Biomarkers in sepsis and other severe infections

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Abstract

Infectious diseases are a major global health problem. The presenting clinical picture results from a mixture of direct toxic actions by the microbiological agent, and the immune response mounted by the host. There is often a rapid onset, which may constitute a diagnostic and therapeutic challenge, whereas in other cases an extensive investigation over a long time can fail to identify a causal microbial agent.

The aim of this thesis was to study the cellular immune response with phenotypic assays in patients with severe infections, focusing on sepsis. We assessed whether our findings could serve as biomarkers and provide valuable diagnostic and possibly also therapeutic information. In the first part (paper I), we examined surface markers on white blood cells from patients with severe infections. In some instances, our analysis could differentiate between infections of bacterial and viral origin. In the second part (papers II–IV), we examined the incidence and nature of the immune alterations found in patients with sepsis and septic shock. We identified a protein (Wnt5a) that inhibited differentiation of monocytes to monocyte-derived myeloid dendritic cells (Mo-mDCs), which may play a role in the DC depletion often seen in sepsis. Also, as indicated by cell-surface phenotype, a large inter-individual variation in immune activation and immunosuppression was detected in patients with sepsis, with predominance of immunosuppression in patients with septic shock. Finally, different types of immature myeloid immunosuppressive cells, myeloid-derived suppressor cells (MDSCs), were found in patients with sepsis; Mo-MDSCs were preferentially expanded in patients with gram-negative sepsis whereas granulocytic MDSCs (PMN-MDSCs) accumulated in patients with gram-positive sepsis.

We conclude that the immune response during severe infections shows large inter-individual variation and biomarker-guided therapy could be useful in individualised treatment.

Key words: Biomarkers, sepsis, monocytes, dendritic cells, T cells, myeloid-derived suppressor cells, immunosuppression, flow cytometry

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To my family

Contents

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Lymphocyte and monocyte flow cytometry immunophenotyping as a diagnostic tool in uncharacteristic inflammatory disorders. Janols H, Bredberg A, Thuvesson I, Janciauskiene S, Grip O, Wullt M. BMC Infectious Diseases. 2010; 10: 205.

II. Wnt5a inhibits human monocyte-derived myeloid dendritic cell generation. Bergenfelz C, Janols H, Wullt M, Jirström K, Bredberg A, Leandersson K. Scandinavian Journal of Immunology. 2013; 78(2): 194-204.

III. Heterogeneity among septic shock patients in a set of immunoregulatory markers. Janols H, Wullt M, Bergenfelz C, Björnsson S, Lickei H, Janciauskiene S, Leandersson K, Bredberg A. European Journal of Clinical Microbiology and Infectious Diseases. 2014; 33(3): 313-324.

IV. A high frequency of myeloid-derived suppressor cells in sepsis patients, with the granulocytic subtype dominating in gram-positive cases. Janols H, Bergenfelz C, Roni Allaoui, Larsson A-M, Rydén L, Björnsson S, Janciauskiene S, Wullt M, Bredberg A, Leandersson K. Submitted manuscript.

Abbreviations

APC	antigen-presenting cell
CARS	compensatory anti-inflammatory response syndrome
CD	cluster of differentiation
$CD4^+$	T lymphocyte bearing CD4 receptor
$CD8^+$	T lymphocyte bearing CD8 receptor
CRP	C-reactive protein
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen-4
DAMP	damage-associated molecular pattern
DC	dendritic cell
G-CSF	granulocyte colony-stimulating factor
HLA-DR	human leukocyte antigen-DR
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
Mo-M	monocyte-derived macrophage
Mo-mDC	monocyte-derived myeloid DC
Mo-MDSC	monocytic-MDSC
NK cell	natural killer cell

NKT cell	natural killer T cell
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PD-1	programmed cell death-1
PMNC	polymorphonuclear cell
PRR	pattern-recognition receptor
SIRS	systemic inflammatory response syndrome
TGF-β	transforming growth factor-beta
Th	T helper
TIMP-1	tissue inhibitors of metalloproteinase-1
TCR	T cell receptor
TLR	toll-like receptor
TNF-α	tumour necrosis factor-alpha
WBC	white blood cell count

Background

An introduction to sepsis

Infectious diseases are a global health problem, causing many deaths per year. Respiratory infections as well as diarrhoea, malaria, measles, and HIV/AIDS are major causes of morbidity and mortality worldwide. Sepsis is also one of the world's leading causes of death with at least 19 million cases every year, the majority in low- and middle-income countries (1, 2). The incidence is rising for various reasons. Even with appropriate antibiotics, immediate fluid resuscitation, and intensive care, patients can quickly deteriorate into septic shock—leading to multiple organ failure and death. Some patients die within the first days of the early acute inflammatory phase, but the majority die after several days from secondary infections caused by profound immunosuppression. The pathophysiology of sepsis, where many different immune cells, inflammatory mediators, and coagulation factors are involved, remains incompletely understood (3).

Many of the signs and symptoms that are associated with infectious diseases are a direct manifestation of the host immune response. For thousands of years, physicians have recognised the hallmarks of a localized bacterial infection: dolor, rubor, calor, tumour, and functio laesa. These signs result from different leukocytes and their metabolites in the immune system, which attempt to kill the invading pathogen. For the host, the challenge with infections is to recognise the foreign invaders and to direct the appropriate immune response effectively without inflicting self-damage. The body uses many different mechanisms to avoid such inappropriate responses, but occasionally this mechanism fails—causing severe tissue damage and death (4).

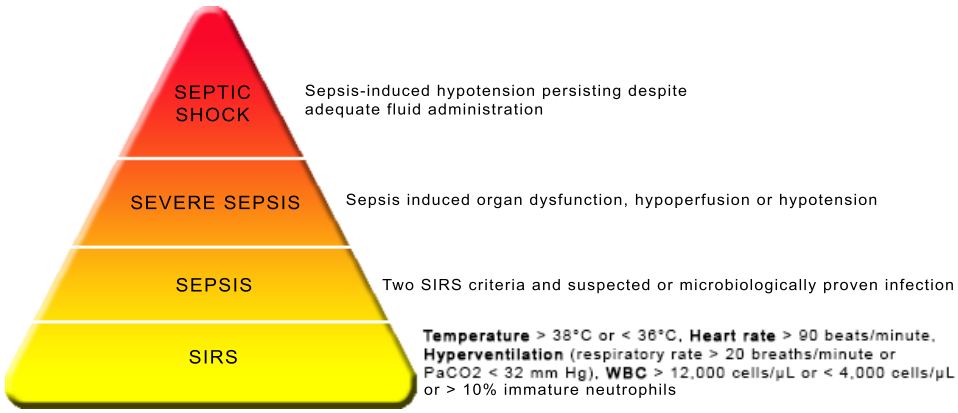
Definition

Sepsis was first mentioned in Homer's poems around 2,700 years ago (5). The word "sepsis" comes from the word σῆψις, (sipsi), which in original Greek means decomposition of organic matter (6). During the late 19th and the 20th centuries, sepsis was described as a systemic infection supposedly caused by the invasion of the blood stream by pathogenic microorganisms. However, patients still died of sepsis even when the mi-

croorganisms had been eradicated with antibiotics. In 1985, a hypothesis was proposed that it was the host immune response to the pathogen that was important in the pathogenesis of sepsis (7). In 1992, a first consensus panel convened by the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) introduced criteria for sepsis and systemic inflammatory response syndrome (SIRS) (8).

However, soon after, the SIRS criteria were found to be unspecific and the definitions have been under debate ever since (9). Thus, the SIRS and sepsis criteria from 1992 are still being used to enrol patients in sepsis trials (10).

Figure 1. Definition of SIRS, sepsis, severe sepsis and septic shock.



Definitions adapted from Bone et al 1992 (8).

Morbidity and mortality

Sepsis is a major cause of morbidity and mortality, and the incidence is rising, probably due to the growing elderly population, antibiotic resistance, immunosuppressive medication and, invasive surgery (11). In a well-cited US study, the incidence of severe sepsis was estimated to be 300 per 100,000 inhabitants with a mortality rate of almost 30% (12). The incidence of sepsis in Sweden is not known, but the incidence of severe sepsis is estimated to be at least 200 per 100,000 inhabitants and that of septic shock to be more than 30 per 100,000 inhabitants (13). According to the annual report Cause of Death in Sweden published by the National Board of Health and Welfare, 1,042 individuals died from sepsis during 2012. This number is probably an underestimation.

Deaths during the first days are usually due to the hyper-inflammatory immune response, characterised by refractory septic shock. However, the majority of patients survive this

phase and if the sepsis persists, the patient may enter an immunosuppressive phase with risk of secondary infections and death (14-16). The mortality rate has declined over the years, but even if the patients do not directly die of sepsis, the survivors have an increased risk of death in the following 5 years and may suffer from persistent physical and cognitive dysfunction (4, 17, 18). The reasons for this are not known, but they are probably multifactorial. The species of microorganism that causes the sepsis may also affect mortality, as gram-positive sepsis tends to have higher mortality than gram-negative sepsis (19). The site of infection also has an influence on outcome, as pneumonia, for example, has a higher mortality rate than urinary tract infection (20).

The clinical picture

Etiology

Humans face a constant threat from potentially pathogenic microorganisms. Our survival depends on different physical barriers, which resist pathogens entering the body, and also on a rapid response from the innate immune system. There are various mechanisms that discourage pathogenic colonization, including the epithelium covering the outer body surface, the mucous lining of the body cavities, and anti-microbial peptides (21). When these defensive mechanisms are broken, microorganisms can enter the body either by directly contaminating tissue or by diffusing through blood or lymphatic fluid and causing sepsis.

Pneumonia is the most common infection leading to sepsis, followed by urinary tract infections and abdominal infections. These infections are usually localized and controlled by the immune system, but they can sometimes spread and cause sepsis. In other cases, a fulminant meningococcal sepsis can occur before meningitis is established. Sepsis often progresses when the host cannot contain the primary infection, which is often related to high microorganism burden and strong virulence factors. The mechanisms of bacterial virulence also vary depending on the bacterial species and strain (22). However, the immunopathology behind these different mechanisms is not well understood (23).

Gram-positive and gram-negative bacteria are the main causes of sepsis (Figure 2), but viruses, fungi, and protozoans are possible pathogens as well (24). The causes of sepsis have changed over time, and now gram-positive bacteria are the most common cause of sepsis, but gram-negative bacteria still account for most cases of sepsis in the intensive care unit (ICU) (11, 24). Endotoxin is a crucial virulence factor for gram-negative bacteria. Gram-positive bacteria instead possess exotoxins, which can also initiate an immune response after binding to pattern-recognition receptors (PRR) (25, 26). Some bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes* also produce superantigens, which cause a non-specific activation of T cells, leading to profound pro-inflammatory cytokine production with risk of rapid progress into septic shock (27). A more pro-inflammatory cytokine profile has been found in gram-negative sepsis, suggesting that different bacteria may elicit different immune responses (26). Nevertheless, in a newly published paper, no difference in cytokine gene expression profiles was observed between gram-negative and gram-positive sepsis (28).

Blood cultures are positive for bacteria or fungi in one-third of patients (12). The most important pathogens for community-acquired sepsis are bacteria with high virulence factors such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria meningitis*, β-hemolytic streptococcus, *Escherichia coli*, and *Klebsiella* spp. (4). Microorganisms that cause opportunistic or nosocomial infections are, however, non-pathogenic organisms, such as *Pseudomonas aeruginosa*, coagulase-negative staphylococci, *Acinetobacter*, *Stenotrophomonas maltophilia*, and *Candida albicans (24)*. However, in about one-third of the cases, an etiological microbial agent is never found (24). The culture-negative sepsis patients have milder illness with lower mortality compared to culture-positive sepsis (29).

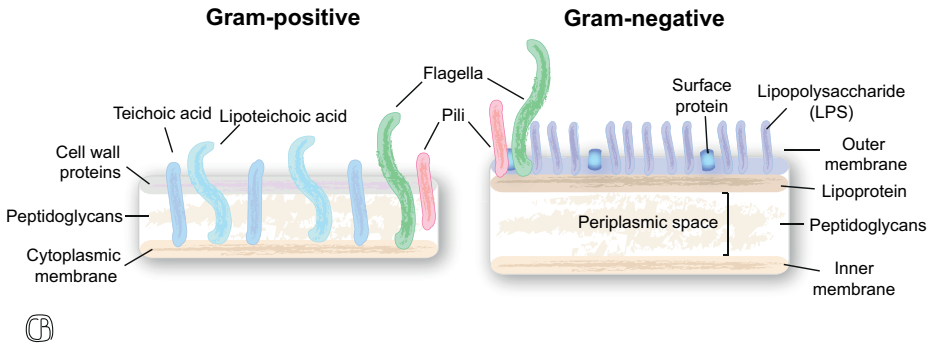


Figure 2. Gram-positive and gram-negative bacteria. Bacteria can be divided into two main groups based on differences in the structure of the cell walls and their gram-stain retention. Gram-positive bacteria have a thick peptidoglycan layer that contains teichoic and lipoteichoic acid. Gram-negative bacteria have a thin petidoglycan layer and an outer membrane that contains lipopolysacharide.

Symptoms and signs

The clinical symptoms and signs of sepsis can be highly variable due to age, co-morbidities, genetically predisposing factors, site of infection, burden and type of microorganism, presence of strong virulence factors, and duration of illness. Fever and chills are common symptoms, although 10% of septic patients are afebrile. Some patients can develop hypothermia, and they have worse prognosis (30, 31). Other symptoms are related to infected organs such as cough in pneumonia, dysuria in pyelonephritis, and redness of the skin in erysipelas. Signs of sepsis include changes in mental status caused by septic encephalopathy, tachypnea caused by metabolic changes or acute lung injury, and development of renal failure with oliguria and uraemia (32).

