial clearance, apoptotic neutrophils are removed by macrophages through phagocytosis. This process, called *efferocytosis*, naturally hinders the extracellular release of neutrophil derived DAMPs but the activity itself also leads to a shift towards M2 macrophages through the induced secretion of TGF-β [108]. Moreover, the natural progression of LPS-stimulated M1 macrophages from an early pro-inflammatory phase to a later, IL-10 secretory phase, dampens inflammation and promotes alternative differentiation in an autocrine/paracrine fashion [104]. In addition, production of IL-4 by basophils, mast cells and recruited Th2-cells may further strengthen the shift from classical to alternative macrophage activation [94, 98]. Naturally, these changes in the macrophage population are not necessarily limited to the active reprogramming of individual M1 cells into M2. Transitions in overall phenotype can also be a result of newly recruited monocytes encountering a changed microenvironment of the inflamed tissue, leading to a higher proportion of M2.

Thus, the buildup of M2 macrophages that occurs in the later stages of the inflammatory response is believed to be a prerequisite for inflammation to resolve and

for the promotion of wound healing [108]. By predominantly secreting high levels of anti-inflammatory cytokines such as IL-10 and IL-1ra, M2 macrophages down-regulate the release of pro-inflammatory mediators from surrounding cells and tissues [71]. Further, they effectively phagocytose dead cells, necrotic tissues and degraded components of the extracellular matrix, factors that would otherwise promote continued M1 differentiation by acting as DAMPs [42, 47]. In addition, M2-related production of TGF-β and platelet-derived growth factor (PDGF) stimulates the differentiation and proliferation of fibroblasts into myofibroblasts which, in turn, synthesize collagen and regenerate the extracellular matrix, both important for restoring tissues and regaining homeostasis [106]. Moreover, M2 macrophages also produce various other factors such as vascular endothelial growth factor (VEGF) and special types of chemokines that recruit regulatory T cells. Finally, M2 macrophages help repair destroyed tissues by secreting products that regulate important metabolic functions [106, 108]. Once inflammation has subsided and tissues have been healed, macrophages either undergo apoptosis or leave the site through the lymphatics [109].

2.4 MONOCYTE HETEROGENEITY

Given the diversity of macrophages and the wide range of activities these cells engage in during both homeostasis and infection, it has also been natural to look at monocytes not just as a pool of precursor cells for macrophages but as cells with their own level of heterogeneity and distinct effector functions [110]. Indeed, already at the morphological level monocytes show variations in both size and nuclear appearance. During the 1980s, differences in density were used for dividing monocytes into a dominant population of large monocytes and a corresponding minor population of small monocytes [11]. By using flow cytometry, Ziegler-Heitbrock and colleagues demonstrated in 1988 that the population of small monocytes could be distinguished from other monocytes based on their expression of the Fcγ-III receptor, CD16 [111]. Conversely, the dominant population of large monocytes was CD16 negative but expressed significantly higher levels of the surface marker CD14 [112]. Thus, based on these two markers, monocytes could be divided into two subsets defined as the classical $CD14^{high}CD16^{-}$ subpopulation, comprising 85-95% of total monocytes, and the $CD14^{low}CD16^{+}$ subpopulation making up the remaining 5-15% of cells [11].

In the following decades, much work was focused on identifying the immunological and functional features of these two monocyte subsets and several investigators found differences in their expression of cell surface markers connected to cell trafficking and chemotaxis [113]. For example, $CD16^{+}$ monocytes were found to have a high expression of CX_3CR1 and low expression of CCR2 and they migrated in response to the chemokine CX_3CL1 [114, 115]. By contrast, the classical monocytes displayed significant expression of CCR2 and CD62L, but little or no CX_3CR1 and migrated in response to CCL2 [116, 117]. These variations in cell surface markers and behavior suggested a possible tissue-specific surveillance and that the extravasation of each subpopulation might be regulated by alternative scenarios of inflammation [118]. Moreover, $CD16^{+}$ monocytes were identified as primarily pro-inflammatory due to their high production of TNF-α and low production of IL-10 in response to LPS [119, 120]. Compared to the classical subset, $CD16^{+}$ monocytes were also considered as more differentiated and macrophage-like [121] and increased in numbers during various inflammatory conditions [122-124]. They also displayed higher antigen presenting capacity [125] and in an *in vitro* model of transendothelial migration they were seen to develop into dendritic cells [126]. As a consequence, $CD16^{+}$ monocytes were hypothesized to have a unique role in innate immunity.

2.4.1 Three monocyte subsets and their production of cytokines

While the division of monocytes into two subsets was the standard for a long time, it eventually became apparent that further diversity existed within the $CD16^{+}$ subpopulation [114, 127, 128]. Based on the same defining markers of CD14 and CD16, the $CD16^{+}$ monocytes could thus be separated into two subsets of $CD14^{high}CD16^{+}$ and $CD14^{low}CD16^{++}$. This additional level of monocyte heterogeneity

was in 2010 incorporated in the official nomenclature of human monocytes, now dividing them into three major subpopulations: the classical ($CD14^{high}CD16^{-}$), the intermediate ($CD14^{high}CD16^{+}$) and the non-classical ($CD14^{low}CD16^{++}$) monocytes [129]. While this definition of a third subset has not rendered previous reports on monocyte heterogeneity invalid, it has on the other hand raised questions as to how earlier features ascribed to the whole population of $CD16^{+}$ monocytes should be separated between the intermediate and non-classical subsets [130].

In order to further elucidate these questions, a number of studies have recently reevaluated monocyte heterogeneity taking into account all defining subsets and using several cell surface markers, gene expression analysis and TLR-induced cytokine production assays [127, 131, 132]. Due to the fact that the classical subset has remained intact in this new paradigm, much of the data from earlier studies have been reproduced and for instance, classical monocytes are still distinguishable from other monocytes by their high expression of CCR2 and CD62L. However, for the intermediate and non-classical monocytes, further phenotypic differences have been demonstrated as exemplified by a lower expression of CX_3CR1, comparably higher expression of CCR5 and more significant levels of cell surface markers connected to antigen presentation in the intermediate subpopulation [127, 132, 133]. With regard to cell surface markers, intermediate monocytes appear to be wedged between the classical and non-classical monocytes, suggesting that they may represent a transitional stage between these two subpopulations [127, 132]. The notion that the three subsets represent a continuum of differentiation with the intermediate monocytes being the "gradual link" between the other two has been corroborated by results from gene expression analysis [132] and in the treatment of patients using recombinant M-CSF, where an initial rise in the classical subpopulation of monocytes is followed sequentially by increases in intermediate and finally the non-classical subset [134]. In addition, purified preparations of classical monocytes that are kept in culture spontaneously acquire attributes of intermediate and non-classical monocytes while simultaneously down-regulating markers associated with their original classical phenotype [132, 133, 135]. However, despite this type of circumstantial evidence, there is still a possibility that monocyte heterogeneity represents an early commitment by progenitors in the bone marrow and that tissue specific or inflammatory signals account for the recruitment of these different populations and for their varying numbers in peripheral blood [11].

Although the differential expression of surface markers has been critical for the recognition and definition of different monocyte subsets, cytokine production is supposedly an important function of these cells *in vivo*. Diversity in this regard has usually been evaluated *in vitro* through TLR-ligation and determinations of the cytokine profile of monocyte subsets isolated by flow cytometric cell sorting [127, 131, 132]. However, in contrast to the relative consensus regarding their expression of cell surface antigens, studies of cytokine profiles in the different monocyte subsets have generated conflicting results. For example, Cros *et al* found intermediate monocytes to be the main producers of IL-6, TNF-α and IL-1β in response to LPS whereas the non-classical monocytes were described as poor LPS-responders [131]. Instead non-

classical monocytes preferentially secreted high levels of pro-inflammatory cytokines in response to TLR7/8-ligation, possibly indicating a special role of this subset in the immunity against viruses. However, these results do not correspond to those of Skrzeczynska-Moncznik *et al*, who a few years earlier had identified the non-classical monocytes as not only capable of responding to LPS but also being the main producers of TNF-α [127]. In addition, the intermediate monocytes were here defined as the main producers of IL-10 and not the classical subpopulation as suggested by Cros *et al*. In 2011, Wong *et al* described yet another relationship for the cytokine secreting profiles of the three monocyte subsets. Using a virtually identical experimental setup stimulating the different monocyte subsets with increasing doses of LPS, the intermediate monocytes were here distinguished as the cells with the lowest production of IL-6, TNF-α and IL-1β [132]. Moreover, the non-classical monocytes responded very well to LPS and were defined as the subset producing the highest levels of IL-1β and TNF-α. In addition, the classical monocytes were recognized for having the broadest range of cytokine secreting capacity overall, producing high to moderate levels of all cytokines tested. A summary of these findings is found in Table 3.

Table 3. Detected concentrations of secreted cytokines in cell supernatants (ng/ml) derived from LPS-stimulated (0.5-5 ng/ml, 18-24h) classical, intermediate and non-classical monocytes adjusted to 1 million cells/ml. Red markings indicate the main producing monocyte subset as reported by each author. Data taken from [127, 131, 132].

		Cros *et al* (2010)	Skrzeczynska-Moncznik *et al* (2008)	Wong *et al* (2011)
Classical $CD14^{high}CD16^{-}$	IL-6	120	-	[440]
	TNF-α	6	0.5	152
	IL-1β	6	-	14
	IL-10	[2.1]	0.2	[0.7]
Intermediate $CD14^{high}CD16^{+}$	IL-6	[140]	-	280
	TNF-α	[55]	3	60
	IL-1β	[67]	-	12
	IL-10	1.3	[0.7]	0.2
Non-classical $CD14^{low}CD16^{++}$	IL-6	15	-	320
	TNF-α	18	[8]	[200]
	IL-1β	3	-	[23]
	IL-10	0.05	0.2	0.3

2.4.2 Methodological considerations

The apparent inconsistencies between studies investigating the same cytokines, in spite of using similar experimental approaches and conditions, have been suggested to stem from minor differences in the methods employed [132]. This appears likely considering that monocytes are easily influenced by differences in *in vitro* handling [11]. For instance, some of the anti-CD14 antibodies that are commonly used to isolate monocytes have been reported to attenuate LPS responses by disturbing the function of CD14 as an essential co-receptor in the activation of TLR4 [136]. As other anti-CD14 antibodies have been claimed not to affect LPS activation, this may well explain many of the inconsistencies in cytokine levels reported by different investigators [137, 138]. Furthermore, when using flow cytometry, the gating process is both critical and difficult especially when considering that the current definition of monocyte subpopulations relies on relatively minor differences in the expression of CD14 and CD16. Thus, while the intermediate and non-classical monocytes are supposed to be distinguished by variable levels of CD16 expression ($CD16^{+}$ versus $CD16^{++}$) this difference is not taken into full account by all groups when subpopulations are acquired [127, 133]. Finally, cytokine determinations have mostly been performed using ELISA or Luminex assays. While both are sensitive techniques, they are also susceptible to the interference by soluble cytokine receptors and the uptake of cytokines by receptor-bearing cells in the cultures. Such "consumption" of released cytokines has been reported previously for T-cell cytokines and is likely to represent an important factor in the investigation of monocyte-derived cytokines as well [139]. The level of cytokine consumption is, in turn, affected by a number of factors such as the composition of cells and the extent of cellular contact during the culture, the propensity of each monocyte subset to "consume" cytokines as well as the ability of the antibody pairs that are used in the assay, to recognize free cytokine contra the same cytokine bound to soluble receptors. Thus, reported levels of TLR-induced cytokines produced by classical, intermediate and non-classical monocytes may not necessarily reflect their individual ability to synthesize and release these mediators.