The mechanisms causing multiple organ failure have only been partly elucidated. One cause is impaired tissue oxygenation leading to global tissue hypoxia. Many factors contribute to the reduced oxygen delivery, including hypotension due to peripheral dilatation, myocardial depression, and increased metabolism as well as impaired red-cell deformability and thrombosis in the microvascular circulation. The inflammation itself also causes macrocirculatory endothelial lesions, resulting in intravascular depletion and subcutaneous oedema. The dysfunctional epithelial barriers also predispose to secondary infections (33-36). Severe sepsis and septic shock is often associated with altered coagulation, leading to disseminated intravascular coagulation (DIC). This dysregulated coagulation and fibrinolysis cascade results in widespread clotting and subsequent bleeding (37, 38). The early stage of septic shock usually comes after the onset of fever. It is usually associated with a warm stage with peripheral vasodilatation and a hyperkinetic circulation. Without treatment, the warm phase is followed by a cold stage, which is characterised by vasoconstriction and high mortality (39).

Diagnosis and treatment

The diagnosis of sepsis is based on evaluation of the patient's history, the clinical symptoms and signs, biochemical abnormalities, and culture of blood. In 1896, Dr Emanuel Libman introduced blood culture in clinical practice and it is still our most reliable and frequently used technique (40). New methods to rapidly identify microorganisms in the bloodstream such as MALDI-TOF are used nowadays in some clinics, and other non-culture methods are under development (41). Physiological scoring systems such as APACHE and SOFA are sometimes used to determine the degree of illness, but they are not specific for sepsis (42).

The key to management of sepsis is early treatment, and clinicians speak of the critical "golden hours" when recognition of sepsis and accurate treatment can truly affect outcome. It started in 2001, when the trial of early goal-directed therapy was launched, which led to a crucial improvement in treatment of septic shock patients. This resuscitation therapy included crystalloid resuscitation to restore preload, vasopressors to maintain mean arterial pressure, and administration of blood or dobutamine to obtain an adequate central venous oxygen saturation—all within 6 hours at the emergency department (43). Apart from supporting therapy, antibiotics are the basis of sepsis treatment. Patients who are initially given inappropriate antibiotics have an increased risk of dying (44). Another study has identified the antibiotic time delay as the single strongest predictor of outcome. Every hour the antibiotic administration was delayed led to an increased mortality of almost 8% in septic shock patients (45). This finding stresses the importance of quickly killing bacteria to reverse a deteriorating condition.

Different sepsis trials with immunomodulating drugs (e.g. anti-tumour necrosis factor-alpha (TNF-α) and anti-interleukin (IL)-1) have failed during the years (46, 47). The only immunomodulating drug that is recommended today is a short therapy with hydrocortisone for patients with refractory shock. This therapy is given in order to substitute for a relative adrenal insufficiency, but the evidence for this has been questioned (4).

Introduction to the immune system

Myelopoiesis

Leukocytes originate from haematopoietic stem cells (HSCs) in the bone marrow in a process in which a complex network of different cytokines, intercellular interactions, and an intricate regulation of transcription factors orchestrate the lineage commitment processes of the HSCs (48). HSCs subsequently develop into multipotent progenitors and into lineage-restricted progenitors (Figure 3), (49, 50). During severe infections such as sepsis, there is an increased consumption of and subsequent requirement for myeloid cells. Both immature and mature cells of the myeloid lineage are mobilised from the bone marrow into the peripheral blood. This is called emergency myelopoiesis, and this "left shift" is a sign of underlying severe pathology (51).

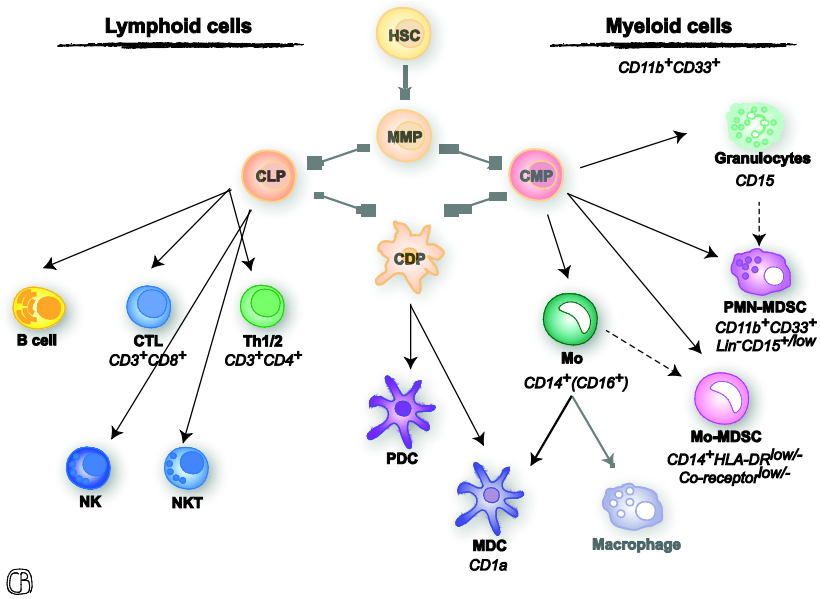


Figure 3. Schematics of haematopoietic differentiation. Haematopoietic stem cells (HSCs) in the bone marrow develop into multipotent progenitors (MMP) and into lineage-restricted progenitors: common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). They can both develop into common dendritic cell progenitors (CDP).

The adaptive and the innate immune systems

The immune system is divided into two categories: the innate and the adaptive immune systems. The innate immune system is also known as the non-specific immune system and operates in both vertebrates and invertebrates. It is orchestrated mainly by cells of myeloid origin i.e. neutrophils, monocytes/macrophages, and dendritic cells, and it is the first rapid line of defence against microorganisms. These immune cells induce phagocytosis of microorganisms, remove debris, and activate the complement cascade and subsequently the adaptive immune system (52).

The adaptive immune response arose less than 500 million years ago in our vertebrate ancestors. The adaptive immune system is also known as the acquired or the specific immune system. It is built up of lymphocyte interactions to provide recognition of foreign invaders, i.e. antigens with perfect specificity and diversity, and provides a long-lasting immunological memory (52, 53). The adaptive immune response is, however, delayed—and kicks in after about three days (53). Different lymphocyte populations play a part in the adaptive immune response. Lymphocytes are generally divided into three main cell populations: T lymphocytes, B lymphocytes, and natural killer (NK) cells. Below, some cells important for my thesis are briefly described.

Impact on some immune cells in sepsis

Neutrophils

The neutrophils are polymorphonuclear (PMN) short-lived cells that die within one hour after they have engulfed bacteria. They are very common, comprising 40–75% of all leukocytes in the circulation. Neutrophils play a crucial role in the first line of defence against invading pathogens. They kill microorganisms with anti-microbial peptides, by oxidative burst, or by producing neutrophil extracellular traps, so-called NETosis. These NETS are made from extracellular DNA and granular proteins. Although their functions are not completely understood, they are thought to augment the killing of microorganisms.

Neutrophils have been regarded as a double-edged sword in sepsis. They are essential for the eradication of microorganisms, but the release of anti-microbial peptides is responsible for organ injury. Pro-inflammatory cytokines give neutrophils a prolonged lifespan, which is attributed to delayed apoptosis. This prolonged inflammatory response augments cell injury. Studies have discovered that these neutrophils have an altered function, which includes impaired chemotactic activity, reduced clearance of

microbes, and reduced production of reactive oxygen species (ROS) (54). Neutrophils mainly secrete cytokines with chemotactic effects, such as IL-8, to recruit additional neutrophils (55).

During an infection, the number of neutrophils rises due to increased synthesis and release from the bone marrow as well as mobilisation from the marginating pool (51, 56). However, leukopenia can occur in severely ill patients and is caused by sepsis-mediated bone marrow suppression (8). In sepsis, a subset of neutrophils with suppressive function has been discovered and these neutrophils produce significant amounts of IL-10 (57). Another study also found a subset of mature neutrophils that suppressed T cell function when healthy volunteers were injected with low-dose endotoxin (58).

Monocytes

Monocytes are innate immune cells originating from the myeloid precursors in the bone marrow. They are a heterogeneous group of mononuclear cells and make up between 3% and 10% of all leukocytes in the blood. They play an important role in fighting infections, controlling inflammation, and promoting tissue repair. The monocytes circulate in the bloodstream and upon activation by either pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), they increase their phagocytosing properties and start producing pro-inflammatory cytokines. Monocytes can be antigen-presenting cells (APCs) and may promote an adaptive immune response by presenting antigens to T cells (59-61). The monocytes are also recruited to the site of infection/inflammation where they can differentiate into either dendritic cells or tissue macrophages. The mechanisms behind the recruitment and migration are thought to be the same as for neutrophils: rolling, adhesion, transmigration, and by the release of different kinds of cytokines (62).

The monocytes are defined by their typical large unilobar bean-shaped nuclei seen in microscopy or by expression of typical cell-surface markers and light-scattering properties found in flow cytometric analysis. Three subsets of monocytes have been identified based on expression of the lipopolysaccharide (LPS) receptor, cluster of differentiation 14 (CD14), and the Fc-γ receptor III (CD16) (Figure 4), (60). CD14 functions together with TLR-4 and MD-2 as a co-receptor for detecting LPS. The subsets are (1) the *classical* $CD14^{++}CD16^{-}$ *monocytes*, (2) the *intermediate* $CD14^{++}CD16^{+}$ *monocytes*, and (3) the patrolling *non-classical* $CD14^{+}CD16^{++}$ *monocytes.* The different monocyte subpopulations have different functions. For example, in elderly individuals, there is an increased proportion of the $CD16^{+}$ subset (63).

Monocytes are probably the most thoroughly studied cells in sepsis. As mentioned previously, they are mobilised to the site of infection with a predominance of the $CD16^{+}$ monocyte population seen in peripheral blood. Several studies have shown that expression of the co-receptor CD40 is unaltered or even enhanced on monocytes in

sepsis patients, which indicates that the immunosuppressive effects of these monocytes in sepsis are not only due to reduced CD40 expression (64, 65). Monocytes also down-regulate expression of CD14 during sepsis, which is seen as a hallmark of monocyte apoptosis in sepsis and endotoxin tolerance (as discussed below). It has been postulated that Tregs may play a role in the increased monocyte apoptosis in sepsis, but the mechanism behind this is still not known (66).

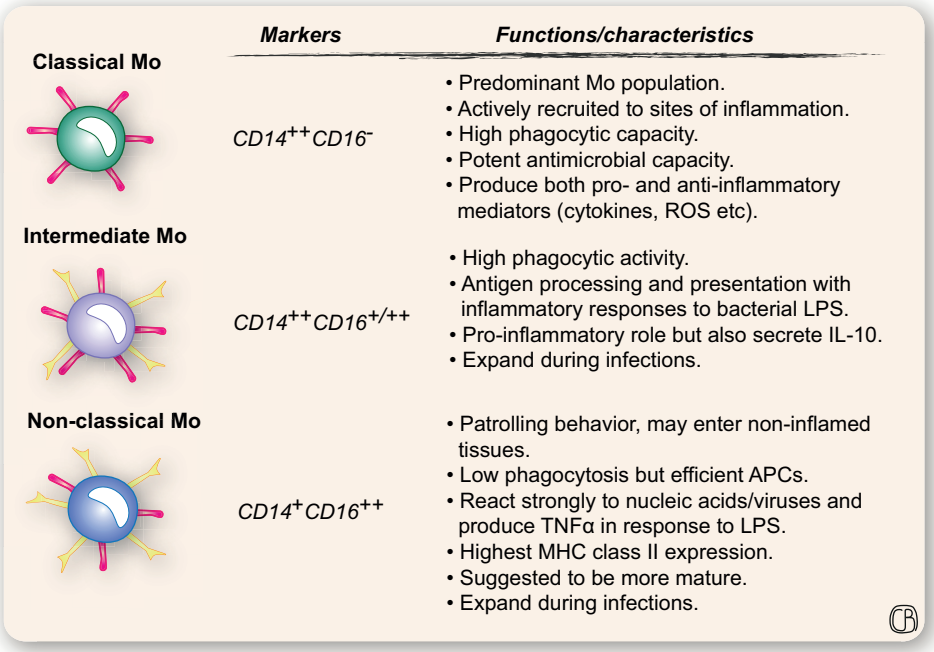


Figure 4: A summary of the main characteristics of the three monocyte subsets. Reprinted with kind permission from Caroline Bergenfelz.

Dendritic cells

Dendritic cells (DCs) are derived from progenitor cells in the bone marrow or from monocytes, so-called monocyte-derived DCs (Mo-mDCs). They are present both in tissue and in the circulation. They are potent APCs and their main function is to capture, process, and present antigen to T cells (naïve and memory T cells), thereby bridging the innate and the adaptive system. The immature DCs are characterised by high phagocytic capacity of antigens, but low T cell activation potential. The immature DCs are constantly sampling the environment for different PAMPs using PRRs.

The DCs mature in response to PAMPs, DAMPs, or pro-inflammatory cytokines. This activation leads to an up-regulation of cell-surface receptors (human leukocyte antigen-

DR (HLA-DR), CD80, CD86, and CD40), up-regulation of the chemokine receptor CCR7, and increased cytokine production. This may lead to activation of other innate immune cells and also cause the DCs to migrate to the lymph node where they activate lymphocytes (67).

Three main subsets of DCs have been found in peripheral blood, and they express different cell-surface receptors and have diverse functions. Two *myeloid DC subsets* (MDCs; MDC1 and MDC2) and one *plasmacytoid DC* (PDC) have so far been identified (68, 69).

In sepsis, a reduced number of dendritic cells both in the circulation and in the spleen have been found (70). Both myeloid DCs and plasmacytoid DCs are affected, and the functional impairment is long-lasting (71). The reduction in circulating DCs has been shown to correlate with disease severity and increased mortality in sepsis (72, 73). The DCs in sepsis express lower levels of HLA-DR and produce increased amounts of IL-10 (71). This leads to a reduced capacity to induce a strong T cell response, and results in T cell anergy or Treg proliferation instead (74).

MDSCs

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of plastic myeloid cells that originate from immature myeloid precursor cells in the bone marrow. The MDSCs are rare in healthy individuals, but are known to expand in various conditions. The MDSCs were first discovered in cancer patients, but recent studies have revealed that these cells also expand in other conditions such as sepsis (75-78). The generation of MDSCs is not fully understood, but IL-6, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), and toll-like receptor (TLR) signalling are probably involved (77).

Human MDSCs exist as at least two main subsets: one monocytic subset *(mo-MDSC)* and one polymorphonuclear or granulocytic sub-population *(PMN-MDSC or G-MDSC)*. The Mo-MDSCs are characterised as more mature and express $CD14^{+}HLA\text{-}DR^{low/-}$Co-$receptor^{low/-}$ on their cell surface. The PMN-MDSC is believed to be more immature and to express $CD33^{+}$ and/or $CD11b^{+}$ on the cell surface, but it lacks all lineage markers (Lin^{-}). However, expression of CD15 may be observed (79).

MDSCs can employ various kinds of immunosuppressive mechanisms that lead to reduced T cell activation. Induction of immunosuppressive factors (e.g. transforming growth factor-beta (TGF-β) and IL-10) and T regulatory cells (Tregs) induction, which leads to antigen-specific T cell suppression and polarisation of the T cells towards a Th 2 adaptive immune response, are some examples (80-85).

MDSCs have been suggested to increase in sepsis, and in this setting they may have a more beneficial role in dampening the extensive immune response by reducing tissue

damage (76, 86). However, the role and the regulation of MDSCs in sepsis are not completely understood, and it is not clear whether the MDSCs in sepsis are similar to the MDSCs found in cancer or whether they are a myeloid variant with similar characteristics (Figure 5), (77-79).

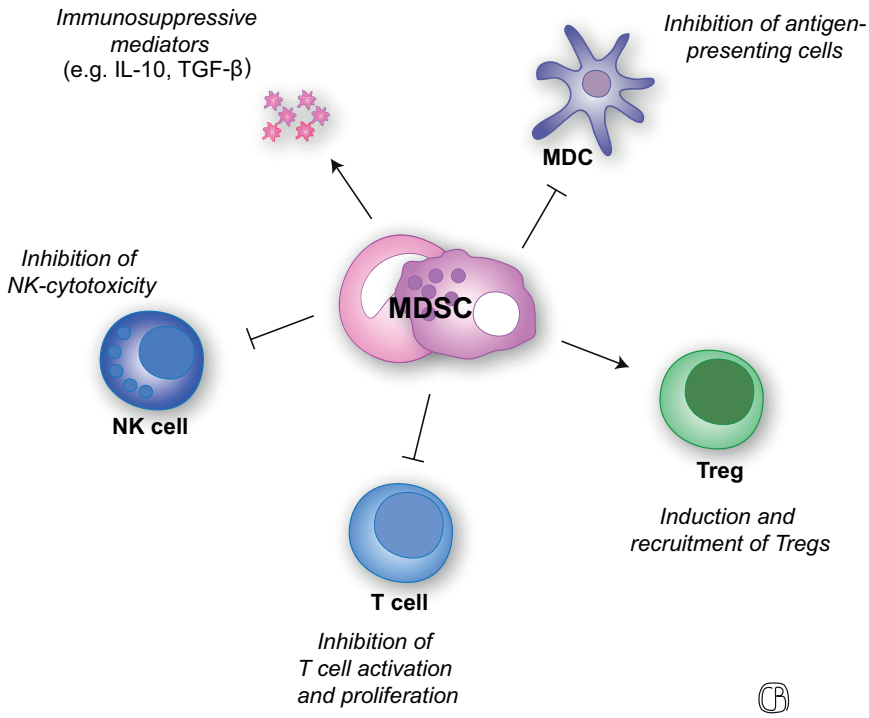


Figure 5. The function of MDSC-mediated immunosuppression in sepsis. MDSCs are potently immunosuppressive through multiple mechanisms. They produce immunosuppressive mediators, which lead to antigen-specific T cell suppression, polarisation of T cells towards a Th 2 adaptive immune response and induce Tregs. They also cause a depletion of ariginine and iNOS, which are essential for T cell and NK cell function. Reprinted with kind permission from Caroline Bergenfelz.

T cells

The most thoroughly studied cell population in the adaptive immune response is probably the T cells, as they are required for almost all adaptive immune responses. There are different classes of T cells: T helper cells (Th), cytotoxic T cells (CTLs), Tregs, and unconventional T cells (γ δ T cells and natural killer T cells (NKT cells)). These cells will be mentioned briefly below.

T cell activation occurs by engagement of the T cell receptor (TCR) with a processed non-self molecule from the pathogen (antigen), which is presented in the major

histocompatibility complex (MHC) on an APC. In order for this activation to take place, the MHC class molecule II must bind to the glycoprotein CD4 predominantly expressed on Th cells and Tregs and the MHC class I must bind to CD8, which is mainly expressed on CTLs. The T cell response is regulated by co-stimulatory receptors (CD28) and co-inhibitory receptors (i.e. cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1)).

Th cells are $CD4^+$ T cells that are important for activation of B cells and CTLs, hence the name "helper" T cells. The Th cells can only help other immune cells after they have been activated themselves and have thus developed into an effector Th cell. When Th cells become activated, they proliferate and mature into different Th cell subtypes (Th1, Th2, Th9, Th17, and Th22), each producing a different set of effector cytokines. The cytokines delivered by the activating APCs, or by other cells in the environment, determine which Th subset will predominate (87). The Th1 cells are mainly involved in the defence against intracellular microorganisms by activating macrophages, DCs, and CTLs through secretion of various cytokines (e.g. IL-2, IL-12, IFN-γ, and TNF-α). These cytokines also increase the generation of monocytes and the expression of cell adhesion molecules on the endothelium, which causes inflammatory cells to migrate into the infected tissue. Th2 cells are involved in the defence against extracellular pathogens, especially parasites, and they also stimulate B cells to produce antibodies by secreting cytokines (e.g. IL-4, IL-6, and IL-10). Once a Th1 or Th2 response develops, it inhibits the differentiation of the other Th cell group by inducing different cytokines.

CTLs are $CD8^+$ T cells; they play a pivotal role in the elimination of infected or damaged cells. They are also involved in preventing reactivation of latent viruses. Naïve $CD8^+$ T cells require Th1- or Th17-mediated APC activation in order to differentiate into effector cells (88). When CTLs are activated, the infected cells are delivered the "kiss of death" (i.e. IFN-γ or perforin secretion, or by induced apoptosis through Fas signalling). Some of the lymphocytes are retained as memory cells.

In the pathogenesis of sepsis, there is a shift from the Th1 cell-mediated immune response with pro-inflammatory cytokines towards a Th2 cell-mediated immune response with immunosuppressive cytokines (89). The factors that determine which Th response is generated are many, including the type and location of the infection (3). Th1, Th2, and Th17 cells are all affected during sepsis, with a subsequent decrease in the production of pro-inflammatory cytokines and suppressed T cell function (90). The reduction in, for example, the Th17 response could contribute to the increased susceptibility to secondary infections with fungi (91).

Tregs

Tregs are immunosuppressive cells characterised by expression of CD4, CD25, and Forkhead box transcription factor 3 (FoxP3). Tregs suppress the immune response mainly through IL-10 and TGF-β secretion, and they require IL-2 for IL-10 production and for their own maintenance (92). Tregs have also been shown to collaborate with MDSCs (80). The activated Tregs can contract to form a pool of memory cells after the infection subsides, which then expands upon a second infectious challenge (93).

Studies have shown that there is an increased percentage of Tregs in sepsis patients. The increase was relative, and was due to the decrease in T cell number rather than an increase in actual Tregs (94). However, many other studies have in fact shown an increase in actual Treg cell numbers in sepsis patients (75, 95, 96). It has been speculated that Tregs could be more resistant to apoptosis, perhaps due to increased expression of the anti-apoptotic protein Bcl-2 (75). The increase of Tregs in sepsis is devastating, and is associated with reduced T cell function and proliferation (93, 95). Tregs have also been shown to suppress the function of other immune cells such as neutrophils, monocytes, and NKT cells (66).

γ δ T and NKT cells

Unconventional γ δ T cells are a subset of T cells that usually lack both CD4 and CD8 co-stimulatory molecules and recognise antigens via a γ δ T cell receptor on the cell surface. They reside in large numbers in the intestinal mucosa and in other mucosal surfaces, but they are also found in the blood. Their reactivity pattern is broader, less specific, shows cytotoxic capacity, and favours reactions to some microorganisms by releasing various pro-inflammatory cytokines (97, 98). Some of the γ δ T cells are retained as memory cells. γ δ T cells have been implicated as one of the major sources of IL-17 production during sepsis, mediating recruitment of neutrophils, macrophages, and DCs (99). The number of γ δ T cells usually falls in sepsis and this is associated with increased mortality (97, 100). The apoptosis of these lymphocytes in the intestinal mucosa may increase the risk of translocation of bacteria into the bloodstream, which could lead to secondary infections with gram-negative bacteria (97, 101-103). The functionally related NKT cells also lack expression of CD4 and CD8; they express an invariant TCR and are important in the immune response, as they rapidly release various pro-inflammatory or anti-inflammatory cytokines. NKT cells are thought to be important regulators of the immune response in sepsis; however, their role is still not defined (104).

B cells

The main function of B cells is to produce antibodies against antigens, to develop into memory B cells after antigen interaction, and to function as APCs. The antibodies secreted are important in the immune defence, as they inactivate the microorganism by binding to it, so-called steric hindrance. The antibodies can also bind to the Fc receptor on phagocytic cells, so-called opsonisation, or activate the complement system.

Immunoglobulin levels usually fall in sepsis, and the mechanism behind this is multifactorial. One mechanism is the apoptosis of B cells (105). Sepsis is also associated with increased antibody consumption due to neutralisation of endotoxin and exotoxin and also clearance of bacteria. Another explanation for the reduced circulation of immunoglobulin is vasoleakage into the tissue. These findings appear to be associated with increased mortality in septic shock (106). Treatment with intravenous immunoglobulin has been tested as an adjunctive treatment in sepsis and septic shock, but the efficacy is still under debate (107).

NK cells

Natural killer (NK) cells account for approximately 10% of peripheral blood lymphocytes and are important regulators of the innate immune response. The NK cells do not express a TCR, but recognise loss of MHC class I molecules on virus-or tumour-infected cells. They mediate spontaneous cytotoxicity through perforin release. They secrete IFN-γ, which contribute to pathogen control including recruitment of other innate immune cells such as macrophages. NK cells also have a role in the adaptive immune response by activating DCs and by producing different cytokines, which polarises the T cell response towards Th1 (108).

The main subsets of NK cells are usually reduced in sepsis, and the reduced numbers are associated with increased mortality (109). Both the cytotoxic function of NK cells and their cytokine production (IFN-γ) are impaired in sepsis patients. This hypo-responsiveness may play a part in the endotoxin tolerance phenomenon also seen in monocytes (110). NK cells play a pivotal role in viral infection, and impaired function of NK cells may lead to the reactivation of latent viral infections often seen in severely ill sepsis patients (111).

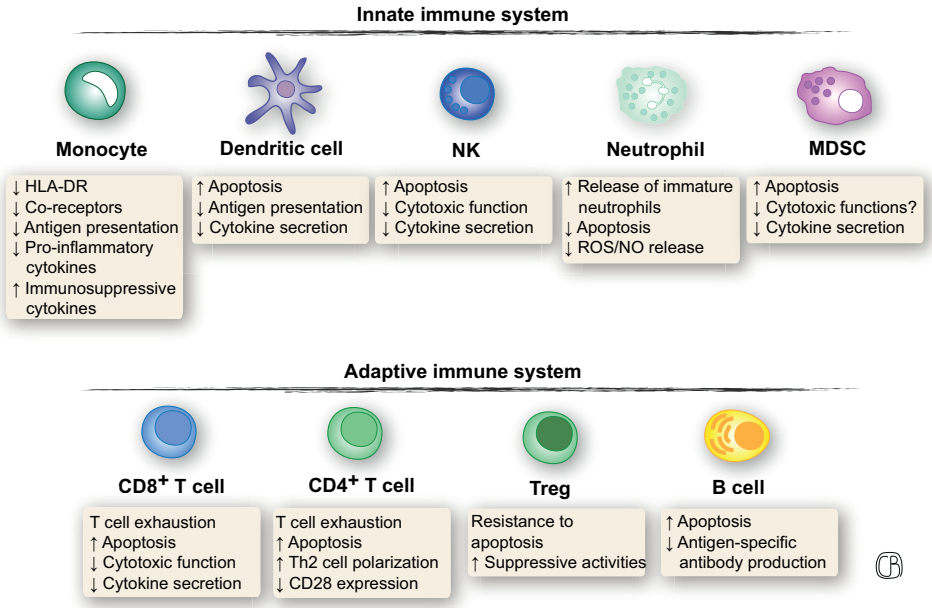


Figure 6. Sepsis has a profound effect on immune cells of both the innate and the adaptive immune system. Sepsis induces apoptosis of different cells including DCs, NK cells, T cells, B cells and MDSCs. The apoptosis in neutrophils is delayed and Tregs are more resistant to apoptosis leading to an increase in Treg numbers. HLA-DR is down-regulated on APCs leading to impaired antigen presenting capacity. Adapted from Hotchkiss et al 2013 (75).