Due to the unique properties of the ELISpot/FluoroSpot assays, used in this study, many of these interfering factors can be reduced or eliminated. Thus, in both assays, the cytokine is captured close to its source of release, which minimizes its binding to receptors on neighboring cells and the risk of interference by soluble receptors.

2.5 THE PATHOPHYSIOLOGY OF SEPSIS

The events of the inflammatory response described in earlier sections of this thesis are under normal circumstances beneficial to the host as they promote local coagulation and confinement of pathogens to the site of infection [140]. Here, the destructive powers of infiltrating monocytes and neutrophils can be insulated and channeled towards eradicating the intruder while simultaneously limiting self-inflicted damages to a compartmentalized part of the host [64]. However, in rare instances, the systemic response against a disseminated bacterial infection becomes dysregulated [141] and extends beyond the barriers of a beneficial containment. The result is a widespread TLR-activation of innate immune cells and the initiation of the so-called systemic inflammatory response syndrome (SIRS). During this hyperinflammatory phase, cytokines like TNF-α, IL-1β, IL-6, IL-8 and IFN-γ are excessively released from several cellular sources including monocytes/macrophages, neutrophils, lymphocytes and endothelial cells. These mediators promote increased activity of several important effector functions like cellular activation, recruitment, extravasation and degranulation. For example, IFN-γ induces enhanced cytokine production by monocytes/macrophages, whereas TNF-α and IL-1β prime neutrophils for increased production of ROS and nitric oxide. Simultaneously, soluble elements of the innate immune system become activated, leading to a powerful increase in the complement split products C5a and C3a. These anaphylatoxins, in turn, induce chemotaxis and cause systemic effects by binding to specific receptors on mast cells. Elevated levels of HMGB1 follow this initial phase, exacerbating various aspects of inflammation by acting as a DAMP and forming synergistic, immunomodulating complexes with other cytokines [142, 143]. Depending on the overall severity of this response, it can lead to profound vascular dilatation, capillary leak, cell death, tissue injury and organ dysfunction [144-147]. Put in other words, many of the cells and factors that give rise to the signs of inflammation (swelling, redness, heat and pain) under normal, compartmentalized conditions are suddenly unleashed systemically, leading to an exaggerated and dysregulated response [148-150]. In this situation it is the exaggerated response and not the bacteria themselves, that becomes the primary threat to the host [151].

The current clinical criteria for patients suffering from SIRS were established in 1992 [152] and include two or more of the following 4 symptoms or signs:

- Body temperature below 36°C or higher than 38°C.
- Heart rate above 90 beats per minute.
- Respiratory rate higher than 20 breaths per minute or an arterial partial pressure of carbon dioxide less than 4.3 kPa.
- In the blood: Leukocytes <4000 cells/mm^3 or >12000 cells/mm^3 or >10% immature neutrophils.

When SIRS is induced by a known or suspected infection, this is defined as sepsis [152]. Severe sepsis occurs when the inflammatory cascade of sepsis progresses and gives rise to organ dysfunction not associated with the original septic focus or due to an

underlying chronic disease. Finally, septic shock is the outmost dangerous state and is a term used for patients who, in addition to symptoms of severe sepsis, also display an abnormally low blood pressure despite being given adequate fluid resuscitation [147, 153]. Overall, the mortality rate of sepsis is high, ranging from 18-50% depending on stage of sepsis and study cited [154-156]. In one recent review comprising more than 11000 patients diagnosed with severe sepsis upon inclusion, a substantial proportion (49.7%) died before the study was completed [147]. Among the study participants, gram-negative and gram-positive bacteria accounted for 57% and 44% of confirmed infections respectively, whereas fungi were found as the causative agent in 11% of infections. As indicated by the figures several patients suffered from multiple infections. The lung (47%) followed by the abdomen (23%) and the urinary tract (8%) were the most common primary sites of infection. In general, the incidence of sepsis is increasing due to an aging population, increased use of immunosuppressive medicine, and more patients undergoing complicated surgery [144].

On the molecular level, the identification of PRRs has generated much insight into how the immune system reacts and how cytokine responses are initiated by infection [35]. However, the exact mechanisms that give rise to the "inappropriate" host response in the beginning of the septic process are still poorly understood [141]. Yet, as touched upon above, the aggressiveness of the bacterium, the size of the inoculum and the immune competence of the affected individual are all known to influence the outcome [151]. Moreover, since the onset of sepsis primarily involves cells belonging to the innate immune system, genetic predisposition in TLRs is likely to be a significant factor [157]. Indeed, studies of identical twins have demonstrated a significant contribution of genetics in the likelihood of dying from an infectious disease [158] and deficiencies in TLR-signaling are associated with increased susceptibility to acquire sepsis [159, 160].

2.5.1 Role of monocytes in sepsis

Due to sepsis being recognized as a systemic disease where cells in the circulation are supposed to propagate the spreading of an uncontrollable inflammation, monocytes have long been on the "list of suspects" when trying to explain the underlying pathophysiology [161, 162]. Monocytes express high levels of most TLRs [50] and TLR-activated monocytes *in vitro* are the main producers [163] of the cytokines that are typically found at elevated levels in the plasma of septic patients (e.g. IL-6, TNF-α, IL-1β, IL-8) [145, 164, 165]. Furthermore, monocyte-induced activation of the coagulation system, combined with a general cytokine-mediated impairment of anti-coagulation mechanisms, cause small capillaries to be blocked by accumulative numbers of microthrombi [144, 147]. In severe cases, this can lead to disseminated intravascular coagulation (DIC), and in turn, the disruption of normal coagulation, as platelets and coagulation proteins are consumed [166]. Pro-inflammatory cytokines also induce nitric oxide, which contributes to causing tissue hypoxia, a fall in blood pressure and ultimately the generation of organ dysfunction when sepsis progresses into

severe sepsis and septic shock [147, 148]. Finally, a link between elevated levels of monocyte-derived cytokines and sepsis-like symptoms has been demonstrated in mice using intravenous injections of TNF-α [167]. The prompt neutralization of such injected TNF-α prevented these symptoms from occurring, despite the same mice having an ongoing bacteremia [168].

2.5.2 The anti-inflammatory phase of sepsis

While universally accepted that the onset of sepsis embraces an intense pro-inflammatory response, it is also well documented that, as sepsis progresses, the immune system can become overly anti-inflammatory [144, 149, 169]. This parallel counteraction, also referred to as the compensatory anti-inflammatory response syndrome (CARS) has been the focus of much attention in the last 15 years and many researchers believe that the immunological consequences of CARS are the main obstacles in reducing the high mortality rate associated with sepsis [170]. Although a small group of patients, suffering from particularly aggressive forms of infections, will rapidly die due to an early, one-sided phase of "cytokine storm", this is not the typical sepsis scenario [151]. Instead, the majority of patients survive the initial phase only to, a few days later, transit into an anti-inflammatory state generated to counterbalance the initial, pro-inflammatory environment [144, 151]. As a consequence, most septic patients will with time display features that are indicative of being immunosuppressed, unable to eradicate their primary infection and susceptible to new, often hospital-acquired secondary infections [144]. The exact mechanisms behind this state of hypo-inflammation, also referred to as "immunoparalysis", are currently not understood although they appear to involve an increased production of cytokines such as IL-10, IL-13 and TGF-β [149, 171]. Nonetheless, a number of immunological defects have been reported to coincide with its manifestation and some are suggested to act as contributing factors in its development. Firstly, based on the findings in autopsies performed 30-90 minutes post mortem, death in a late stage of sepsis appears to be linked to a widespread apoptosis in the spleen of mainly $CD4^+$ T cells and B cells [169]. This loss supposedly contributes to immunoparalysis by depleting necessary effector functions of adaptive immunity during a time period when it is especially needed. In addition, surviving immune cells, such as macrophages, are suggested to further add to the suppression through phagocytosis of the apoptotic cells left behind, a process that, as described in earlier sections, pushes these cells toward an M2 phenotype and the production of more anti-inflammatory cytokines [108]. Secondly, monocytes isolated from septic patients have been shown to display a diminished *in vitro* capacity to secrete pro-inflammatory cytokines (IL-6, TNF-α, IL-1β) in response to TLR-ligands [172-178]. Interestingly, this reduced capacity reportedly coincides with an increased or unaffected capacity for secreting anti-inflammatory cytokines such as IL-10 [176-178]. As a result, septic monocytes are said to have been reprogrammed into a state of "endotoxin tolerance" that reduces the patient's ability to combat microorganisms [60]. The degree of this impairment may reflect sepsis-induced immunoparalysis and has been linked to a poor outcome/prognosis [173]. Thirdly, in

addition to an altered cytokine secretion profile, monocytes from septic patients are impaired in terms of antigen presentation as indicated by their diminished expression of the MHC class II molecule HLA-DR [179-181]. A persistent reduction in the expression of HLA-DR (<40% positive monocytes, compared to normal values of >90%) has been correlated with higher mortality and with an increased likelihood of developing secondary nosocomial infections [182, 183].

3 AIMS OF THESIS

With this background in mind we initiated a project to investigate TLR-induced cytokine secretion by monocytes in septic patients and healthy controls using ELISpot and (or) the fluorescence based FluoroSpot technique. Due to the capacity of these methods to measure and establish cytokine secretion at the single cell level, we hypothesized that they would provide us with a novel perspective on monocytes and neutrophils and the role of these cells in the pathophysiology of sepsis.

Specific aims:

I. To determine the TLR4-induced cytokine profile of isolated granulocytes and mononuclear cells derived from healthy donors using ELISpot.

II. To elucidate the relationship between monocyte populations isolated from healthy individuals secreting different cytokines in response to TLR-ligation using the FluoroSpot technique.

III. To analyze if circulating monocytes contribute to the elevated plasma cytokine levels in septic patients and to evaluate the possibility of using ELISpot as a tool in the diagnosis and stratification of sepsis.

4 MATERIALS AND METHODS

A description of the methods used in paper I-III is given below. All studies were approved by the ethics committee at the Karolinska Institutet.

4.1 ISOLATION OF PBMC (PAPER I-II)

Blood samples from healthy donors were collected by venipuncture into citrate blood collection tubes and subsequently mixed with an equal volume of PBS. The mixture of blood/PBS was layered on top of Ficoll-Paque PLUS and centrifuged 400×g for 30 minutes. PBMCs were retrieved from the interface, washed twice in cell culture medium and diluted to the desired cell concentration.

4.2 ISOLATION OF GRANULOCYTES (PAPER I)

Following density gradient separation of PBMC using Ficoll-Paque PLUS, the remaining cell pellet was resuspended in PBS to its original starting blood volume and layered on top of Polymorphprep. After centrifugation at 600×g for 30 minutes the band of granulocytes was retrieved, mixed with two volumes of PBS and washed twice in cell culture medium. Following the removal of erythrocytes through hypotonic lysis, cells were again washed twice and suspended in cell culture medium. Viability was checked using trypan blue exclusion and purity was assessed by light microscopy of Türk stained cells. In addition, the granulocytes were checked for purity in flow cytometry using forward side scatter analysis. Cells suspended in cell culture medium were added to the flow cytometer and 50000 events were acquired. Based on cellular size and granularity, an estimated number of contaminating PBMC within the granulocyte preparation was obtained.