Immunopathology in sepsis

SIRS and CARS

The pro- and anti-inflammatory phase

For a long time, sepsis has been postulated to be a host response to an infection and deregulation of this immune response is known to contribute to the pathogenesis. For many years, two phases of sepsis have been described: first the systemic inflammatory response syndrome (SIRS), with production of pro-inflammatory cytokines (TNF-α, IL-6, IL-8, IL-1β, and IFN-γ), from which the term "cytokine storm" arose (112). This phase is followed by a secondary compensatory anti-inflammatory response syndrome (CARS) with secretion of anti-inflammatory cytokines (IL-10, TGF-β and IL-1 receptor antagonist (IL-1ra)) in an attempt to restore immunological equilibrium (75, 113, 114). The prolonged state of immune suppression is nowadays referred to as sepsis-induced immunoparalysis, which is characterised by an impaired innate and adaptive immune response (115). This sepsis model of SIRS and CARS has been modified with time, and now there is growing consensus that the production of pro-inflammatory and immunosuppressive cytokines begins immediately after onset of sepsis, to balance the host's need to maintain defence while minimising self-induced tissue damage (75). However, the net effect of these two competing responses is usually an initial dominant hyper-inflammatory phase and a secondary immunosuppressive phase (Figure 7).

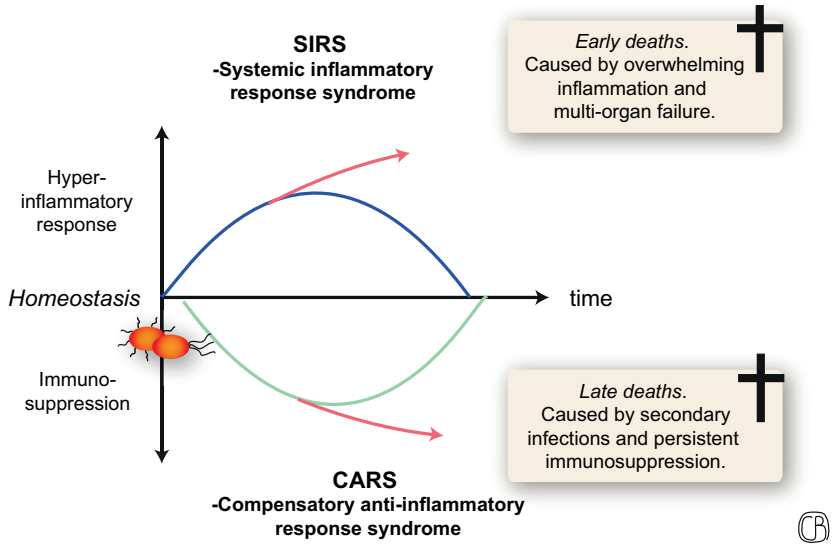


Figure 7. SIRS and CARS. The activation of the pro-inflammatory immune response and the anti-inflammatory immune response begins after onset of sepsis. Deaths during the first days are usually due to the hyper-inflammatory immune response. However, the majority of patients survive this phase. If the sepsis persists, patients may succumb in a later phase to secondary infections due to profound immunosuppression. Adapted from Hotchkiss et al 2013 (75).

Some of the mechanisms behind immunosuppression

Evidence of immunosuppression

There is profound evidence that immunosuppression occurs in sepsis (75, 101). The inability to secrete pro-inflammatory cytokines combined with the enhanced expression of inhibitory receptors and ligands suggests that clinical immunosuppression is present in the majority of sepsis patients. One post-mortem study revealed that 80% of patients who died from sepsis/septic shock had unresolved septic foci despite appropriate antibiotic and source control regimens. One reason for failing to eradicate the infection or for developing secondary infections is thought to have been their immunosuppressive status (116). Many critically ill patients have reactivated latent cytomegalovirus (CMV) or herpes virus infections. The majority of these patients most likely do not have a clinically significant viral infection, but these findings support the concept that critically ill patients become profoundly immune-compromised, which allows reactivation of a

latent viral infection (111, 117). As mentioned briefly earlier, many sepsis patients die from secondary infections caused by relatively non-virulent microorganisms (118).

Apoptosis of many different immune cells

Apoptosis of different immune cells is a major mechanism of cell death in sepsis. Several post-mortem studies of patients who have died from septic shock have shown an apoptosis-induced loss of cells of both the innate and the adaptive immune system. The apoptotic immune cells are $CD4^+$ T cells, $CD8^+$ T cells, B cells, DCs and NK cells (70, 103). The apoptosis plays a potentially important role in immunosuppression due to an impaired immune response. Both the extrinsic death-receptor pathway and the intrinsic mitochondria-mediated pathway are involved in the apoptosis (119). The exact cause of the loss of the immune cells is, however, not known (105). In addition to the apoptosis, the immunosuppression is enhanced by an inhibitory anergic effect on phagocytes. This effect is induced by the release of the anti-inflammatory cytokine IL-10 (120).

T cell exhaustion

Another mechanism of immunosuppression in sepsis involves T cell function, so-called T cell exhaustion. This phenomenon is characterised by reduced T cell proliferation, failure to produce pro-inflammatory cytokines, and reduced capacity to initiate cytotoxic cell death. These T cells also have an increased tendency to undergo apoptosis (121). In sepsis, T cell exhaustion is mediated by increased expression of PD-1 and CTLA-4 on both $CD4^+$ and $CD8^+$ T cells. At the beginning of sepsis, there are few changes in the expression of these inhibitory molecules, but eventually there is increased expression of PD-1 and CTLA-4 (122-124).

Endotoxin tolerance

One of the most important mechanisms against a deregulated pro-inflammatory cytokine storm is a homeostatic programme called endotoxin tolerance. This feedback mechanism basically shuts off the immune response, and the monocytes enter into a transient unresponsive state after a second exposure to endotoxin, i.e. LPS, various microbacterial products, and other TLR agonists (125). The striking sign of endotoxin-tolerant monocytes is their reduced capacity to release pro-inflammatory cytokines (TNF-α, IL-1β, IL-12, and IL-6) and they become strictly anti-inflammatory cells with an increased or unimpaired production of IL-10, TGF-β, and IL-1ra (125-127).

The other main feature of endotoxin tolerance is down-regulation of HLA-DR on monocytes (128). Loss of HLA-DR is detected early, is observed in the majority of sepsis patients, and reverses when the patient is recovering (129, 130). The reduced

expression of HLA-DR correlates with mortality, and can be used as an independent predictor of death (131, 132). The monocytes show a retained or even enhanced phagocytic activity, but the reduced expression of HLA-DR together with the down-regulated expression of the co-receptor CD86 hinder the monocytes to activate T cells (64, 133, 134). This results in difficulties in clearing infections despite appropriate antibiotic treatment, in risk of developing secondary infections, and in reactivation of latent viral infections (101). Endotoxin-tolerant monocytes often up-regulate the expression of genes that are associated with cell matrix generation, which makes these cells resemble M2 macrophages (134).

Activation of the innate immune response

Cell recruitment and migration

The cells that are involved in the innate immune system are neutrophils, eosinophils, basophils, mast cells, platelets, monocytes/macrophages, NK cells, and unconventional T cells. At the site of an infection or injury, resident immune cells or parenchymal cells produce early inflammatory mediators. These mediators lead to chemotaxis of recruited innate immune cells to the inflammatory site.

Pathogen recognition

The PRRs are expressed on epithelial cells and all the cells of the innate immune system. They act as sentinels against invading organisms by recognising conserved "non-self " molecules, so-called PAMPs, and "self" molecules, so-called DAMPs (135). PAMPs refer to exogenous molecules from invading microorganisms. These molecules include LPS in gram-negative bacteria, lipoteichoic acid, peptidoglycans and flagellin in gram-positive bacteria, and viral nucleic acid in viral infections, and fungal cell wall components in fungal infections. DAMPs are endogenous molecules released from necrotic tissue or apoptotic cells due to chronic inflammation associated with sepsis, tumours, or trauma. These DAMPs consist of intracellular proteins or heat shock proteins, uric acid, S100 proteins, fibrinogen, fibronectin, hyaluran, and high-mobility group box-1. The immune reaction to these molecules may explain the SIRS symptoms in non-infectious conditions such as burns or trauma, but can also augment the symptoms in sepsis (136-138).

The PRRs recognise a broad range of these common structures shared by a vast majority of molecules of threats using only a confined numbers of receptors. This mechanism is what makes the innate immune response so rapid. There is overlap between signalling pathways, and one microorganism can trigger pro-inflammatory signals from different kinds of PAMPs, which can amplify the inflammation (139). Upon binding with a

ligand, the TLR complex goes through a conformational change and specific adapter molecules are recruited to the cell surface. The nuclear factor-kappa B (NFκB), mitogen-activated protein kinase, and/or interferon regulatory factor pathways are activated, which leads to the production of pro-inflammatory cytokines and type-I IFNs within hours of onset of infection (140, 141).

The pattern-recognition receptor family

The TLRs are probably the best known of the PRRs, as they are central to initiation of the immune response. To date, 10 different TLRs have been identified in humans, (Figure 8). They are classified according to their location in the cell, since some microbes are extracellular, some are present in internal compartments in the cytosol, and some are cytoplasmic (142, 143). The discovery of different TLRs has provided evidence that the intracellular pathways of gram-positive and gram-negative bacteria are different (144).

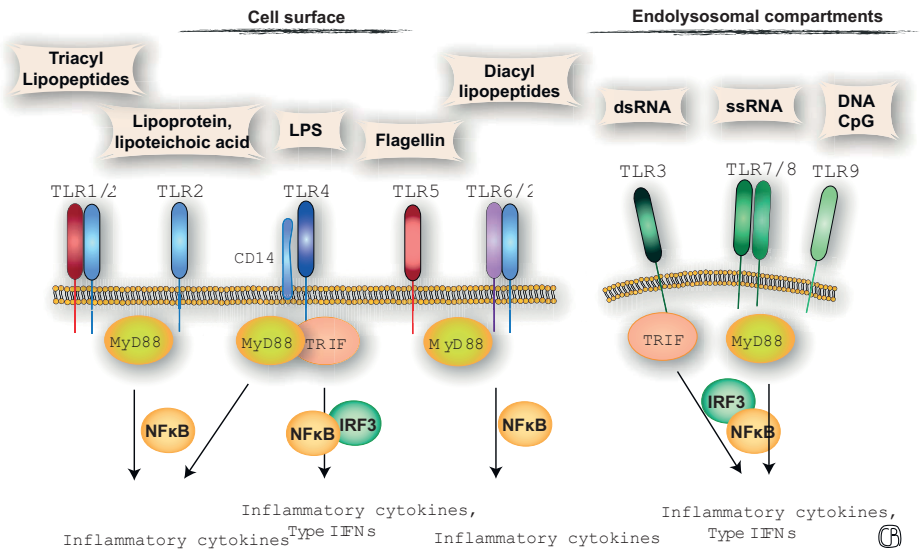


Figure 8. The main characteristics of the Toll-like receptor family. TLRs can be either extracellular, which recognise bacterial and fungal PAMPS and DAMPS or intracellular, which recognise viral and DAMPs. Upon activation, downstream signalling is mediated either through MyD88 (via NFκB) or TRIF (via NFκB or IRF3), which induces inflammatory cytokines or type-I IFNs. Reprinted with kind permission from Caroline Bergenfelz.

Wnt5a

Wnt5a is a secreted glycoprotein that belongs to the non-canonical Wnt protein family. It induces specific calcium-dependent signals by binding to certain cell-surface receptors (Frizzled receptors) on different kinds of cells. Wnt5a proteins are essential for development processes such as cell differentiation, cell adhesion, and cell polarity. The role of Wnt5a in infectious diseases and its effects on immune cells are less known. Wnt5a has been suggested to inhibit T cell development as the APCs start to produce Wnt5a, which subsequently causes inhibition of a Th1 response and promotion of a Th2 response. This mechanism has been shown to be dependent on TLR and NF-κB signalling (145, 146). Wnt5a has been suggested to be up-regulated in monocyte-derived DCs, and upon TLR signalling in macrophages (146). Thus, Wnt5a expression is increased on macrophages in active tuberculosis and in sepsis, but the function is unclear (147, 148).

Biomarkers

Sepsis is still defined by unspecific changes in clinical parameters and laboratory tests rather than specific diagnostic biomarkers. The failure of former sepsis trials has led to the development of novel strategies where immunostimulatory rather than immunosuppressive drugs could play a role. A requirement for applying immunotherapy is a proper selection of patients, and there is a need for therapeutic biomarkers (149). A biomarker is defined as a marker that is "objectively measured as an indicator of a normal biological process, pathogenic process, or pharmacological response to a therapeutic intervention" (150). A biomarker may serve different functions. (Table 1).

Table 1. The use of biomarkers.

Screening:	To identify patients at risk of adverse outcome, to perform prophylactic intervention or further diagnostic test.
Diagnosis:	To establish a diagnosis to enable a treatment decision, and to do so more reliably, more rapidly, or more inexpensively than with available methods.
Risk stratification:	To identify subgroups of patients within a particular diagnostic group who may experience greater benefit or harm with therapeutic intervention.
Monitoring:	To measure response to intervention to permit the titration of dose or duration of treatment.
Surrogate endpoint:	To provide a more sensitive measure of the consequences of treatment that can substitute for a direct measure of a patient-centred outcome.

Definition adapted from Marshall et al 2009 (151).

Sepsis causes changes in the expression and activity of many different endogenous molecules. The majority of these markers both contribute to changes in the immune response and reflect the level of immune activation or immunosuppression. Many of these mediators may reflect the immune status only at a given moment, and their expression must be interpreted cautiously. They may serve as potential biomarkers, but the heterogeneity of the immune response has made research difficult. More than 170 different molecules have been proposed as biomarkers for sepsis. However, none of them have yet proven to be sufficiently sensitive and specific to be widely used for diagnosing sepsis, but many could be used to identify a critically ill patient (152, 153). Other biomarkers have been proposed to identify immunosuppression, but it is still not clear which and how many of these immunomarkers are needed to diagnose a profound immunosuppression and what patients might benefit from immunotherapy.

Biomarkers of the pro-inflammatory phase

Acute-phase mediators

White blood cell count, neutrophil count, C-reactive protein (CRP), and procalcitonin (PCT) are biomarkers in peripheral blood. They are established biomarkers for infection and inflammation and are often used together with other parameters to diagnose sepsis and to evaluate treatment response (154). A leukocytosis with predominately neutrophils is often found early in patients with sepsis. However, leukopenia can occur in severely ill patients and is related to increased mortality (8). Both CRP and PCT rapidly increase in septic patients, and the debate goes on about which is the best biomarker for sepsis (154). However, PCT is now used in some clinics as a tool to determine when antibiotics may be discontinued (155).

Cytokines

Cytokines play an important part in the features of sepsis and are potential biomarkers. Cytokines are molecules that are secreted by a wide range of immune cells including monocytes, macrophages, and lymphocytes—but also by endothelial cells, fibroblasts, and stromal cells. They act through receptors and are important for cell signalling, which influences the immune responses in complex ways. They have a short half-life of a few minutes to a few hours.

TNF-α is one of the most studied pro-inflammatory pleiotropic cytokines. It is an acute-phase reactant and is rapidly released by activated macrophages and other immune cells, and its levels in blood are increased in sepsis (156, 157). TNF-α promotes inflammation by inducing gene expression of specific proteins/mediators. Many of the main characteristics of inflammation can be attributed to the effect of TNF-α (156). TNF-α production, for example, leads to vasodilatation and shock through production of iNOS and COX-2—and stimulation of endothelial adhesion molecules (158). Increased levels of TNF-α are associated with mortality in sepsis (159).

IL-1β shares many properties with TNF-α, and increased blood levels are also found in sepsis (156, 157). It is secreted by activated monocytes, macrophages, lymphocytes, fibroblasts, endothelial cells, and DCs. IL-1β contributes to the pain hypersensitivity during inflammatory conditions and acts as a major pyrogen in fever. It is produced as a pro-interleukin and is cleaved to the active form by caspase-1. Caspase-1 is activated by a molecular intracellular platform called the inflammasome, which is composed of PRR in the nucleotide-binding oligomerization domain-like receptor family. IL-1β can be used as an indicator of early mortality in sepsis (160).

IL-6 is a pleotropic cytokine with both pro-inflammatory and anti-inflammatory properties. It is produced by a variety of cells—especially macrophages, fibroblasts, and smooth muscle cells—in response to stimuli from IL-1β, TNF-α, and LPS. IL-6 mediates an acute-phase response with fever and leukocytosis, and it releases complement factors

and acute-phase proteins from the hepatocytes (140). IL-6 is associated with increased severity and mortality in sepsis (161, 162).

IL-8 is secreted by macrophages, neutrophils, and other cell types such as endothelial cells. It is also known as a neutrophil chemotactic factor, because it promotes migration of primarily neutrophils to the site of infection and it also induces phagocytosis (163). Increased levels of IL-8 are observed in sepsis and in other inflammatory conditions (164).

IL-12 is mainly produced by phagocytes and dendritic cells and it induces T cells and NK cells to produce IFN-γ, which directly activates macrophages. IL-12 also stimulates the differentiation of naïve CD4 T cells into Th cells and increases the proliferation of haematopoietic progenitors. Increased levels of IL-12 have been found in neonatal sepsis, but the role of IL-12 in sepsis still remains unclear (165, 166).

IL-18 is a pro-inflammatory cytokine produced by several cell types including macrophages, DCs, and other immune cells and it induces the release of IL-1β, TNF-α, and IL-8. It contributes to the host defence against various microorganisms through synergism with other cytokines including IL-12 and IL-15, and it stimulates T cells and NK cells to release IFN-γ or type-II interferon. This cytokine appears to have a role in sepsis, and increased levels have mostly been seen in patients with gram-positive sepsis (167).

Cell-surface markers

Many markers are used to determine the activation status of neutrophils, monocytes, and T cells. The most intensively studied myeloid marker is probably CD64, which has been proposed to be an early biomarker for neonatal sepsis (168). However, it has been shown to have low sensitivity and specificity in distinguishing between bacterial and viral infections (169). CD11b is another myeloid marker that increases in sepsis (170). Different monocyte activation markers have also been studied, including CD14, the lipopolysaccharide-binding protein (LBP), and the receptor for RAGE (154). Perhaps the most promising monocyte marker is soluble CD14, which has been identified as a promising marker for sepsis and severity of sepsis (171, 172). Expression of CD40, CD11c, CD163, and soluble CD163 are other myeloid activation markers used in the context of sepsis (65, 173-175). To measure T cell activation, different markers can be used (e.g. CD45RA/CD45RO ratio, CD69, CD71, and HLA-DR or co-stimulatory molecules (CD152, CD27, CD28, and CD134) (176).

Biomarkers of the immunosuppressive phase

Cytokines

IL-6 has also been shown to have anti-inflammatory characteristics, whereby it inhibits the release of TNF-α and IL-1β and stimulates the release of IL-10 and cortisol (177). As mentioned previously, IL-6 is associated with increased sepsis severity and mortality (161, 162).

IL-10 is mainly produced by macrophages, dendritic cells, B cells, and T cells. MDSC is also a producer of IL-10. IL-10 suppresses the gene expression and synthesis of pro-inflammatory cytokines but increases IL-1ra production, thus reducing circulation of IL-1β (163). IL-10 also inhibits the expression of HLA-DR and co-stimulatory molecules on monocytes and macrophages. IL-10 promotes the differentiation of Tregs and inhibits the proliferation of $CD4^+$ T cells. Increased levels of IL-10 correlate with sepsis mortality (178). However, its effect is not universally anti-inflammatory, as it enhances B cell proliferation and immunoglobulin secretion, and can also enhance the development of $CD8^+$ T cells (92, 179). The plasma levels of IL-10 are elevated in severe sepsis and this is associated with increased mortality (178).

Tissue inhibitors of metalloproteinase-1 (TIMP-1) is a glycoprotein with cytokine-like activities; it is expressed by a variety of cell types including neutrophils and monocytes. It functions as an inhibitor of matrix metalloproteinases (MMPs), which degrade the extracellular matrix and play a role in facilitating recruitment of leukocytes from the circulation in sepsis (180). Recent studies have identified higher levels of TIMP-1 in patients dying from sepsis than in sepsis survivors, but the role of MMPs/TIMP-1 in sepsis is still unclear (181).

Cell-surface markers

Expression levels of HLA-DR on monocytes remain one of the best studied immunosuppressive biomarkers; they provide valuable clinical information, such as risk of secondary infection and death (182, 183). The expression levels of HLA-DR can be measured either as mean fluorescence intensity (MFI) or as percentage of a certain cell population. Down-regulation of activation markers is also a sign of immunosuppression (i.e. CD14, CD40, and CD11c) (65, 173, 184). Monocytes also have increased expression of the negative co-stimulatory molecule PD-1L on the cell surface, with their counterparts PD-1 and CTLA-4 on T cells (122-124). T cells often show reduced expression of the co-stimulatory molecule CD28 (75). Until now, flow cytometry has been the golden standard for this assay, but the blood sample has to be analysed quickly in a flow cytometry laboratory and cannot be stored for later handling, which complicates clinical usage.

Other biomarkers of interest

At the moment, the most frequently used biomarker for determination of organ dysfunction in sepsis is lactate (185, 186). Many hospitals use lactate levels with a cut-off level of 4 mmol/L to screen for sepsis. However, new reports have revealed that this level is probably set too high, and that sepsis patients with lactate levels above 1.5 mmol/L have an increased mortality rate (185). In some studies, microarray analysis comparing sepsis patients with healthy controls has been able to identify gene expression markers associated with innate and adaptive immune function specific for early sepsis, but the clinical significance of these findings needs to be studied further (187, 188). Other biomarkers have been suggested as possible diagnostic markers of sepsis, but they are not yet used in clinical practice (152, 154).

It will be challenging to find reliable biomarkers that can be used easily in routine clinical practice. It is unlikely that only one biomarker would help clinicians to determine the immune status of a patient, not in the near future anyway. As clinical studies have revealed that expression of both pro- and anti-inflammatory cytokines is elevated early in sepsis, probably a combination of specific biomarkers reflecting both phases would be most suitable (189).

Possible immunotherapy in the future

Sepsis and cancer share many immunosuppressive mechanisms, which include increased MDSCs, increased Tregs, and T cell exhaustion. Their shared immunosuppressive mechanisms could in part be due to the fact that both diseases may evolve into states of low-grade chronic inflammation and ongoing antigen exposure. During recent years, immunotherapy in cancer has developed markedly, and has led to a totally different way of treating cancer. Instead of directly affecting the tumour, treatment is developed to target the immune system itself. Antibodies to specific receptors on T cells are administered, which leads to activation of the formerly suppressed T cells. This antibody treatment has led to a breakthrough in the treatment of some forms of cancer. The journal Science chose cancer immunotherapy as the year's breakthrough in science in 2013 (190). However, not all patients respond to immunotherapy and the reason for this is not known.

Immunotherapy may also have beneficial effects in sepsis (191). A newly published paper has shown that blocking of inhibitory molecules in peripheral blood mononuclear cells (PBMCs) from sepsis patients *in vitro* inhibits T cell apoptosis and restores the production of cytokines. These results strengthen the idea that immunotherapy could be a possible way forward in the development of new sepsis treatments (192). Possible therapeutics to consider are the cytokines IL-7 and IFN-γ, granulocyte colony-stimulating factor (G-CSF), GM-CSF, or antibodies against specific co-stimulatory molecules such as PD-1 and CTLA-4 (182, 193-195). Clinical trials are now taking place in different countries and hopefully the results will be successful.

The present investigation

Aims

The general objective of this thesis was to study the cellular immune response with phenotypic and functional assays in patients with severe infections, focusing mainly on sepsis.

The specific aims were:

Paper I	To determine whether an extended immunophenotyping of monocyte and lymphocyte markers could define disease-specific patterns in patients with fever due to various infectious diseases.
Paper II	To explore the function of Wnt5a and its role in monocyte-derived dendritic cell differentiation in sepsis patients.
Paper III	To study the incidence and nature of the immune alterations caused by different microbial organisms found in sepsis and septic shock patients.
Paper IV	To investigate the presence and generation of myeloid-derived suppressor cells in peripheral blood in sepsis caused by different microbial agents.

Material and methods

Subjects

All the patients included in the studies were admitted to the emergency department at Skåne University Hospital in Malmö, Sweden. The patients who presented signs of sepsis according to Bone's criteria (8) were eligible for all studies. In all four studies, the majority of sepsis patients—including those with septic shock—were included prospectively within two days of admission. Patients were only recruited in daytime, as the time between sample collection and laboratory analysis was less than six hours. To stratify the patients according to sepsis severity, the same diagnosis criteria were used. However, due to the small number of patients with severe sepsis, these patients were included in the sepsis group. In paper I, the patients with infectious diseases other than sepsis were included at their first consultation. Only adults were included in the studies. Healthy volunteers were recruited from the hospital and the research staff, and blood donors were used as healthy controls in paper II.

Methods

Data collection

Age, sex, and significant co-morbidities (chronic viral infections, renal failure, pulmonary disease, diabetes mellitus, cardiovascular disease, and malignancies) were recorded. Duration of ventilation and length of stay in the ICU, length of stay in the hospital, and 28-day mortality were recorded in the data set as well as the microbial findings and the final diagnosis set by the attending clinicians.

Laboratory methods

Routine samples and microbial cultures (papers I-IV)

Blood was drawn from all the patients for anaerobic and aerobic blood cultures. Urine for culture and where relevant, nasopharyngeal swabs were taken. Analysis of WBC, differential count, and CRP was also done routinely. For research purposes, blood for cytokine assays and surface and functional phenotypic assays was drawn at the same time point. All microbiological specimens were analysed at the medical microbiology laboratory and the routine laboratory assays were done at the clinical chemistry laboratory. Accredited hospital routine methods were used throughout.

Flow cytometry (papers I–IV)

This method simultaneously examines different physical and chemical properties of individual cells based on how the cells scatter incident light from a laser beam. Different detectors provide information about the cell volume (displayed as forward scatter; FS), the cell complexity i.e. granularity (displayed by side scatter; SS), and the fluorescence intensity of fluorochrome-conjugated antibodies used to detect specific CD receptors on the cell surface. The fluorescence-activated cell sorter (FACS) is a specialized flow cytometry machine, which was used in paper IV to sort out specific cell populations in a mixture of cells. The leukocytes obtained for cytometric analysis were obtained from whole blood in two different ways. The non-Ficoll-enriched method was used in paper I, in paper III (except CD163), and partly in paper IV. The CXP software program was used for data processing. The Ficoll-enrichment method was used in paper II, paper III (monocyte CD163), and partly in paper IV (see below). The Cell Quest software program was used to analyse this dataset.