4.3 DEPLETION OF CD14 POSITIVE CELLS (PAPER I)

In order to investigate the cellular source of cytokines secreted by mononuclear cells in response to LPS, CD14-positive monocytes were depleted from PBMCs using BD IMag™ anti-human CD14 magnetic particles. In brief, freshly isolated PBMCs were centrifuged and incubated with the anti-CD14 magnetic particles for 30 minutes. The mixture was then carefully diluted in 2ml of cell culture medium and placed in the BD magnet. After 10 minutes, unbound cells depleted of monocytes, were carefully removed and washed in cell culture medium. For maximum depletion, the process was repeated once.

4.4 ISOLATION OF MONOCYTES (PAPER II)

Monocytes were enriched using the RosetteSep isolation technique from Stemcell™ Technologies. Freshly drawn, citrated blood was incubated for 10 min with the monocyte enrichment cocktail containing bifunctional antibody complexes with a combined specificity for glycophorin A expressed on erythrocytes and cell surface markers expressed on T cells (CD2, CD3, CD8), B cells (CD19), NK cells (CD56, CD2), granulocytes (CD66b) and dendritic cells (CD123), respectively. Following incubation at room temperature, the mixture was layered on top of Ficoll-Paque PLUS and centrifuged 1200×g for 20 minutes. Through the formation of antibody crosslinks between glycophorin A on the erythrocytes and CD markers on the unwanted cells, non-monocytic cells were removed as heavier units of "immunorosettes" by density gradient separation. The enriched monocytes were retrieved from the Ficoll interface, washed twice and suspended in cell culture medium.

4.5 ISOLATION OF $CD16^+$ MONOCYTES (PAPER II)

$CD16^+$ monocytes were isolated in a two-step procedure using a specialized kit ($CD16^+$ Monocyte Isolation Kit) from Miltenyi Biotech based on antibodies conjugated to magnetic MicroBeads. First, PBMCs were incubated with a mixture of buffer (PBS/0.5% BSA/2mM EDTA), FcR-blocking solution and magnetic MicroBeads coated with antibodies specific for granulocytes (anti-CD15) and NK-cells (anti-CD56). After incubation for 15 minutes at 8°C, cells were washed in buffer and added to a prepared LD column placed in the Miltenyi magnet. PBMC depleted of NK-cells and granulocytes, were collected in the flow-through fraction. After centrifugation, these cells were then further incubated with a mixture of buffer and MicroBeads coated with anti-CD16 antibodies. After incubation for 15 minutes at 8°C, cells were again washed in buffer and added to a newly prepared MS column. This time, the cells of interest, i.e. $CD16^+$ monocytes, were retained on the column, whereas cells passing through consisted of classical monocytes ($CD14^+CD16^-$) and remaining T and B cells. After rinsing with buffer, the column was removed from the Miltenyi magnet and placed in a suitable collection tube. Using 1ml of added buffer $CD16^+$ monocytes were then pushed into the collection tube using the supplied plunger. In order to obtain a higher level of purity, the cells were again added to a freshly prepared MS column and the procedure repeated. Finally, the $CD16^+$ monocytes were washed twice and suspended in cell culture medium to the desired concentration.

4.6 ISOLATION OF LEUKOCYTES USING DEXTRAN (PAPER III)

In paper III, cells were isolated by mixing citrated whole blood with an equal volume of PBS containing 2% dextran. After 15 minutes of incubation at room temperature a majority of red blood cells had sedimented to the bottom of the tube. The top buffy coat, containing total leukocytes, was retrieved and cells were counted in Türk and in

trypan blue. After centrifugation for 8 min at 250×g, the supernatant was removed and stored for further analysis by Luminex, and the cell pellet was resuspended in cell culture medium to the desired concentrations of 2.5×10^6 and 1.5×10^5 cells per ml. Cells were then added to the ELISpot plate and incubated overnight in the presence or absence of stimuli.

4.7 ELISPOT ASSAY (PAPER I-III)

The ELISpot technique was employed for detection of cytokine secreting cells in isolated populations of human PBMCs, granulocytes, enriched monocytes and total leukocytes, respectively. Ethanol pre-treated polyvinylidene fluoride membrane plates were coated with antibodies recognizing the cytokine of interest. Isolated cells were subsequently added at desired cell concentrations to wells containing medium, with or without stimuli, and incubated for 20 hours at 37°C in 5% CO_2. The next day, cells were removed by washing the plates in PBS and the secreted cytokines were detected using biotinylated detection antibodies in combination with streptavidin (SA) conjugated to alkaline phosphatase (ALP) and finally developed with substrate. After drying the plates, spots, each corresponding to one single secreting cell, were counted using an ELISpot reader.

4.8 ELISA ASSAY (PAPER I)

Concentrations of IL-6, TNF-α and IL-1β were measured by ELISA using supernatants derived from LPS-stimulated granulocytes cultured at varying cell concentrations in uncoated ELISpot plates. The ELISpot plates had prior to the experiment been activated by ethanol, washed five times in sterile water and finally blocked in cell culture medium for 30 minutes before the isolated granulocytes were added. After 20 hours of incubation, supernatants were retrieved and analyzed in pre-coated ELISA kits according to the manufacturer's instructions.

4.9 FLUOROSPOT ASSAY (PAPER II)

The FluoroSpot technique was used to analyze single cells secreting two cytokines simultaneously. Ethanol-activated, low-fluorescent polyvinylidene fluoride membrane plates were incubated overnight with either a single or a combination of two capture antibodies directed against the following cytokines: IL-6, MIP-1β, TNF-α, GM-CSF, IL-1β, IL-10, IL-12p40. The next day, plates were washed five times with PBS and after blocked with cell culture medium, fresh medium with or without ligands for TLR2 (LTA) or TLR4 (LPS) was added. Finally, enriched monocytes were added to the plates at desired cell concentrations and incubated for 20h at 37°C in 5% CO_2. The following day, cells were removed by washing five times in PBS and secreted cytokines were detected using FITC-conjugated detection antibody to IL-6 or IL-1β in

combination with biotinylated antibodies against MIP-1β, TNF-α, GM-CSF, IL-1β, IL-6, IL-10 or IL-12p40. After 2 hours of incubation, plates were washed as above and incubated with a mixture of streptavidin conjugated with red fluorophore and anti-FITC antibody conjugated with green fluorophore. Following incubation for 1 hour, plates were again washed and the plastic underdrain lining the backside of the FluoroSpot plate was removed. Fluorescence enhancer was added for 10 minutes after which the plates were carefully emptied and left to dry protected from light. Analysis and counting of spots were performed using a two-filter FluoroSpot reader.

4.10 FLOW CYTOMETRY (PAPER II)

Monocytes, depleted of contaminating erythrocytes and resuspended in cold FACS buffer (PBS supplemented by 0.02% NaN_3 and 0.5% FCS), where analyzed for expression of CD3, CD14, CD19, CD56 and CD16 by incubating 4×10^5 cells in 50µl of cold FACS buffer mixed with either 10µl of anti-human CD3-PE, 20ul of anti-human CD14-PE, 20µl of anti-human CD19-PE, 10µl of anti-human CD56-PE or 5µl of anti-human CD16-Alexa488. The double staining for CD14 and CD16 was performed by combining 20µl anti-human CD14-PE, 5µl anti-human CD16-Alexa488 and 25µl of FACS buffer. The level of non-specific staining was checked using matched isotype controls. In case of the double staining, the principle of "fluorescence minus one" was used, i.e. the substitution of only one isotype control at a time [129]. After 15 minutes of incubation at 4°C protected from light, cells were washed twice in cold FACS buffer, put on ice and analyzed in a flow cytometer.

4.11 LUMINEX ASSAY (PAPER III)

Plasma samples from the 38 enrolled patients, 32 healthy controls and 9 human endotoxemia volunteers were analyzed for concentrations of IL-6, TNF-α, GM-CSF, IL-1β, IL-10 and IL-12p40 using a Milliplex kit from Millipore based on the multiplex Luminex™ technology. The analyses were carried out by an outside commercial provider according to the manufacturer's protocol.

4.12 INJECTION OF ENDOTOXIN INTO HEALTHY VOLUNTEERS (PAPER III)

Healthy male volunteers (n=9) were administered an intravenous bolus injection of purified endotoxin at a dose of 40 EU/kg body weight. Blood samples were retrieved before, 30 min and 150 min post injection. Total leukocytes were isolated using dextran sedimentation, plasma saved for Luminex analysis and cells analyzed in ELISpot as described previously.

4.13 PATIENTS (PAPER III)

38 patients with suspected sepsis and 32 healthy controls were enrolled into the study. For three of the patients, a definitive diagnosis of sepsis could not be made and were therefore excluded. In addition, a differential cell count could not be established for three patients and two controls. Thus, 32 septic patients, categorized as having sepsis (6%), severe sepsis (19%) and septic shock (75%) were included in the study in paper III. The median age of these patients were 65 years (range 27-86 years) with an approximately equal distribution of men (49%) and women (51%). For all patients, the estimated time period between the first signs of sepsis and study blood sampling ranged from 9-337h (median 48h).

4.14 STATISTICAL ANALYSIS (PAPER I-III)

In paper I and II, differences between unstimulated and TLR-stimulated cells were evaluated by applying the non-parametric Wilcoxon signed rank test using SPSS 16.0 software. In paper III, statistical analysis was performed using Graphpad Prism v.5 software and the Mann-Whitney U test. In addition, Spearman or Pearson's method was used for correlation analysis. Differences were in all cases considered significant for $p<0.05$.

5 RESULTS AND DISCUSSION

5.1 PAPER I - ELISPOT ANALYSIS OF LPS-STIMULATED LEUKOCYTES: HUMAN GRANULOCYTES SELECTIVELY SECRETE IL-8, MIP-1BETA AND TNF-ALPHA

The coordinated production of cytokines following TLR4 activation has been studied previously in both PBMCs and granulocytes [184-190]. While earlier reports have typically employed cytokine detection techniques such as ELISA, intracellular cytokine staining in combination with flow cytometry (ICS) and RT-PCR, we here used the ELISpot technique. Principally, this technique combines the advantage of ELISA, by looking at what is truly secreted from the cells, with that of ICS, which permits analysis at the single cell level [191, 192]. Thus, the technique provides information not possible to obtain by the other assays. Moreover, as the cytokine of interest is captured immediately at the site of the producing cell, ELISpot is, compared to ELISA, less impaired by factors such as soluble receptors, protease activity and uptake of cytokines by surrounding receptor-bearing cells [193]. Finally, the ELISpot assay typically requires fewer cells for analysis, and data from studies of antigen-specific T cells have often demonstrated a very high sensitivity [139].

With the ultimate intention to use this assay to analyze samples from sepsis patients, we first needed to establish the cytokine profiles of the blood cells most likely to become activated under septic conditions, i.e. monocytes and granulocytes. Like many others, we used LPS as a model PAMP for TLR4 receptor stimulation. For the investigation, PBMC (5000 cells per well) were incubated for 20 hours in ELISpot plates with or without LPS followed by an analysis of cytokines known to be produced in response to LPS (IL-1β, IL-6, IL-8, IL-10, IL-12p40, TNF-α, MIP-1β and GM-CSF).