Isolation of cells for functional assays (papers II and IV)

PBMCs were isolated by the Ficoll-enrichment method, which is a rapid method to isolate mononuclear cells from whole blood using density-gradient centrifugation. In this method, blood is layered on a special solution called Ficoll and then centrifuged. The erythrocytes and the granulocytes with high-density sediment to the bottom of the tube and the purified mononuclear cells (including monocytes and lymphocytes) with lower density are collected at the interface between the plasma at the top and the Ficoll-Paque™ below. The viability of the cells was checked by trypan blue staining.

In papers II and IV, further discrimination of immune cells was performed by using a magnetic separation kit (MACS) to obtain highly purified cell populations. Freshly isolated Ficoll-enriched leukocytes were incubated with a cocktail of primary antibodies directed against unwanted cells (such as B cells and NK cells), followed by incubation with magnetic bead-conjugated antibodies against the primary antibody. The cell sus-

pension was then put through a magnetic column where the unwanted cells linked to magnetic beads were retained in the column, but the unlabelled, wanted cells (naïve T cells and monocytes) passed through.

Western blot (paper II)

This is a method to detect specific proteins in a sample using gel electrophoresis. The monocytes were washed with PBS and then lysed in Laemmli sample buffer supplemented with dithiothreitol (DTT). The proteins were then run on a 10% SDS-PAGE gel and transferred to a PVDF membrane, where they were stained with specific antibodies using PBS-Tween-BSA and enhanced chemiluminescence (ECL). This method was performed to assess the effect of Wnt5a and IL-6 on ERK1/2-phosphorylation in monocytes.

Cytometric Bead Array analysis (paper IV)

Cytometric Bead Array analysis is a flow cytometric method where up to six different cytokines can be analysed in a single sample. Less blood is required and it is less time-consuming than ELISA. IL-8, IL-1β, IL-6, IL-10, and TNF-α were analysed after the sorted cells had been cultured and the supernatants had been collected for analysis.

Enzyme-linked immunosorbent assay (ELISA) (papers I–IV)

This is a method to detect and quantify soluble mediators such as cytokines present in cell cultures or serum. A capture antibody directed at the specific antigen of interest is used to coat wells in a microplate. The sample is added and the antigen binds to the immobilized antibody. Subsequently, a chemical reaction with the secondary antigen-specific enzyme-linked polyclonal antibody is supplemented for recognition. In the final step, a substrate produces a detectable signal, which is proportional to the amount of antigen of interest. In all papers, plasma from whole blood was immediately frozen at -20°C and later analysed for different cytokines (IL-6, IL-8, IL-10, IL-12, sTNFRI, and sTNFRII). ELISA was also done in paper II (IL-6, IL-10, and IL-12) using the supernatants from freshly prepared monocytes and the DC differentiation cultures stimulated with rWnt3a, rWnt5a, or LPS.

In vitro culture (papers II and IV)

MLR (mixed lymphocyte reaction) is an *in vitro* assay to measure the proliferative or the suppressive response of cells from one individual (the responder) to cells from another individual (the stimulator). The incorporation of ^{3}H-thymidine into the DNA as a measurement of lymphocyte proliferation was determined in a Microbeta counter. In paper II, monocytes from healthy controls and sepsis patients, separated by MACS, were induced to differentiate in a DC or a macrophage differentiation culture con-

taining GM-CSF and IL-4 or only GM-CSF. In paper IV, freshly sorted low-density granulocytes from healthy volunteers and sepsis patients, obtained by the Ficoll-enrichment method, were sorted out in the FACS Aria cell sorter. In the suppression assay, sorted cells were cultured together and allowed to suppress allogeneic naïve T cells from healthy blood donors. The T cells were activated with CD3/CD28 dynabeads.

Pinocytosis assay (paper II)

This method was used to assess the effect of Wnt5a on pinocytic activity *in vitro* by measuring the uptake of dextran. The monocytes were differentiated in DC medium and Wnt3a and Wnt5a were added. The differentiated cells were incubated with FITC-dextran and HLA-DR-APC for 20 minutes, and the uptake of dextran was analysed by flow cytometry.

Statistics

Statistical analyses were performed using Excel (Microsoft Excel version 14.3.8), SPSS (SPSS version 20.0), and Graphpad Prism 6. In papers I, II, III, the Mann-Whitney test was used due to non-parametric data and a small data set. In paper II, Student's t-test was used when comparing normally distributed variables. In paper III, Pearson's correlation coefficient was used in order to measure the linear dependence between CD40 monocyte expression and T cell activation. The immune response index was based on standard deviation because the healthy controls were thought to represent a normal distribution. In paper IV, the Kruskal-Wallis multiple comparison test was used for non-parametric data. The cytokines were analysed using Student`s t-test or One-way ANOVA test.

Ethical considerations

An ethical permit to collect blood samples from patients with different kinds of severe infections and from breast cancer patients was granted by the Local Ethical Committee at Lund University (Dnr 288/2007, Dnr 2010/477, Dnr 2010/135, and Dnr 2013-358). The participating patients gave oral and a written consent. If the patient was not in a condition to provide informed consent herself/himself, the relatives were asked for consent.

Results and discussion

Paper I—Immunophenotyping in patients with fever

Background and results

Patients sometimes suffer from prolonged fever of unknown origin (196). After extensive investigation, the majority of them are diagnosed with an infection, malignancy, autoimmune disease or inflammatory disease. However, some patients never receive a diagnosis, and there is a need for improved diagnostic tools (197).

In this study, we wanted to determine whether an extended immunophenotyping of leukocytes would define disease-specific patterns, and whether these findings could provide valuable diagnostic information. We included patients with gram-negative sepsis, neuroborreliosis, active tuberculosis (TB), mononucleosis, influenza, mixed connective tissue disorders (consisting of patients with autoimmune inflammatory diseases), and healthy controls. Peripheral blood was analysed for relative frequencies of lymphocytes and monocytes and also their activation status, using flow cytometry.

Patients with mononucleosis caused by Epstein-Barr virus (EBV) infection had lymphocytosis and a significantly reduced CD4/CD8 T cell ratio. Patients with mixed connective tissue disorders also presented with lymphopenia and a significantly decreased CD4/CD8 ratio.

T cell activation was measured by analysing HLA-DR expression on the cell surface. Other T cell markers (CD69, CD71, and CD152) were only expressed at low levels and did not add any additional information. EBV patients had increased expression of HLA-DR on both $CD8^+$ and to a lesser degree $CD4^+$ T cells. TB patients and patients with mixed connective tissue disorders were found to express enhanced HLA-DR on $CD4^+$ T cells.

Other lymphocyte populations were also analysed. We found an increased proportion of NK cells in the influenza group, but this did not reach statistical significance. The fraction of NK cells was significantly reduced in gram-negative sepsis. When we analysed

B cells, we found a significant decrease in the B cell population in EBV patients. No additional information was obtained from analysing a subset of B and T cells.

Total monocyte count was elevated in the majority of patients, but with large variations. When analysing the monocyte subsets, we found that the non-classical monocyte subset ($CD14^+$ $CD16^+$) expanded in infections with gram-negative sepsis, TB, EBV, and influenza. To determine the activation status of monocytes, we examined the surface expression of CD40. The classical monocytes ($CD14^+$ $CD16^-$) had significantly increased CD40 expression in all groups, most pronounced in gram-negative sepsis and EBV infection. This effect was also seen in the non-classical monocytes, but with larger variations.

Soluble TNF-α receptors are discarded following inflammatory stimuli, and can bind to circulating TNF and prevent its functions (198). Levels of soluble TNF-α receptors were significantly increased in gram-negative sepsis. The level of CRP was elevated in most patients, but negative in some patients with active TB, neuroborreliosis, and EBV infection. We also analysed other cell-surface markers indicating unconventional T cell subsets, plasmacytoid DCs, and Tregs (from CD25 and FoxP3 expression), but they did not give any informative results.

Discussion

The reduced ratio of $CD4^+$/$CD8^+$ T cells found in EBV patients emphasises the importance of interpreting results measured as fraction of cells cautiously. In this case, the reduced ratio only represents an increased proportion of $CD8^+$ T cells and not a real reduction in lymphocytes. The EBV patients were younger than the other patients, which could explain the greater T cell activation observed. Immunosenescence is a well-known phenomenon, and there is evidence to suggest that the innate immunity remains mostly intact during life, but the adaptive immune response is affected by increasing age, with impaired T cell function (199, 200).

Lymphopenia is a common feature of mixed connective tissue disorders and apoptosis of $CD4^+$ T cells has been shown to correlate with disease severity (201). The reduced ratio of $CD4^+$/$CD8^+$ T cells in this group could be explained by a true reduction in $CD4^+$ T cells. It has become evident in recent years that one of the mechanisms driving autoimmune diseases is an immune response initiated by activated $CD4^+$ T cells (202). We could confirm increased activation of $CD4^+$ T cells in patients with mixed connective tissue disorders.

T cell-mediated immune responses and mainly $CD4^+$ T cells play a crucial role in the pathogenesis of *Mycobacterium tuberculosis* infections. The type of T helper response generated influences the course of the disease (203). We observed that $CD4^+$ T cells in the TB patients also showed increased expression of HLA-DR as a sign of activation.

Early during acute viral infections such as influenza, the NK cells are activated to elicit effector functions such as pro-inflammatory cytokine production and lysis of infected cells. The majority of the influenza patients had an increased proportion of NK cells, except one patient who had low levels. The disparity between these results might be due to differences in duration of the illness and in where in the course of disease the blood sample was drawn (acute versus recovery). This stresses the importance of taking a proper medical history before interpreting the results.

In contrast, the reduced fraction of NK cells observed in gram-negative sepsis patients is probably a direct effect of NK cell reduction commonly seen in sepsis patients (110). The cause of the reduced number of NK cells in sepsis is not known, but it may be attributable to migration of NK cells into infected tissue or apoptosis (204). The reduced fraction of B cells observed in EBV patients most probably only reflects the increased $CD8^+$ T cell population.

Monocytes are important mediators of the innate immune response. They are very plastic in nature and change their phenotype in response to stimuli from the environment. During an infection or inflammation, monocytes increase in numbers and are mobilised to the infection site with a predominance of the $CD16^+$ monocyte subset (205). Monocytosis and an increased fraction of the non-classical monocytes were indeed observed in the majority of patient groups. It is, however, interesting to mention that even if patients with an EBV infection had a normal monocyte count, they showed an increase in the non-classical monocyte subset together with a strong degree of monocyte activation. The classical $CD16^-$ and the non-classical monocytes were activated in all patient groups, but to different degrees, suggesting the importance of both monocyte subsets in various diseases.

Pro-inflammatory cytokines are important mediators in the pathogenesis of sepsis and some of them have natural inhibitors. The level of soluble TNF receptors was only increased in gram-negative sepsis. This may not be so surprising, as sepsis is the prototype of disease with abundant pro-inflammatory cytokine release. The CRP could not be used to discriminate between bacterial and viral infections, as it showed a wide range. In some infections even presenting normal values.

When combining specific markers, characteristic patterns were revealed, which could—in some specific diseases—differentiate between infections of bacterial and viral origin. This flow cytometric method might be suitable for clinical diagnostic laboratories, and may help the clinicians in the diagnostics of patients with fever of unknown origin.

Paper II—Wnt5a inhibits the generation of Mo-mDCs

Background and results

Immunosuppression is a common feature of sepsis, contributing to secondary infections and death. Several changes in different immune cell populations have been identified, including changing numbers of monocytes and DCs. Monocytes can differentiate into myeloid DCs, which are pivotal cells for pathogen sensing and T cell activation, bridging the innate and the adaptive immune system.

Monocytes can roughly be divided into three subsets depending on CD14 and CD16 expression. The $CD16^+$ monocytes are known to expand during sepsis (86). Peripheral blood DCs can be divided into two types of myeloid DCs (MDC1 and MDC2) and one type of plasmacytoid DCs with different properties. In sepsis, a marked depletion of DCs has been identified, which may weaken the B and T cell response and thereby contribute to immunosuppression (70). The data on how the different subsets are affected are limited and somewhat contradictory (71, 206). The reason for the reduction in DCs is not known, but may involve increased apoptosis, altered migration to other compartments, or other unknown mechanisms.

Wnt5a is a protein that is expressed at increased levels in myeloid cells upon activation, such as in sepsis and breast cancer, but the reason for this is still not clear. In a previously published paper, we showed that in a pro-inflammatory environment, Wnt5a inhibits the M1 cell generation from human monocytes (207). Whether or not Wnt5a can also affect the differentiation of monocytes to dendritic cells (Mo-mDC) had not been explored. A previous study had investigated the gene expression profile of monocyte-derived dendritic cells and found Wnt5a to be highly up-regulated during this process compared to monocytes and monocyte-derived macrophages (Mo-M) (146). We therefore wanted to study whether Wnt5a could promote the differentiation of Mo-mDCs *in vitro*.

First, we decided to study the monocyte and DC population compartment found in the peripheral blood of patients with sepsis. We found a significant increase in both the $CD16^+$ non-classical population and the intermediate monocyte population, and the main circulating MDC1 population was slightly reduced compared to healthy controls.

We then verified the increased expression of Wnt5a in human Mo-mDCs compared to human monocytes, which has been validated in another study (146). We let human monocytes differentiate in *in vitro* Mo-mDC cultures treated with Wnt5a, and we found that Wnt5a inhibited the generation of Mo-mDCs ($CD14^{+/low}CD209^+$) while

promoting generation of $CD14^{++}CD209^{+}$ $CD16^{+}$ monocytes. When we stimulated these Mo-mDCs with LPS, we found an additional increase in $CD14^{++}CD1a^{-}$ $CD206^{+}$ monocytes. The monocyte sub-population generated showed inefficient pinocytosis and relatively effective antigen presentation. The cells produced both pro- and anti-inflammatory cytokines. This phenotype displayed characteristics more of $CD14^{+/++}CD16^{+}$ monocytes than of Mo-mDCs. Together, these results indicated that Wnt5a prevents the generation of Mo-mDCs while promoting the generation of monocytes. We also found that when monocytes from sepsis patients were cultured in Mo-mDC medium, these cells preferably differentiated into $CD14^{+}/^{++}$ HLA-DR^{++} monocytes as compared to healthy controls.