As seen in Figure 5, the frequency of secreting cells varied considerably depending on what cytokine that was analyzed. IL-6, IL-8, TNF-α and MIP-1β secreting cells displayed the highest frequencies after stimulation (average range 9.6-10.5% of PBMC). In contrast, IL-1β and GM-CSF were produced by smaller populations (5.5-6.3%) whereas IL-10 and IL-12p40 were produced by only a small fraction of the cells (1.2-1.6%). These frequencies of secreting cells were surprisingly consistent between different donors suggesting the existence of a relatively fixed population of cells that responded in a predefined manner.

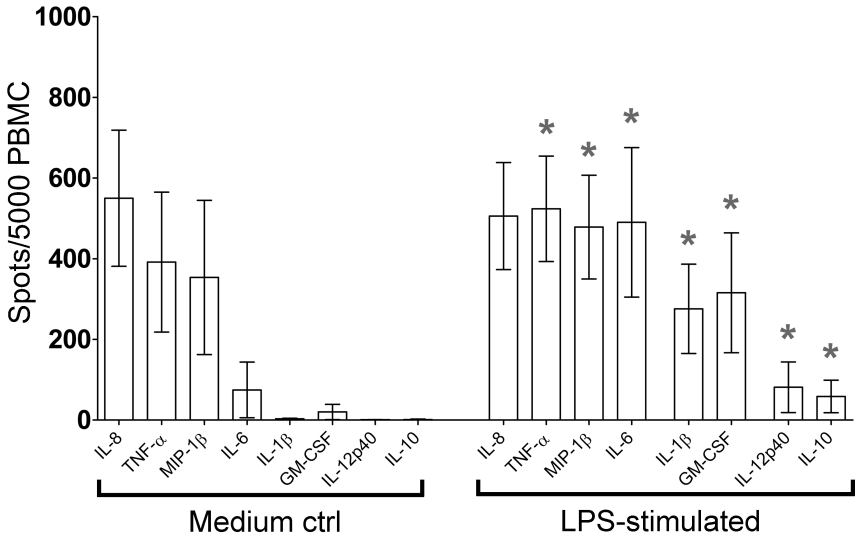

Figure 5.

ELISpot analysis of isolated PBMC (5000 cells/well) incubated for 20 hours in the absence or presence of LPS (100 ng/ml) and analyzed for the secretion of IL-8, TNF-α, MIP-1β, IL-6, IL-1β, GM-CSF, IL-12p40 and IL-10. Values represent the mean ± range of six individuals. Differences were considered significant for p<0.05 (*).

In contrast to the potent cytokine secretion seen by the LPS-stimulated PBMC, little or no spontaneous production was observed for several of the cytokines when cells were cultured in medium alone (IL-1β, IL-6, IL-12p40 and GM-CSF). Also in the instances where a cytokine was secreted by a substantial number of unstimulated cells (IL-8, MIP-1β and TNF-α), LPS had a clear stimulatory effect as reflected by the increased size and intensity of spots.

With LPS being recognized as a potent activator of monocytes and with the maximum number of secreting cells (i.e. around 10% for IL-8, TNF-α, MIP-1β and IL-6) roughly corresponding to the expected frequency of monocytes in PBMC, we repeated the experiments after having removed the monocytes using anti-human CD14-coupled magnetic beads. This way we could confirm that monocytes were indeed responsible for all or almost all of the cytokine secretion observed. With this knowledge and the fact that the frequencies of secreting cells showed little variation between individuals, we assumed that it would be quite possible to sort monocytes into subpopulations based on their cytokine profiles. This aspect was further studied in paper II.

While PBMC is the leukocyte fraction most commonly used in immunological research, granulocytes comprise the bulk of leukocytes in the blood and consist of a dominating population of neutrophils (≥95%) and smaller populations of eosinophils and basophils. Neutrophils have typically been described as short-lived phagocytic effector cells with an abundance of pre-stored anti-microbial factors but with limited capacity to synthesize new proteins [81]. In line with this, neutrophils harbor a comparatively small endoplasmic reticulum and contain fewer ribosomes in their cytoplasm than most other cell types [86]. Nonetheless, during the 1990s, neutrophils were shown to possess *de novo* cytokine-synthesizing capabilities *in vitro* in response to pathogenic factors such as LPS [82]. Although it was recognized that the amount of

cytokine produced by each neutrophil was limited, their presence in large numbers at infectious sites suggested that they could be a much more influential and immunologically important cell type than what had previously been acknowledged [75]. However, from a methodological viewpoint, these discoveries have remained controversial and further reports on cytokine production by neutrophils have been inconsistent [86]. Since neutrophils produce low amounts of cytokine per cell, large numbers of isolated granulocytes must be cultured together with LPS in order to accumulate amounts of cytokines measureable by ELISA, the technique most frequently used. As a result, the analysis becomes highly susceptible to the presence of contaminating cells (e.g. monocytes), something that is not easily discernible with the ELISA technique.

Based on previous experiences of the ELISpot technique in analyzing T cells, we thought that this would present an ideal method as, instead of measuring the accumulated amount of cytokine in a cell supernatant with the potential drawbacks discussed above, it provides information about the frequency of secreting cells. To test this, granulocytes were purified and 5000 cells per well were incubated with or without LPS for 20 hours in ELISpot plates and analyzed for the same panel of cytokines as previously done for PBMC. As expected, a large proportion of the added cells secreted IL-8 and MIP-1β, two chemokines known to be secreted by neutrophils [86]. TNF-α-producing cells were also detected, although at lower frequencies (Figure 6). In fact, the percentages of TNF-α spots were so low that we could not exclude that the production could originate from the minor populations of eosinophils and/or basophils [194, 195]. However, apart from a few strong spots, reminiscent of those seen for monocytes, we were unable to detect granulocyte-derived secretion of IL-1β, IL-6, IL-10, IL-12p40 or GM-CSF (Figure 6).

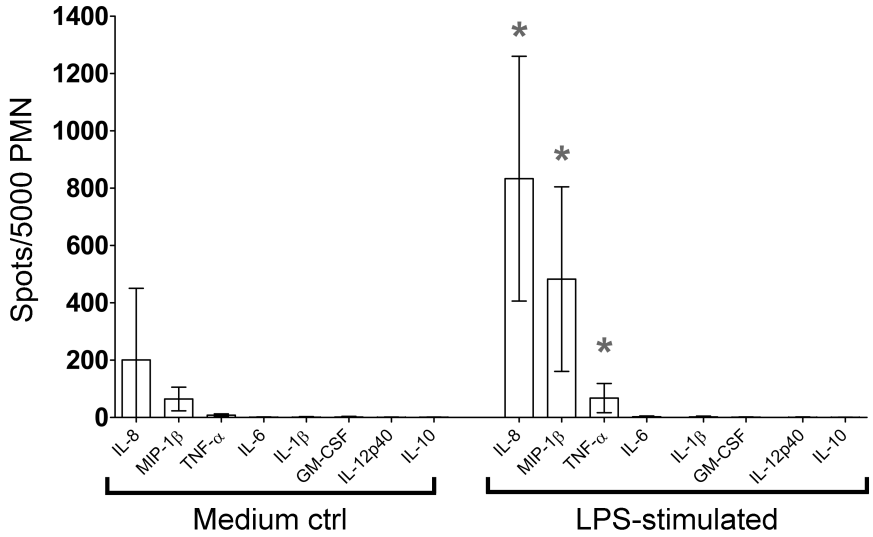

Figure 6.

ELISpot analysis of isolated PMN (5000 cells/well) incubated for 20 hours in the absence or presence of LPS (100 ng/ml) and analyzed for the secretion of IL-8, TNF-α, MIP-1β, IL-6, IL-1β, GM-CSF, IL-12p40 and IL-10. Values represent the mean ± range of six individuals (IL-8, TNF-α, MIP-1β, IL-6, IL-1β, GM-CSF), five individuals (IL-10) and four individuals (IL-12p40). Differences were considered significant for $p<0.05$ (*).

While these observations differ from several reports describing a multitude of cytokines being produced by granulocytes, they are in agreement with reports showing a much more restricted cytokine secretion profile for these cells [86, 189]. These two latter reports both used highly purified preparations of granulocytes in combination with careful interpretations of their ELISA measurements. Similar to us, these investigators have demonstrated a lack of IL-6 and IL-1β production by granulocytes. However, they also disqualified the small amounts of TNF-α detected in their granulocyte cell supernatants as being derived from contaminating mononuclear cells. To elucidate this further and to highlight possible differences in outcome between ELISA and ELISpot we investigated the production of TNF-α as well as IL-6 and IL-1β using both methods in parallel. For this purpose, isolated granulocytes were incubated for 20 hours with or without LPS at increasing cell numbers in ELISpot plates. The plates used for ELISpot were run as normal whereas the plates used to generate supernatants for ELISA measurements were without coating antibody. As expected, ELISpot was able to detect granulocyte-derived TNF-α spots already at 1000 cells per well. In contrast, IL-6 and IL-1β were either not detected at all or only in the form of a few strong spots. In ELISA, however, detectable amounts of cytokine in the cell supernatants were found first at $\geq$50.000 cells per well. Moreover, at this concentration, an interesting dichotomy in results between ELISA and ELISpot became apparent. Thus, while IL-6 and IL-1β were secreted by a very limited number of cells, much higher spot numbers were seen for TNF-α. However, the results from the ELISA were essentially reversed with the highest concentrations observed for IL-6 followed by IL-1β and lastly TNF-α. Accordingly, had the experiment only been interpreted based on the ELISA measurements, one had most likely arrived at a different conclusion regarding the cytokine-secreting capacity of granulocytes.

In summary, the findings in paper I demonstrated that LPS stimulation of granulocytes resulted in a differential production of cytokines. By using the ELISpot technique we could demonstrate that several cytokines previously reported to be produced by granulocytes were likely to be the result of contaminating monocytes. The fact that two of the granulocyte-produced cytokines, IL-8 and MIP-1β are potent chemokines indicates that the primary function of granulocytes may indeed be to attract other immune cells to the infectious site and hereby promote the inflammatory process [88]. We also showed that monocytes are the principal cells responsible for the cytokine secretion observed in LPS-stimulated PBMC. While the cytokine profile of stimulated monocytes contained a number of both pro- and anti-inflammatory cytokines it was at the same time clear that they displayed a quite consistent pattern among different donors regarding the frequency of cells secreting certain cytokines. This may suggest that monocytes can be split into subpopulations based on their cytokine profiles. The broad cytokine producing capacity of monocytes on the other hand shows that these, in addition to displaying chemotactic activity, also serve as stimulatory and/or inhibitory regulators of other immune cells. Thus, monocytes/macrophages seem to be likely candidates to participate in creating the imbalance in the production of cytokines, suggested to be associated with sepsis.

5.2 PAPER II- FLUOROSPOT ANALYSIS OF TLR-ACTIVATED MONOCYTES REVEALS SEVERAL DISTINCT CYTOKINE-SECRETING SUBPOPULATIONS

Despite the great functional diversity of cells originating from monocytes (e.g. macrophages, dendritic cells and osteoclasts), only three distinct subpopulations of monocytes have been clearly defined. As previously mentioned these are the classical, non-classical and intermediate monocytes [129]. While this division has been universally accepted as the basis of monocyte heterogeneity, it has been difficult to ascribe any clear *in vivo* function to these subsets [196]. It is also evident from other studies that monocytes display further phenotypic heterogeneities, both in their expression of cell surface markers and also in their capacity to produce and secrete various cytokines [132, 197].

In view of these results and with cytokines playing such a critical role in all aspects of immunity, we wanted to further investigate the cytokine secretion profile of monocytes to see if we could find a consistent pattern of secretion that could provide a basis for a functional division of these cells. Such cytokine-based subclassification has been done for T cells and has been instrumental for the understanding of the functional differentiation between T cell subsets [23].

To investigate this, monocytes from healthy volunteers were enriched using negative selection. While this approach resulted in a lower purity of isolated monocytes than what is typically achieved with positive selection, it has the significant advantage of leaving cells "untouched". Thus, compared to positive selection, no bias in selection of a particular type of monocyte or steric hindrance of the TLR co-receptor CD14, the most common target for monocyte isolation, is introduced [127, 131, 132] [137]. Consequently, the monocytes investigated in paper II consisted of all three subpopulations, i.e. the classical, the intermediate and the non-classical monocytes, in essentially the same proportions as found in blood. Furthermore, we extended the number of stimuli and, besides LPS (TLR4), we also included LTA, a TLR2 ligand derived from gram-positive bacteria. For the analysis we used the novel FluoroSpot assay as this allowed the simultaneous analysis of more than one cytokine and hereby provided a more informative picture of the cytokine profiles [198].

When we analysed the enriched monocytes in a single-color FluoroSpot, a similar pattern of cytokine secretion was observed as was previously seen for PBMC. Thus, following stimulation with either LPS or LTA, the cells produced IL-6 (average percentage of responding cells: 32.5%), TNF-α (39.2%), MIP-1β (30.1%), IL-1β (25.9%), GM-CSF (9.1%), IL-10 (1.3%) and IL-12p40 (1.2%) (Figure 7).

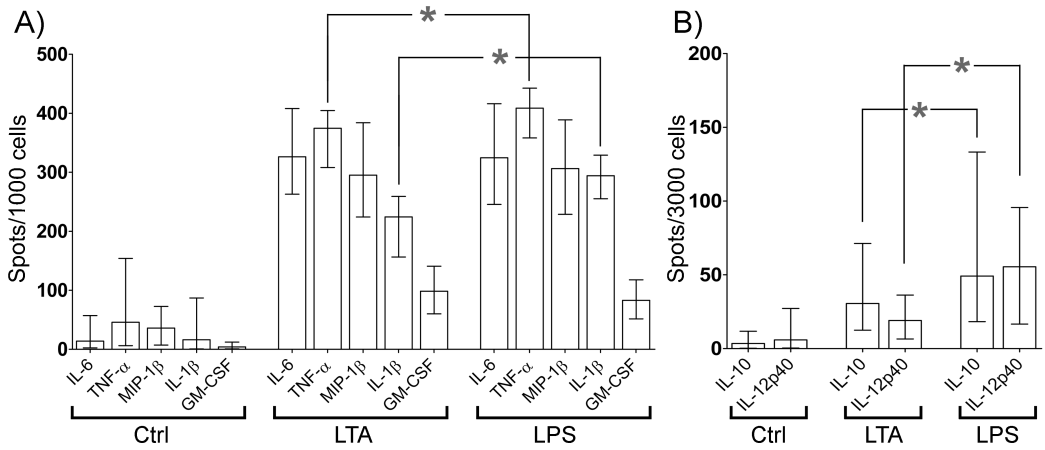

Figure 7.

(A) Single-colour FluoroSpot analysis of enriched monocytes (1000 cells/well) incubated for 20 hours in the absence or presence of LTA (500ng/ml) or LPS (50ng/ml) and analyzed for the secretion of IL-6, TNF-α, MIP-1β, IL-1β and GM-CSF using 1000 cells/well (A) or for the secretion of IL-10 and IL-12p40 using 3000 cells/well (B) Values represent the mean ± range of six individuals analyzed (n=6). Differences were considered significant for p<0.05 (*).

Based on the number of secreting cells, a tentative division into three subgroups composed of high frequency responders (IL-6, IL-1β, TNF-α and MIP-1β), moderate frequency responders (GM-CSF) and low frequency responders (IL-10 and IL-12p40) could be made. When comparing responses to the two different TLR-ligands the number of monocytes secreting IL-6, MIP-1β and GM-CSF were similar independent of whether LTA or LPS was used for stimulation. However, for the cytokines IL-1β, TNF-α, IL-10 and IL-12p40 a modest but significant difference was observed, i.e. LPS generally stimulated more cells compared to LTA (Figure 7). As TLR4 is unique by signaling through both the Myd88- and TRIF-dependent pathways [41], these results may reflect the activation of the two intracellular pathways in parallel as compared to the single Myd88-dependent activation achieved through TLR2. Interestingly, this additional signaling only seemed to pose a positive effect on the secretion of some cytokines (IL-1β, TNF-α, IL-10 and IL-12p40) but not on others (IL-6, MIP-1β and GM-CSF).

In our study, antibodies to IL-6 and IL-1β were available for detection with green fluorescence and each of these could be combined with red fluorescence detection for all the other cytokines. This way, we could investigate to what extent monocytes secreting IL-6 or IL-1β also secreted TNF-α, MIP-1β, IL-1β, GM-CSF, IL-10 or IL-12p40. In total, the pattern of co-secretion was evaluated through eleven different combinations of two-color FluoroSpot.

In the case of monocytes secreting TNF-α, the great majority also simultaneously produced IL-6 and IL-1β, displaying a secretory overlap ranging from (on average) 65-83% in response to both LTA and LPS. However, when IL-1β and IL-6 were tested in combination with the two cytokines MIP-1β and GM-CSF a different relationship was observed. While the majority of IL-6-secreting monocytes also secreted MIP-1β (>75% average) only a minority of the IL-1β-secreting cells secreted this cytokine (<38%

average). Similarly, of the GM-CSF-secreting monocytes the great majority (>83% average) also secreted IL-6 while less than 30% co-secreted IL-1β. This observation of a high percentage of co-secretion for IL-6 and a low percentage for IL-1β was similar in relation to IL-10 and IL-12p40. Finally, when combining IL-6 and IL-1β, a minority of the cells secreting IL-6 also secreted IL-1β (average range 23-40%). Figure 8 below summarizes the relationship in FluoroSpot for IL-6/GM-CSF, IL-1β/GM-CSF and IL-6/IL-1β.

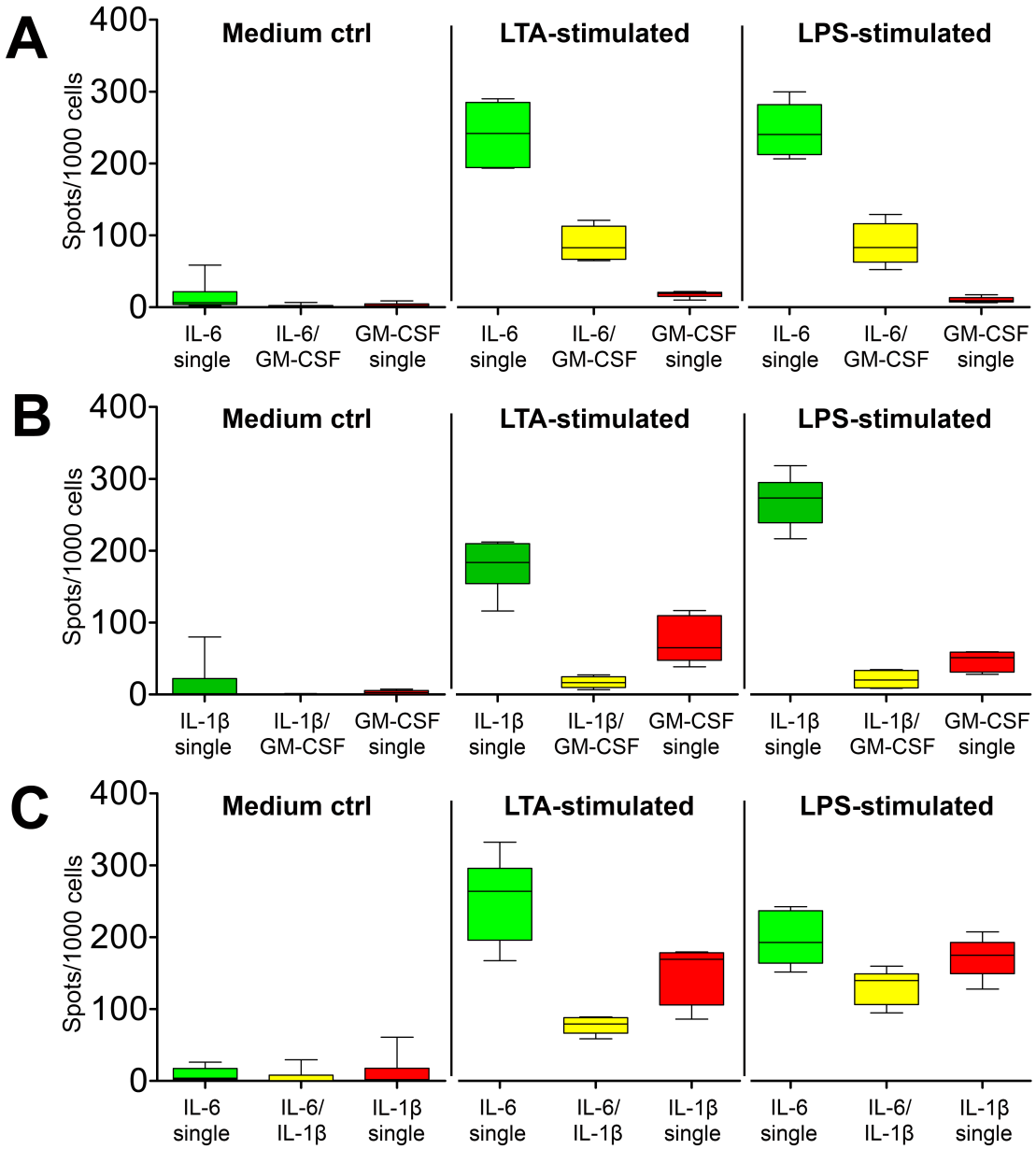

Figure 8.

FluoroSpot analysis of cytokine co-secretion by enriched monocytes. Cells (1000/well) were incubated for 20 hours and analyzed for the co-secretion of (A) IL-6/GM-CSF, (B) IL-1β/GM-CSF and (C) IL-6/IL-1β in response to LPS (50 ng/ml) or LTA (500 ng/ml). By the use of FluoroSpot, each cytokine combination revealed three distinct cytokine-secreting subpopulations: monocytes secreting either cytokine (FITC or Cy3 labeled; green or red boxplots) or monocytes secreting both cytokines (yellow boxplots). Boxplots represent minimum, first quartile, median, third quartile and maximum and are based on six individual donors (n=6).