To clarify the mechanisms behind this, we investigated the effects of Wnt5a on monocytes from healthy controls. Other studies have previously revealed that IL-6 can inhibit Mo-mDC differentiation by activating STAT-3, but enhance Mo-mDC differentiation by activating ERK1/2 (208, 209). We discovered that monocytes stimulated with rWnt5a produced significantly more IL-6 and also showed delayed ERK1/2 activation. We could show that when IL-6 was blocked in a Wnt5a-treated Mo-mDC culture, the generation of Mo-mDCs was to some extent restored. These results were confirmed in another setting when monocytes were allowed to differentiate into Mo-mDCs in conditioned medium from an IL-6- producing breast cancer cells line (MDA-MB-231) that was stimulated with rWnt5a.

Discussion

In sepsis, the DCs are reduced and usually the intermediate and the non-classical monocyte populations are increased. We also found a small reduction in the MDC1 population and an increase in the $CD16^{+}$ monocyte population in peripheral blood from sepsis patients.

There is now increasing evidence that Wnt5a signalling may play a role in sepsis. In Mo-mDC cultures, we observed a small decline in Mo-mDCs, with a subsequent increase in the $CD14^{++}$ monocytes when rWnt5a was supplemented, and the effect was dependent on IL-6. This finding indicates that rWnt5a may play a role in the $CD14^{+/++}$ $CD16^{+}$ monocyte accumulation often found in sepsis. Cancer patients also show changed levels of DCs and monocyte subsets, and we could confirm that Wnt5a indeed increased the effect of monocyte generation in conditioned medium from rWnt5a-stimulated breast cancer cell lines producing IL-6.

The IL-6 level is normally increased in sepsis patients, and we could confirm this in our study. IL-6 has been shown to inhibit Mo-mDC differentiation through activation of the STAT 3 pathway, and a direct correlation has been observed between increased levels of IL-6 and declining levels of dendritic cells. We found that Wnt5a inhibits ERK1/2 and delays IL-6 induced ERK 1/2 activation in monocytes, while activating STAT3 (207).

Peripheral blood monocytes can differentiate into either myeloid DCs or macrophages in tissue. Monocytes from sepsis patients differentiate preferentially into immature CD1a-negative DCs, and these cells induce T cell anergy, suggesting a tolerogenic property of the immature DC (74). This supports our findings that Wnt5a may be involved in the inhibition of Mo-mDC differentiation both in sepsis and in breast cancer patients. Finally, another study has shown that the Mo-mDCs that are still generated in Mo-mDC cultures under rWnt5a-stimulating conditions are altered towards a tolerogenic state with reduced capacity to induce a proper Th1 response (210). We did not address this particular question in our study, but it strongly supports our finding that Wnt5a is indeed an immunoregulatory factor.

With these findings, we proposed that Wnt5a induces a negative homeostatic feedback loop during monocyte activation and differentiation, leading to the accumulation of $CD14^{+}/^{++}$ $CD16^{+}$ monocytes and a decline in the mDC population found in patients with sepsis.

Paper III—Large inter-individual variation in immune markers

Background and results

The pro-inflammatory immune response in sepsis is immediately followed by a counteracted anti-inflammatory phase with risk of immunosuppression. The pro-inflammatory phase of sepsis is quite well understood, but the immunosuppressive phase is not. In this study, we analysed expression levels of immunoregulatory markers on leukocytes in blood from sepsis patients using flow cytometry. The patient groups were sepsis, septic shock, and severe virosis.

In an attempt to illustrate the innate immune response, we first studied markers of monocyte activation because their levels of expression are known to reflect activation status. We found that the majority of patients with septic shock and gram-positive sepsis had significantly reduced expression of HLA-DR compared to gram-negative sepsis. We found an increased proportion of HLA-$DR^{-/low}$ monocytes in peripheral blood from septic shock and gram-positive sepsis patients. We found CD14 to be significantly reduced on monocytes in septic shock and in gram-positive sepsis. The expression of CD11c on monocytes was significantly reduced in septic shock and in gram-positive sepsis patients, but it was strongly enhanced in the majority of gram-negative sepsis patients. We found that CD40 expression showed large variation, with only increased expression observed in gram-negative sepsis. We could confirm that there was an increased proportion of CD163-expressing monocytes in septic shock patients compared to healthy controls.

The adaptive immune response is also affected during sepsis (75). In an attempt to study some of these changes, we examined the activation status of T cells by their HLA-DR and CD40 expression. We found a large amount of inter-individual variation in T cell activation in the different sepsis groups. The CD40 expression on T cells increased significantly in all sepsis groups. We did not, however, find any correlation between CD40 expression on monocytes and the frequency of activated T cells. When we studied the immunosuppressive Treg population using a PCR approach, we found profound variations.

In order to calculate the net effect of the immune response in every patient, we made an effort to calculate an immune response index. The majority of the septic shock patients and the gram-positive patients showed a rather normal index as compared to healthy controls, although displaying large variation. However, the gram-negative sepsis group showed signs of immune activation.

We finally measured soluble mediators in the blood from the different patient groups and found that the pro-inflammatory cytokine IL-6 was significantly increased in both septic shock and gram-positive sepsis. IL-18 was increased in gram-positive sepsis and TIMP-1 was elevated in septic shock and in gram-negative sepsis.

Discussion

Endotoxin-tolerant monocytes are suggested to be re-programmed HLA-DR$^{-/low}$ cells, which elicit immunosuppressive functions (125). Down-regulation of HLA-DR on monocytes is already an established marker for immunosuppression (183). CD14 expressed on monocytes is a co-receptor that recognises LPS in gram-negative bacteria and that—upon signalling in complex with the TLR and LPS—initiates cell activation and production of pro-inflammatory cytokines. The CD14 expression has been identified to be related to the state of sepsis, as a reduction in CD14 expression is found in the most severely ill sepsis patients (184, 211).

We could confirm that patients with septic shock and gram-positive sepsis had significantly reduced expression of both HLA-DR and CD14. CD11c is expressed on various cells including monocytes, and reduced expression of CD11c has been related to mortality (173). The patients with septic shock and gram-positive sepsis did indeed show reduced CD11c expression. We also found an increased proportion of HLA-DR$^{-/low}$ monocytes in septic shock and gram-positive sepsis, proposing them to be re-programmed endotoxin-tolerant monocytes. This is not fully compatible with what was seen in paper IV, where we analysed monocytes prepared using the Ficoll-enrichment method. There, we found HLA-DR$^{-/low}$ monocytes preferentially in gram-negative sepsis. Also, patients with gram-positive sepsis had reduced levels of monocyte HLA-DR expression, although not significantly. This result indicates that HLA-DR$^{-/low}$ monocytes in gram-negative and gram-positive sepsis may be enriched differently depending on the cell isolation technique.

We then studied the CD40 receptor, which upon ligation to the T cell-derived CD40 ligand (CD40L), causes activation and production of pro-inflammatory cytokines and up-regulation of co-stimulatory molecules (212). A previous study has identified increased expression of CD40 to be a protective phenomenon in sepsis (65). In our study, only patients with gram-negative sepsis had increased CD40 expression, perhaps indicating less immunosuppressive properties. However, there was a marked variation in CD40 expression, indicating an active CD40:CD40L immune activation even in patients with other immunosuppressive phenotypes.

CD163 is a scavenger receptor and serves as a marker on alternatively activated monocytes/macrophages with immunosuppressive properties (213). An increased proportion of monocytes expressing CD163 has previously been observed in sepsis patients, but this was not found to be correlated with clinical outcome (214). We could confirm an increased proportion of CD163 expression monocytes in septic shock, also using the Ficoll-enrichment method. CD163 expression on monocytes appears to be a suitable diagnostic biomarker for re-programmed monocytes. The soluble form of CD163 has previously been evaluated as a biomarker for sepsis and has been shown to correlate with clinical outcome (175).

In paper I, we found a low grade of T cell activation in the gram-negative sepsis patients, by measuring the expression of HLA-DR on the cell surface. In this paper, we found a large inter-individual variation in the different sepsis groups with only signs of activation in septic shock when we analysed both $CD4^+$ and $CD8^+$ T cells. T cell activation may aid bacterial clearance, but may also lead to tissue damage. However, too little activation may hinder bacterial clearance. The marked variation in T cell activation needs to be clarified. Even if CD40 is largely expressed on APCs, it can also be found on a sub-population of activated T cells (215). The CD40 expression on T cells was indeed increased significantly in all sepsis groups, but the clinical significance of these findings needs to be studied further.

Surprisingly, we did not find any correlation between increased monocyte CD40 activation and signs of T cell activation. This finding may be interpreted as monocytes being activated early in sepsis, while the activation of T cells is a later event. Another possible explanation could be that HLA-DR on T cells is not the best activation marker and other plausible markers such as the CD45RA/CD45RO ratio, CD69, CD71, or other co-stimulatory molecules might have been more informative. Immune regulatory cells such as Tregs are usually increased in sepsis patients, and in this study we also found data that supported this (75). Our data were not significant, however, due to the marked variation in the number of Tregs. Tregs are known to increase after 3 days of sepsis and this variation might partly be explained by the difference in duration of illness.

TIMP-1 has previously been associated with severity of sepsis, and we also found that TIMP-1 levels were elevated in septic shock and in gram-negative sepsis (181). IL-6

levels were higher in gram-positive patients, which contradicts previous findings (26). IL-18 has previously been associated with gram-positive sepsis, and we could confirm this result (164). The immune response index indicated that gram-positive sepsis in general showed more of an immunosuppressive state than gram-negative sepsis. It is known that gram-positive sepsis patients have higher mortality than gram-negative sepsis patients (19). This can partly be explained by the emerging multi-resistant bacteria, but it may also be due to differences in host responses and virulence mechanisms. A homogenous immunosuppressive pattern was never found in any of the subgroups, rather the opposite, with mixed phenotypes being observed. Only 22% of the patients showed a homogenous immunosuppressive or immune-activated pattern.

In conclusion, the immune response was shown to be highly individual, with large inter-individual variation. This result suggests that it is important to examine the immune status of each patient before considering immune-modulatory treatment. These findings highlight the complexity of the immune response and emphasise the importance of a more individualised treatment of sepsis, as every patient is unique.

Paper IV—The MDSCs in sepsis patients differ with microbial agents

Background and results

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that potently suppress immune responses in cancer patients. MDSCs also increase in sepsis, but their presence and function in this condition is less clear. It is also not known whether the cancer MDSCs are identical to the MDSCs found in sepsis, or whether their subset varies with the type of microbial agent.

During our work for paper II, we found an enrichment of granulocytic cells in the mononuclear fraction of the Ficoll-enriched PBMCs in patients with sepsis, but not in healthy controls. Both Mo-MDSCs and PMN-MDSCs have previously been identified in this mononuclear fraction (216). To our knowledge, no studies using density gradient-centrifuged blood from sepsis patients had been performed to study MDSCs previously. In the mononuclear fraction from Ficoll-density gradient-centrifuged blood, we found that $CD14^{+}HLA\text{-}DR^{low/-}$monocytes (Mo-MDSCs) were increased both in gram-negative and in gram-positive sepsis while PMN-MDSCs ($CD11b^{+}$ $CD33^{+}$ Lin^{-} $CD15^{+/low}$) were significantly increased preferentially in gram-positive sepsis compared to gram-negative sepsis and healthy controls.

In the mononuclear fraction, we also found a fraction of cells expressing $CD14^{low}CD64^{low}$ in the patients with gram-positive sepsis. This population was found in Ficoll-enriched

samples as well as in whole blood. Except for the $CD14^{low}CD64^{low}$ surface phenotype, these cells showed a surface phenotype similar to that of conventional PMN-MDSCs.

To determine whether these cells were also functionally similar to PMN-MDSCs, we sorted out $CD33^{+}CD14^{low}CD64^{low}$ and $CD33^{+}CD14^{+}$ $CD64^{+}$ cells using the FACSAria cell sorter. In the *in vitro* T cell suppressive assay, we found that the $CD14^{low}CD64^{low}$ population from gram-positive sepsis patients significantly inhibited T cell proliferation by measuring the incorporation of thymidine in T cells co-cultured with antiCD3/CD28 dynabeads. This finding suggests that these cells are also PMN-MDSCs despite a small elevation of CD14 expression. In support of this, IL-8 was the most abundant cytokine secreted. The T cell inhibition was probably not caused by the secretion of immunosuppressive cytokines, since the $CD14^{low}CD64^{low}$ PMN-MDSCs released only tiny amounts of pro- and anti-inflammatory cytokines. However, the IL-10:TNF-α ratio was increased, which might indicate an immunosuppressive response.

Morphologically, the $CD14^{low}CD64^{low}$ PMN-MDSCs from gram-positive sepsis patients were heterogeneous with blasts, but also some cells with ring-shaped nuclei typical for MDSCs in cancer. In non-Ficoll-enriched blood, the $CD14^{low}CD64^{low}$ could only be detected in gram-positive sepsis. CD64 expression on granulocytes was increased in all the sepsis patients and in the patients with viral infections, as compared to healthy controls.