Overall, these results demonstrate that despite being incubated under identical culture conditions, individual monocytes responded differently to TLR2- and TLR4-stimulation by secreting what appeared to be a predisposed set of cytokines. For each individual and combination of cytokines analyzed, a recurring pattern of co-secretion was detected as each responding monocyte could be categorized into one of three cytokine-secreting subpopulations ("FITC single", "FITC+Cy3 double" or "Cy3 single"). Furthermore, although variations existed between individuals and TLR-ligand, percentages of co-secretion were still fairly consistent, suggesting that predetermined subsets of monocytes do exist and that a sophisticated regulation in how these cytokine subsets are maintained within the immune system are in play. However, due to our analysis being limited to the detection of only two cytokines simultaneously, there was a natural restriction in our understanding of how these different subpopulations of monocytes correlated to each other. For example, we could not elucidate how the monocytes secreting GM-CSF related to the ones secreting TNF-α or whether the small population of monocytes co-secreting IL-1β and GM-CSF also secreted IL-12p40. Nonetheless, although the results in paper II only partially reveal what appears to be a complex arrangement, one can still speculate how these cytokine secretion patterns are formed. First, it is possible that monocytes could be marked by differences in their TLR-signal transduction pathways specific for TLR2 and TLR4. As an example, a subgroup of monocytes could harbor an increased quantity of certain MAPKs, allowing these cells to stabilize the translation of a more diversified number of cytokines in response to TLR-ligation [199]. The effect would essentially be monocytes secreting a wider repertoire of cytokines compared to others. Through subtle variations in the starting quantities of the different MAPKs, diversity in cytokine secretion would follow and manifest itself as the monocyte subpopulations observed by us in FluoroSpot. Alternatively, the cytokine subsets could also be the result of direct changes in the cellular expression of the genes coding for the cytokines investigated. In this case, TLR-signaling would pose no distinction in activation in one monocyte compared to another. Instead, individual monocytes would be predisposed towards secreting a particular set of cytokines as a result of direct epigenetic modifications to their genome [200]. In turn, these modifications could originate either at the level of monocyte progenitors in bone marrow, or through continued differentiation in the peripheral blood [11].

Irrespective of the molecular basis behind these cytokine subsets, our results nonetheless indicate a diversity among monocytes that in complexity far exceeds a simple division of the cells into the three subsets of classical (~90%), intermediate (~5%) and non-classical (~5%) monocytes.

In order to more specifically highlight this issue, we also purified the $CD16^+$ monocytes for analysis in FluoroSpot (of IL-6/TNF-α, IL-6/MIP-1β, IL-6/GM-CSF, IL-6/IL-10 and IL-6/IL-12p40). Apart from a small increase in the proportion of TNF-α, the $CD16^+$ monocytes displayed a similar cytokine-secreting profile as the monocyte population as a whole. Simply put, if the intermediate and non-classical monocytes had been the predominant producers of TNF-α and/or IL-10 [119, 127], a much higher

frequency of such cytokine-secreting cells should have been seen when the proportion of $CD16^+$ cells (~10% in total monocytes) was increased to ~88% in the specific experiment described above. Although the $CD16^+$ monocytes were not separated into their respective subpopulations of intermediate and non-classical, our results still holds merit in disputing the practice of relating the expression of CD14 and CD16 to a specific type of TLR4-induced cytokine profile. Instead, we propose that other cell surface markers should be investigated as potential correlates of the cytokine secretion profile of monocytes to provide monocyte researchers with an additional and potentially better tool for exploring the function of these cells than what is currently being utilized.

As touched upon above, a fundamentally important question is whether monocytes are equal when it comes to their ability to secrete different cytokines or whether monocytes, similar to T cells [100], fall into specialized subsets endowed with a particular type of function that is mirrored by their TLR-induced cytokine secreting capacity *in vitro*. Our findings in paper II not only strengthen the argument for a high degree of cytokine specialization within the population of monocytes, but they also contradict the official nomenclature of monocytes as being the functional identity of these cells. Nevertheless, open questions remain regarding the stability of these subsets, how they relate to the development of macrophages and whether or not they can be correlated to surface markers that are separate from CD14 and CD16.

5.3 PAPER III- CIRCULATING MONOCYTES ARE NOT THE MAJOR SOURCE OF PLASMA CYTOKINES IN PATIENTS WITH SEPSIS

While several immunomodulatory therapies for treating sepsis, including the blocking or neutralization of pro-inflammatory cytokines, have been used in the clinic during the last two-three decades there has been little improvement in the rate of fatal outcomes in septic patients [201]. The reasons behind these failures have been suggested to stem from an incomplete understanding of the septic syndrome and by a lack of precise criteria for staging the patients' immune status [202, 203]. One argument has been that while interventions intended to reduce inflammation may be suitable during the initial phases of sepsis, such treatments should not be given to those that have moved into a secondary state dominated by anti-inflammatory mechanisms [151, 169, 201]. In the trials performed, patients have been categorized according to the standard criteria for sepsis, i.e. severe sepsis or septic shock. However, while these definitions are universally accepted, they do not take into full account the differences in immunological status that are likely to exist between patients belonging to the same sepsis category [204]. Although a number of studies have tried to define patients as being in either an inflammatory or anti-inflammatory state based on cytokine levels in their plasma, it has not been possible to find a consensual correlation between a certain set of cytokines or other mediators and the different stages of sepsis [181, 205-208].

The lack of predictive diagnostic markers has typically been explained by the inherent heterogeneity in the etiology of sepsis in different patients and by the difficulties to stratify a disease process based on accumulated levels of cytokines in plasma [181, 203, 209]. Partly due to this and partly due to other potential inadequacies of ELISA and Luminex measurements [139, 193, 209], we wanted to assess cytokine secretion in septic patients by looking at the cellular level. We hypothesized that measuring cytokine secretion at the single cell level directly *ex vivo* as possible by the ELISpot technique would give us a more accurate picture and allow for a better categorization of patients into either a hyper- or hypo-inflammatory state. If correct, ELISpot could this way provide valuable insight into the pathogenesis of sepsis and could potentially be used diagnostically to both identify sepsis at an early stage and to more accurately define the various stages of the disease.

Thus, with the purpose of capturing a "snapshot" of the ongoing cytokine secretion, total leukocytes, isolated by dextran sedimentation, were incubated overnight in the absence or presence of LPS in ELISpot plates coated with capture antibodies against IL-6, TNF-α, IL-1β, GM-CSF, IL-10 and IL-12p40. Apart from being a very gentle method of isolating the cells, this protocol also allowed us to shorten the time needed for setting up our experiments. Counting the time used between drawing of the blood and starting incubation of the cells in the ELISpot plates, this ranged from 45-180 minutes in all individuals analyzed. In total, 35 septic patients, 32 healthy controls and 9 endotoxemia volunteers were evaluated in this study. Plasma samples from each individual were also collected and analyzed in Luminex for the same set of cytokines.

This way we were able to analyze both the spontaneous and the LPS-induced secretion in the different groups as well as compare these to the levels found in plasma.
When first looking at the number of spontaneous spots generated by cells that were cultured overnight in medium alone, we could not observe any difference between patients and the healthy controls for any of the cytokines (Figure 9).

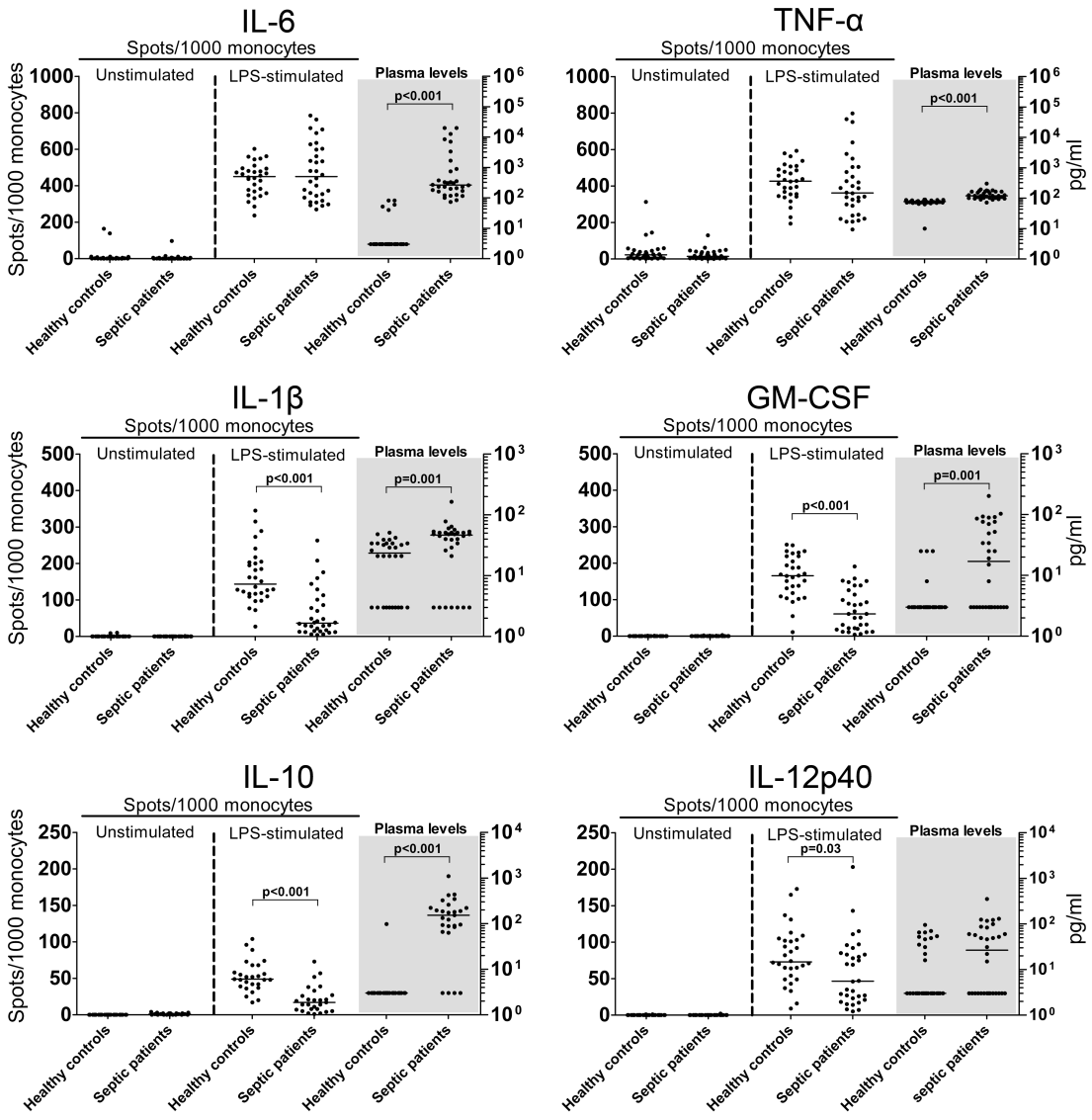

Figure 9.

Cytokine ELISpot and Luminex analysis (IL-6, TNF-α, IL-1β, GM-CSF, IL-10 and IL-12p40) of total leukocytes and plasma isolated from septic patients and healthy controls. The results of ELISpot are presented as the number of spots detected per 1000 monocytes analyzed, whereas concentrations in plasma are shown as pg/ml. Data is presented in the form of dot-plots with the horizontal bars indicating the median value of each group. For 3 patients and 2 healthy controls a differential blood count could not be established and they were therefore excluded from the analysis, resulting in a total cohort of 32 septic patients and 30 healthy controls. In the case of IL-10, the analysis was performed on a reduced number of patients (n=27) and healthy controls (n=27). Differences were considered significant for p<0.05 (*) using the Mann-Whitney U test.

In fact, the number of cytokine-secreting cells in freshly cultured leukocytes was generally very low in both groups. Considering a supposed role of monocytes and their secreted cytokines as principle mediators of the septic syndrome, these results were quite surprising to us, especially considering that the concentrations of the same cytokines in plasma were significantly up-regulated at the time of sampling. Since monocytes can be activated by very low amounts of TLR-ligands [197] and the plasma of many septic patients reportedly contain up to 5 ng/ml of LPS [210], we had envisioned an increased number of *in vivo* activated, cytokine-secreting monocytes in the patients. Earlier studies of septic patients that also failed to detect increased cytokine-secretion in unstimulated cell cultures have typically employed ELISA for their measurements [172, 175, 211, 212]. We had anticipated that, given the higher sensitivity of the ELISpot, and by looking at the single cell level, we would get a result that corresponded better to the increased cytokine levels in plasma. It is of course possible that the monocytes, as a consequence of the *in vitro* handling once taken out of the patient, rapidly ceased their secretion of cytokines. However, we believe this is unlikely since TLR-activation typically elicits very powerful cytokine responses that are not easily terminated [213]. In support of this, we also showed in separate experiments that stimulation of whole blood samples from healthy donors with LPS, followed by leukocyte isolation using dextran, did not affect the capacity of the cells to produce and secrete cytokines (data not shown). Instead, we believe that the results reflect that monocytes, once activated *in vivo,* are quickly removed from the circulation by adherence to the endothelium [214, 215]. Although the monocytes in this state might not instantly extravasate into tissues, we speculate that they are nonetheless inaccessible to blood sampling via venipuncture.

In contrast to the limited cytokine secretion by cells cultured in medium alone, the same cells added to ELISpot wells containing LPS resulted in an increased number of cytokine secreting monocytes both in patients and healthy controls (Figure 9). However, while the number of IL-6 and TNF-α spots/1000 monocytes were similar in the two groups, septic patients responded with a lower frequency of IL-1β, IL-10, GM-CSF ($p<0.001$) and IL-12p40 ($p= 0.03$) secreting monocytes. Although most studies of sepsis have demonstrated a down-regulation in the amount of cytokines secreted by monocytes in response to LPS [172-178], our results in ELISpot highlights new aspects on this refractory or endotoxin-tolerant state. Firstly, the number of LPS-induced IL-6 and TNF-α spots were similar in sepsis patients and controls, demonstrating that the sepsis-derived monocytes were indeed able to respond to LPS. This indicates that the intracellular signaling pathways necessary for secreting these two cytokines had not been significantly affected. In contrast, the frequencies of cells secreting IL-1β, IL-10, GM-CSF and IL-12p40 were lower in the septic patients. Thus, while the isolated population of monocytes had been properly activated through TLR4 to secrete IL-6 and TNF-α, they simultaneously lacked the ability to secrete another group of cytokines, suggesting a deficiency in the TLR4 signal transduction and/or secretory pathways specific for IL-1β, IL-10, GM-CSF and IL-12p40. In theory, increased levels of intracellular negative TLR-regulators described earlier in this thesis could explain this altered repertoire of cytokine secretion. Previous reports on monocytes in sepsis have

come to similar conclusions on monocyte TLR4-responsiveness as being "reprogrammed" [174]. However, while their results, in general, have pointed towards a strengthened anti-inflammatory profile with a decreased production of cytokines such as TNF-α and IL-6 but a maintained production of IL-10 [174], our data indicate the opposite with a sustained TNF-α and IL-6 production but a lowered number of IL-10 secreting cells.

Another hypothetical explanation for the observed changes in LPS-induced cytokine profile in septic patients could involve replacement of the monocyte population by another population of monocytes generated in a second wave of host defense. Recently, mice have been shown to harbor a reservoir of monocytes in the spleen that can serve exactly this purpose by rapidly restoring the monocyte population in situations of systemic inflammation [216]. If present in humans, such newly recruited monocytes would constitute a fresh pool of monocytes not likely to show evidence of exhaustion, but still be marked by changes in the way these cells respond to LPS *in vitro*. An increased proportion of such monocytes, as possibly seen in the samples of our study, could therefore be a sign of stimulated and more cytokine capable monocytes having moved out into tissues. This concept is supported by the fact that the change in the cytokine repertoire of septic blood monocytes could be negatively correlated with the patient's individual SOFA score, suggesting a link between the "functional" composition of the monocytes in circulation and the degree of organ dysfunction (Figure 10).

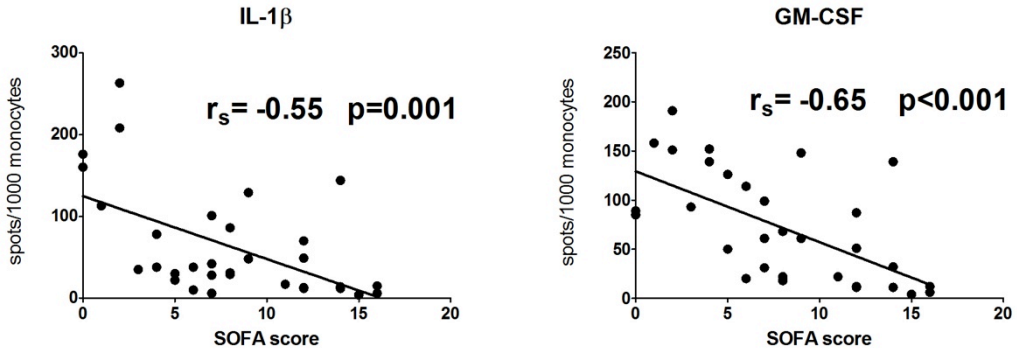

Figure 10.
Analysis of the correlation between the SOFA score and the number of IL-1β - or GM-CSF-secreting monocytes in response to LPS (spots/1000 monocytes). Differences were considered significant for p<0.05 (*) using the Spearman rank correlation coefficient.

During the course of this study, we also had the opportunity to test cells from healthy individuals challenged with low doses of endotoxin (40 EU/kg). Blood samples were collected at three time points; before, 30 minutes after and 150 minutes after LPS infusion. In this way, we could control the starting point of the systemic inflammatory response and thereby increase our chances of "capturing" a population of *in vivo* activated monocytes from the circulation. However, in line with results in sepsis patients, we found few or no cytokine-secreting monocytes when cells were incubated in medium alone (Figure 10). This was in spite of elevated levels of the same cytokines

being readily detected in plasma after 150 minutes. At this time point, we also saw a dramatic reduction in the number of LPS-induced spots which, however, correlated to a corresponding reduction in the number of circulating monocytes (Figure 10).

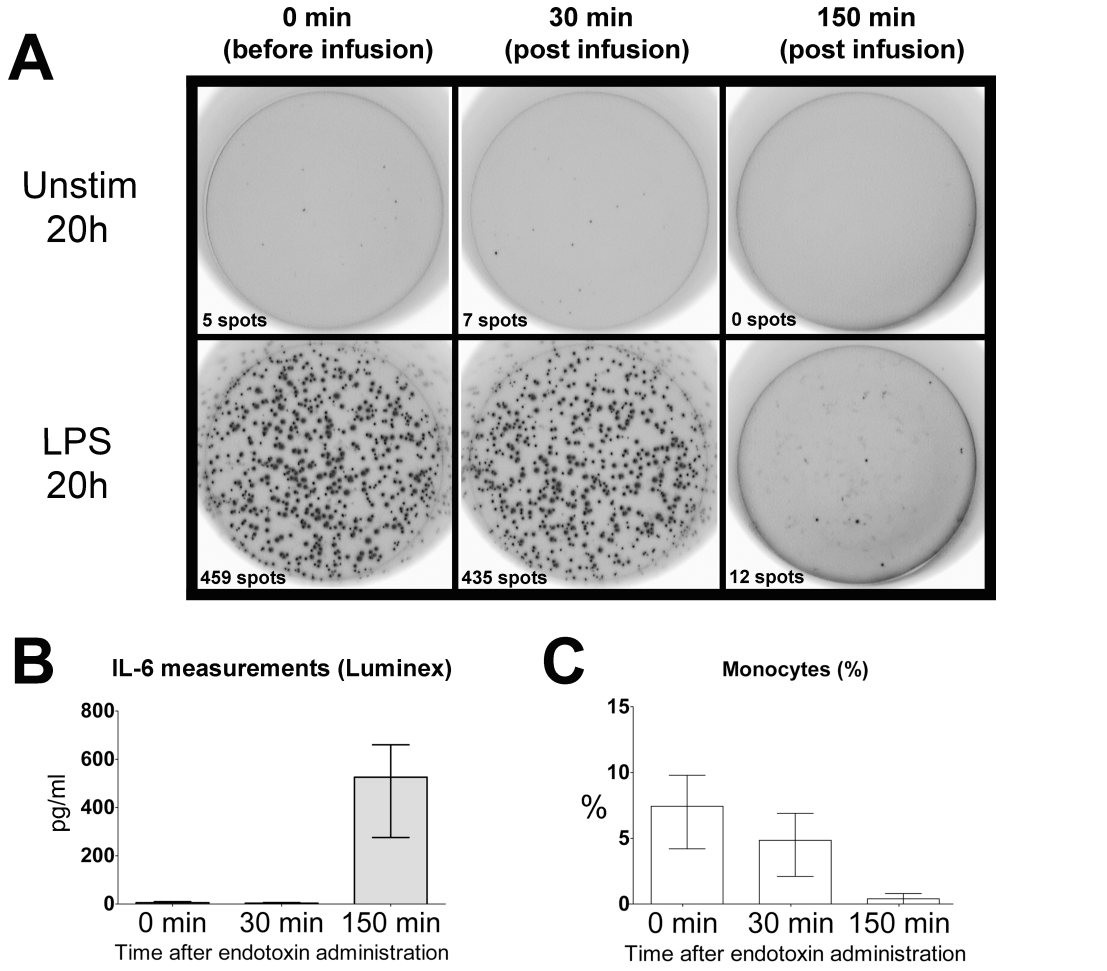

Figure 11.

IL-6 ELISpot of leukocytes, IL-6 Luminex analysis of plasma and differential cell count on the percentage of monocytes in leukocytes isolated from healthy volunteers injected intravenously with low doses of endotoxin (40 EU/kg). Blood samples were retrieved before, 30 minutes and 150 minutes post infusion. (A) Representative examples of IL-6 ELISpot following endotoxin administration. Cells (15,000 leukocytes/well) were incubated for 20h in the absence or presence of LPS. (B) Luminex analysis of IL-6 in plasma following endotoxin administration. (C) Percentages of monocytes in the isolated samples of leukocytes before, 30 minutes and 150 minutes post infusion. Data represent the mean ± range of three individual donors.

Collectively, these results went against our initial expectations, especially considering that blood samples harvested 30 minutes after the injection of LPS at least in theory should contain sufficient amounts of endotoxin to trigger the leukocytes both in the circulation and subsequently in the ELISpot wells. However, previous studies of the levels of endotoxin that remain in circulation post LPS infusion have been evaluated using the Limulus assay [217]. Using an equal dose of injected endotoxin (40EU/ kg) the study by Pajkrt *et al* only found minute amounts of LPS in the blood 30 minutes

post injection. Thus, considering the seemingly rapid removal of activated monocytes from the circulation and the numerous plasma proteins *in vivo* that are known to interact with LPS and modify its stimulatory activity, our negative results are not unreasonable. Furthermore, recent experiments in mice have demonstrated that cells of hematopoietic origin, compared to cells of non-hematopoietic origin, only play a minor contributing role in generating the increased plasma levels of IL-6 following LPS injection [218].