Discussion

In sepsis, a pro-inflammatory phase (SIRS) is elicited to eliminate the pathogen. In order to dampen the excessive immune response, another compensatory anti-inflammatory (CARS) response is generated. Two subsets of MDSCs have been identified in peripheral blood in sepsis patients, but little is known about their role in this disease. MDSCs produce anti-microbial ROS and speculations have been made as to whether MDSCs in sepsis could be beneficial for the patient by both suppressing the immune response and combating the invading microorganism.

Both gram-negative and gram-positive bacteria stimulate pro-inflammatory signals through partly overlapping TLRs. Hypothetically, these signals might induce different types of MDSCs. This is supported by our findings that the PMN-MDSCs increased in gram-positive sepsis and Mo-MDSCs in gram-negative sepsis.

In paper III, using non-Ficoll-enriched blood, we found an increased proportion of $HLA\text{-}DR^{-/low}$ monocytes in septic shock patients and gram-positive sepsis patients, but not in gram-negative sepsis patients. However, in this paper, using Ficoll-density gradients, we found $HLA\text{-}DR^{-/low}$ monocytes preferentially in gram-negative sepsis. Gram-positive sepsis showed reduced levels of monocyte HLA-DR expression, although not significantly. This indicates that $HLA\text{-}DR^{-/low}$ monocytes in gram-negative

and gram-positive sepsis may be enriched differently depending on the cell isolation technique. However, this needs to be investigated further.

PMN-MDSCs have been identified as being positive for lineage cell markers $CD11b^{+}$ $CD33^{+}$ Lin^{-} $CD15^{+/low}$, but in this study we found a fraction of immunosuppressive PMN-MDSCs that expressed low levels of CD14. If CD14 is excluded in the analysis, an underestimation of the number of PMN-MDSCs in peripheral blood could occur.

This cell fraction was preferentially enriched in the low-density granulocyte fraction of Ficoll-treated blood, making it more challenging to find them in samples from whole blood. Non-Ficoll-treated blood samples are often used in the clinic. MDSC populations might therefore be missed when examined. It is likely that by including a Ficoll-density centrifugation step in the analysis, an enhanced detection of the PMN-MDSC population would occur. Despite being poor producers of cytokines, the PMN-MDSCs from the gram-positive patients were potent suppressors of T cell proliferation. The suppression of T cells was caused by a ROS dependent mechanism.

In conclusion, we suggested that different microorganisms can induce different MDSCs in sepsis patients. The $CD14^{low}$ PMN-MDSCs are immature myeloid cells with immunosuppressive characteristics and are primarily induced in gram-positive sepsis.

Conclusions

I. In some patients, immunophenotyping of lymphocyte and monocyte cell-surface markers could provide informative patterns in the assessment of fever of unknown origin.

II. Wnt5a inhibits generation of Mo-mDCs while promoting the generation of $CD14^{+/++}$ $CD16^{+}$ $CD209^{+}$ monocytes; the mechanism is IL-6-dependent.

III. Signs of immunosuppression predominated in septic shock and gram-positive sepsis, whereas immune activation was more prevalent in gram-negative sepsis. A large inter-individual variation in immunoregulatory markers was identified.

IV. Different MDSC subsets are induced depending on the microbial agents. Immunosuppressive Mo-MDSCs prepared using density-gradient isolation are preferentially generated in gram-negative sepsis and PMN-MDSCs are preferentially generated in gram-positive sepsis, including a $CD14^{low}$ PMN-MDSC population.

Final reflections

Sepsis is a serious medical condition with a high mortality rate. However, when diagnosed early and treated correctly, the mortality is reduced.

One challenge is that patients with sepsis are a diverse group when presenting themselves at the emergency department. They come at different stages of the disease, with a wide range of causative microorganisms, and at all ages. Co-morbidities and genetic susceptibility vary, and will most probably influence the immune response. Sepsis is also a dynamic process. The levels of different mediators released from the host or from the pathogens change rapidly during the disease process, along with the clinical picture. The consequence of this is a great individual heterogeneity in the immune response generated, making it difficult to generalise. An individualised approach with the assessment of the immune status of each patient would therefore be preferable before considering immunomodulating therapy.

The mechanism of this complex immune response has been extensively studied in animal models, revealing complicated cascades of immune activation and immunosuppression in lymphocytes, monocytes, and granulocytes. However, our knowledge in the clinical setting is insufficient and more research is needed.

Another problem is the lack of a golden standard for diagnosis of sepsis, and that the present definition of sepsis is too vague. This leads to variations between studies and sepsis trials, hindering comparison and validation. A better definition of sepsis, severe sepsis, and septic shock would facilitate inclusion in sepsis trials and improve care and the development of new biomarkers and treatments.

An ideal biomarker for sepsis should be able to identify the presence or absence of sepsis. It should also facilitate the identification of patients who would benefit from any particular intervention, and preferably render it possible to monitor the response after a given treatment. Advanced high technological research is being carried out in many countries, and hopefully these studies will help to identify better biomarkers and improved treatment options.

One must remember, though, that the majority of those who die from sepsis live in poor countries and that many of them are children (217). It is therefore a necessity to improve sepsis-related healthcare in general, including access to healthcare services, diagnostic facilities, and treatment regimens. It is also important that the biomarkers developed should not only be easy to use, fast, and reliable, but they should also be inexpensive so that they can be available where they are most needed.

Summary in English

It is a challenge to treat patients with severe infections. The infection can be caused by a variety of microorganisms provoking different immune responses. The immune response greatly influences the symptoms, the clinical signs, and the outcome of the patient. In sepsis, the immune response probably also changes over time during the clinical course. In general, it is believed that sepsis initially induces a profound activation of the pro-inflammatory immune system. In cases with excessive and devastating activation an anti-inflammatory negative feedback loop is generated, with a risk for secondary infections and death.

In this thesis we aimed at evaluating if an extended analysis of white blood cells could provide valuable diagnostic and possibly therapeutic information in patients with different severe infections with focus on sepsis.

In the first study we analysed white blood cell-surface markers from patients with sepsis, neuroborreliosis, active tuberculosis, mononucleosis, influenza or mixed connective tissue disorders. We found that some markers indicated a sub-acute bacterial infection or a mixed connective tissue disorder, and that in some cases this analysis could differentiate between infections of bacterial and viral origin. We conclude that this method may help in the diagnostics of some patients with fever of unknown origin.

The second paper was a study of the dendritic cell (DC). It functions as an important bridge between the innate and adaptive immune response as it presents antigens to lymphocytes. We found that the specific protein Wnt5a inhibited the generation of monocyte-derived myeloid DC. We also showed that when monocytes were stimulated with Wnt5a, the production of IL-6 was induced. We suggest that the effect of Wnt5a could be one explanation for the decreased number of dendritic cells seen in sepsis patients.

In our third paper we investigated the immunoactivating (pro-inflammatory) and the immunosuppressive (anti-inflammatory) response in patients with sepsis, septic shock and severe viral infections. We found that signs of immunosuppression dominated in the patients with gram-positive sepsis and septic shock, whereas monocyte activation was common in the gram-negative sepsis patients. The main finding, however, was a profound inter-individual variation. Our data implies that it is important to examine the immune status in each individual sepsis patient before considering giving immunomodulatory treatment.

In our last manuscript, we studied the presence and function of a novel cell population, the myeloid-derived suppressor cells (MDSCs), in sepsis patients. MDSCs are known to suppress the immune response in cancer. We found that different bacteria can induce different kinds of MDSCs. We also found that they can suppress T cell proliferation.

Svensk sammanfattning

Det är en utmaning att behandla patienter med allvarliga infektioner eftersom immunförsvaret reagerar olika på olika mikroorganismer. Immunförsvarets reaktion har i sin tur en stor betydelse för symtombild, kliniska fynd och prognos. Vid blodförgiftning (sepsis) förändras dessutom sannolikt immunsvaret under det kliniska förloppet. Man tror att det inledningsvis vid sepsis sker en aktivering av det proinflammatoriska immunförsvaret. I de fall där aktiveringen blir för kraftig skapas en förödande antiinflammatorisk negativ återkoppling med risk för sekundära infektioner och död.

Den här avhandlingens mål var att undersöka om en utökad analys av vita blodkroppar kan ge information av värde för diagnostik och behandling av patienter med allvarliga infektioner med fokus på sepsis.

I den första studien granskade vi ytmarkörer på vita blodkroppar från patienter med antingen sepsis, borrelia, tuberkulos, körtelfeber, influensa eller bindvävssjukdom. Vi kunde där se att vissa markörer framträdde vid en halvakut bakteriell infektion eller en bindvävssjukdom. Den här typen av analys skulle kanske i en del fall kunna hjälpa till att skilja mellan bakterie- och virusinfektioner. Vår slutsats är att metoden skulle kunna vara av värde i diagnostiken av vissa patienter med oklar feber.

Den andra artikeln var en studie av dendritiska celler som, genom att de presenterar antigener för lymfocyter, fungerar som en viktig brygga mellan det medfödda och det förvärvade immunförsvaret. Vi kunde visa att det specifika proteinet Wnt5a hämmade bildandet av speciella dendritiska celler med ursprung från monocyter. Vi kunde också visa att när monocyter stimulerades med Wnt5a började de tillverka IL-6. Wnt5a's egenskaper skulle kunna vara en av förklaringarna till det minskade antalet dendritiska celler hos vissa patienter med sepsis.

I vår tredje artikel undersökte vi det aktiverande och det hämmande immunsvaret hos patienter med sepsis, septisk chock och allvarliga virusinfektioner. Vi såg att tecken på immunhämning dominerade hos patienter med grampositiv sepsis och septisk chock medan monocytaktivering var vanligt hos patienter med gramnegativ sepsis. Det viktigaste fyndet var dock de uttalade individuella variationerna. Våra data antyder att det är viktigt att undersöka hur immunförsvaret reagerat hos den enskilde sepsispatienten innan man ger behandling för att undertrycka eller stimulera det.

I vårt fjärde arbete undersökte vi förekomsten och funktionen av en särskild sorts immunhämmande celler, myeloid-derived suppressor cells (MDSCs), hos patienter med sepsis. MDSCs är en nyupptäckt celltyp känd för att dämpa immunförsvaret vid cancer. I studien såg vi att olika bakterier kan framkalla generering av olika typer av MDSCs. Vi såg också att MDSCs kan hämma tillväxten av T-celler.

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Appendix- CD markers important for this thesis

CD marker	Expression on cells	Function
CD1a	T cell, B cell, DC, monocyte/macrophage	Antigen presenting
CD2	T cell, B cell, NK cell	Involved in cell adhesion
CD3	T cell	Involved in T cell signal transduction
CD4	T cell, monocyte/macrophage	Initiates the early phase of T cell activation
CD8	T cell, NK cell	Initiates the early phase of T cell activation
CD11b	T cell, B cell, DC, NK cell, monocyte/macrophage, granulocyte	Involved in various adhesive interactions
CD11c	T cell, B cell, DC, NK cell, monocyte/macrophage, granulocyte	Important for cell-cell interactions
CD14	monocyte/macrophage, granulocyte	Mediates the innate immune response to LPS
CD15	stem cell/precursor, monocyte/macrophage, granulocyte	Involved in adhesion and in granulocyte activation
CD16	T cell, DC, NK cell, monocyte/macrophage, granulocyte	Mediates phagocytosis and antibody-dependent T cell mediated cytotoxicity. Binding antibodies and modulates immune response. Low affinity FcR
CD19	B cell, DC, stem cell/precursor	B cell co-receptor. Involved in the proliferation and survival of mature B cells
CD20	T cell, B cell	Involved in the development and differentiation of B cells into plasma cells
CD25	T cell, B cell, NK cell, monocyte/macrophage	IL-2 receptor
CD27	T cell, B cell, NK cell	Required for generation and maintenance of long-term T-cell immunity
CD28	T cell	Co-stimulatory molecule. Required for T cell activation, T cell proliferation
CD33	DC, stem cell/precursor, monocyte/macrophage, granulocyte	Involved in cell-adhesion, cell-cell signalling, apoptosis and inhibitory receptor

CD34	stem cell/precursor	Involved in cell adhesion
CD40	T cell, B cell, DC, stem cell/precursor, monocyte/macrophage	Involved in cell adhesion, cell proliferation, signal transduction
CD45	T cell, B cell, DC, NK cell, stem cell/precursor, monocyte/macrophage, granulocyte	Involved in the regulation of T and B cell antigen receptor signalling
CD56	T cell, NK cell	Involved in cell adhesion
CD64	DC, stem cell/precursor, monocyte/macrophage, granulocyte	Involved in the innate and the adaptive immune reponse
CD69	T cell, B cell, NK cell, monocyte/macrophage, granulocyte, platelets	Involved in lymphocyte proliferation and signal transmission in NK cells and platelets
CD71	stem cell/precursor	Involved in the uptake of transferrin-iron complexes
CD80	T cell, B cell, DC, monocyte/macrophage	Co-stimulatory molecule. Involved in lymphocyte activation
CD83	B cell, DC	Involved in antigen presentation and immune stimulation
CD86	T cell, B cell, DC, monocyte/macrophage	Co-stimulatory molecule. Can bind to either CD28 or CD152 and regulates T cell activation
CD123	DC, stem cell/precursor, granulocyte	Involved in hematopoietic progenitor cell growth and cell differentiation
CD134	T cell	May suppress apoptosis
CD152	T cell, B cell	Involved in T cell inhibition
CD154	T cell	The CD40 ligand. Involved in B cell proliferation and immunoglobulin production
CD163	monocyte/macrophage	Scavenger receptor for the hemoglobin-haptoglobin complexes
CD206	DC, monocyte/macrophage	Involved in antigen endocytosis. Pathogen receptor
CD209	DC, monocyte/macrophage	Involved in DC migration, T cell proliferation. Also serves as pathogen receptor

Paper I-IV