In conclusion and contrary to our expectations, our results indicate that sepsis is not associated with an increased number of cytokine-secreting monocytes in the blood. Instead, the increase in cytokine levels seen in plasma from patients is likely generated by extravasated monocytes, macrophages and/or from other sources including activated endothelial cells and those of non-hematopoietic origin. However, as a manifestation of the disease, we could observe an altered cytokine profile in the septic monocytes after LPS stimulation *in vitro*. This was seen in the form of a lowered frequency of monocytes secreting IL-1β, IL-10, IL-12p40 and GM-CSF. This reduction was shown to correlate to the degree of organ dysfunction as measured by SOFA score. The correlation suggests that ELISpot analysis of these cytokines, similar to what has previously been shown for monocyte HLA-DR [181], could potentially be useful as a clinical tool for determining the immunological status of septic patients.

6 CONCLUSIONS

As a leading cause of morbidity and mortality in critically ill patients, sepsis represents a major burden on healthcare resources worldwide [209]. In fact, medical costs for sepsis are expected to exceed $60 billion per year in the United States alone [219], a staggering amount that will only continue to rise as the population grows older and antibiotic resistance becomes more ubiquitous. Furthermore, there has been a disappointing level of improvement in the treatment of sepsis patients during the last few decades [201]. This is in spite of great efforts having been made to find better techniques for early diagnosis as well as novel strategies for therapeutic intervention, both of which have been key factors in improving the outcome of other disorders such as acute myocardial infarction, stroke, and trauma [220]. By understanding the early pathogenesis of these diseases, it has been possible to develop and test new means of therapy that have ultimately led to significant breakthroughs in treatment. A similar progression in sepsis would be highly valued. However, in the last two decades, the failures of several high-profile trials have made experts realize the need for a re-evaluation of the sepsis syndrome [4]. A better definition and real-time assessment of the immunological status characterizing the different stages of sepsis will here be of critical importance and hopefully allow both for improved diagnosis and treatment of this severe condition [202, 220].

In this thesis, we developed and evaluated the highly sensitive ELISpot and FluoroSpot techniques for the measurement of a number of pro- and anti- inflammatory cytokines. With the ultimate purpose of investigating cells from sepsis patient, we first applied the techniques to study cytokine secretion by normal monocytes and granulocytes in response to TLR-ligation. This way, we could demonstrate the advantages and improved accuracy of establishing cytokine profiles at the single cell level compared to measuring cytokines in cell supernatants. In addition, we showed that monocytes, at least under the experimental conditions of the FluoroSpot assay, can be divided into cytokine-secreting subsets in response to TLR-stimulation and we believe that this type of categorization may represent a more relevant way of defining the heterogeneity and ultimately the function of these cells. Using blood cells from septic patients, we further demonstrated that, despite analyzing cytokine secretion at the single cell level in newly admitted patients presenting with severe sepsis and septic shock and with significantly elevated cytokine levels in their plasma, it was not possible to detect any cells expressing increased production of these biomarkers. We believe that this is due to that, once activated, the cells responsible for cytokine production are rapidly removed from the circulation by adherence to the endothelium and subsequent extravasation. Finally, while ELISpot analysis of leukocytes isolated from septic patients showed no signs of increased spontaneous cytokine secretion directly *ex vivo*, LPS-stimulation of these cells revealed a shift in the composition of the monocytes towards a more pro-inflammatory, less diverse, cytokine-secreting profile. This reduced repertoire of secreted cytokines could be correlated with the degree of organ dysfunction in these patients.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Ett infekterat sår aktiverar kroppens immunförsvar i form av en inflammationsreaktion. Denna mobilisering har som mål att försvara oss mot de bakterier som tagit sig in i såret. Det medfödda immunförsvaret spelar här en avgörande roll då dess immunceller, bland andra monocyter och granulocyter, innehar förmågan att känna igen bakteriefragment via så kallade Toll-lika receptorer och starta den inflammatoriska processen genom att producera viktiga signalproteiner kallade cytokiner. Cytokinerna signalerar till närliggande celler att en infektion påträffats, varvid de klassiska inflammationstecknen uppstår i det infekterade området; svullnad, rodnad och dunkande smärta.

I normala fall läker infektioner av sig själva utan att det uppstår några komplikationer men i sällsynta fall kan bakterier eller fragment av dessa ta sig ut i blodet och orsaka sepsis (blodförgiftning). Detta är ett allvarligt sjukdomstillstånd där det medfödda immunförsvaret överreagerar och producerar för mycket cytokiner när bakterierna sprider sig i blodomloppet. Sepsis kännetecknas initialt av feber, frossa och ett påverkat allmäntillstånd, men kan i allvarligare fall snabbt övergå i livshotande yttringar såsom blodtrycksfall, organsvikt och medvetslöshet. Sjukdomen är komplex då den sägs bestå av två parallella faser där immunförsvaret först överaktiveras, och sedan trycks ner av efterföljande anti-inflammatoriska processer.

Läkare har länge efterfrågat förbättrade möjligheter att tidigt kunna diagnostisera sepsis men även metoder för att i senare skeden av sjukdomsförloppet kunna klassificera sepsis utifrån patientens aktuella immunstatus. Syftet med den här avhandlingen var att undersöka cytokinproduktionen från monocyter och granulocyter, i både friska individer och i sepsispatienter, med hjälp av en känslig metod kallad ELISpot. Denna metod utgår från de isolerade cellerna och mäter frisättningen av cytokin i form av antalet producerande celler och inte, som de flesta andra tester, den totalamängd som sekreterats från ett okänt antal producerande celler. Härigenom kan man uppnå en sorts realtidsanalys på enskild cellnivå av vad som produceras just där och då. Vår förhoppning var att, genom att applicera denna metod på patienter med sepsis, kunna fånga en ögonblicksbild av cytokinproduktionen och på så sätt bättre kunna konkretisera sjukdomens olika faser.

I det första delarbetet undersökte jag och mina kollegor cytokinproduktionen hos isolerade immunceller tagna från friska individer efter stimulation med ett bakteriefragment kallat lipopolysackarid (LPS). Vi behövde veta vilka cytokiner som sekreterades som svar på stimuli liksom vilka celler som stod för produktionen. Detta var viktigt att ta reda på inför testningen av ELISpot-metoden på sepsispatienter. Flera cytokiner undersöktes och ett återkommande mönster kunde urskiljas: Granulocyter producerade framförallt IL-8 och MIP-1β och en mindre andel av cellerna även det proinflammatoriska cytokinet TNF-α men däremot inget IL-6, IL-1β, GM-CSF, IL-10 eller IL-12p40. Denna selektiva cytokinproduktion hos granulocyter skilde sig från

många tidigare publicerade arbeten där andra och mindre diskriminativa metoder använts. Bland de andra immuncellerna var det framför allt monocyter som svarade på LPS-stimulering. Dessa var också mycket mer kapabla än granulocyter och producerade alla de cytokiner som undersöktes. Antalet producerande monocyter skilde sig dock stort beroende på vilket cytokin som analyserades, och till exempel producerades både IL-10 och IL-12p40 av ca 10 gånger färre celler jämfört med TNF-α och IL-6, och ca 5 gånger färre jämfört med GM-CSF och IL-1β. Då dessa förhållanden i antalet cytokinproducerande monocyter var relativt stabila mellan olika individer, tyder detta på att monocyternas förmåga att producera dessa cytokiner var förutbestämt och på något sätt reglerades av immunförsvaret.

I det andra delarbetet användes en ny form av fluorescensbaserad ELISpot-teknik kallad FluoroSpot. Med hjälp av denna kunde vi undersöka två olika cytokiner samtidigt från samma population av isolerade celler, istället för bara en såsom med ELISpot. Utöver LPS undersöktes även monocyternas cytokinproduktion som svar mot ett annat bakteriefragment kallat LTA. Som vi sett förut producerades de olika cytokinerna av olika antal monocyter men tack vare FluoroSpot teknikens fördelar fick vi nu även en inblick i hur monocyternas produktionen överlappade mellan olika cytokiner. Till exempel visade det sig att nästan alla monocyter som producerade GM-CSF även producerade IL-6 medan endast en mindre andel uppvisade en samtidig produktion av IL-1β. Genom att kombinera analyserna från flera olika cytokinkombinationer i FluoroSpot kunde vi snabbt urskilja vissa distinkta och överlappande mönster för hur denna produktion var arrangerad och hur dessa mönster var relativt stabila från individ till individ. Studien ger på detta sätt en ny inblick i monocyternas kategorisering och den komplexitet som finns inbyggd i denna del av det medfödda immunförsvaret.

I det sista delarbetet undersöktes och jämfördes cytokinproduktionen hos immunceller från 30 friska individer och 32 sepsispatienter med hjälp av ELISpot tekniken. Återigen testades samma panel av cytokiner som i de tidigare studierna: IL-6, TNF-α, IL-1β, GM-CSF, IL-10 och IL-12p40. Från det att blodet tappats från patienten till att cellerna isolerats och adderats till ELISpot för analys tog det cirka 1 timme. Vi hoppades därigenom kunna fånga en del av de monocyter som förmodats redan vara aktiverade i sepsispatienterna och på så sätt i realtid kunna studera och jämföra cytokinproduktionen hos patienter och friska individer liksom mellan patienter i olika stadier av sepsis. Trots att vi i flertalet patienter fann kraftigt förhöjda nivåer av fria cytokiner i blodet kunde vi dock aldrig se en höjning i antalet cytokinproducerande celler hos dessa. Det var alltså ingen skillnad mellan helt friska människor och allvarligt sjuka sepsispatienter vilket grusade vår förhoppning att med denna metod kunna konkretisera sjukdomens olika faser. När vi däremot stimulerade cellerna med LPS in vitro, ökade som förväntat antalet cytokinproducerande monocyter. Mönstret från denna cytokinproduktion var dock annorlunda i sepsispatienter jämfört med friska individer. Sålunda var antalet producerande celler signifikant lägre i patienterna för vissa av cytokinerna (IL-1β, GM-CSF, IL-10 och IL-12p40) medan de för andra

cytokiner (IL-6, TNF-α) var desamma i båda grupperna. Denna selektiva minskning visade sig korrelera med nivån av organsvikt bland sepsispatienterna.

Sammanfattningsvis hoppas vi att studierna i denna avhandling kan bidra till en allmänt bättre förståelse av cytokinproduktionen hos såväl granulocyter som monocyter liksom visa på möjligheten att indela monocyter i funktionellt distinkta grupper baserat på vilka cytokiner de har förmågan att producera. Våra resultat från sepsispatienter indikerar vidare att den cirkulerande populationen av monocyter inte är den primära källan av sjukdomsframkallande cytokiner vid sepsis. Mycket tyder istället på att de celler som förmodas aktiveras vid sepsis mycket snabbt och effektivt elimineras från blodbanan genom att adherera till kärlväggarnas endotel och därefter genom s.k. extravasering försvinna ut i kringliggande vävnad. Denna insikt kan vara av betydelse för såväl framtagningen av ny och förbättrad sepsisdiagnostik som för möjligheten att utveckla nya strategier för behandling.

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