Dynamics of Human Leukocyte Antigen-D Related expression in bacteremic sepsis

To my father

Örebro Studies in Medicine 161

SARA CAJANDER

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Title: Dynamics of Human Leukocyte Antigen-D Related expression in bacteremic sepsis
Publisher: Örebro University 2017
www.oru.se/publikationer-avhandlingar

Print: Örebro University, Repro 04/2017

ISSN 1652-4063
ISBN 978-91-7529-191-8

Abstract

Sara Cajander (2017): Dynamics of Human Leukocyte Antigen-D Related expression in bacteremic sepsis. Örebro Studies in Medical Science 161.

Monocytic human leukocyte antigen-D related (mHLA-DR) expression determined by flow cytometry has been suggested as a biomarker of sepsis-induced immunosuppression.

In order to facilitate use of HLA-DR in clinical practice, a quantitative real-time PCR technique measuring HLA-DR at the transcription level was developed and evalutated. Levels of HLA-DR mRNA correlated to mHLA-DR expression and were robustly measured, with high reproducibility, during the course of infection. Dynamics of mHLA-DR expression was studied during the first weeks of bloodstream infection (BSI) and was found to be dependent on the bacterial etiology of BSI. Moreover, mHLA-DR was shown to be inversely related to markers of inflammation. In patients with unfavourable outcome, sustained high C-reactive protein level and high neutrophil count were demonstrated along with low mHLA-DR expression and low lymphocyte count. This supports the theory of sustained inflammation in sepsis-induced immunosuppression. The association between mHLA-DR and bacterial etiology may be linked to the clinical trajectory via differences in ability to cause intractable infection. *Staphylococcus aureus* was the dominating etiology among cases with unfavourable outcome. With focus on patients with *S. aureus* BSI, those with complicated *S. aureus* BSI were found to have lower HLA-DR mRNA expression during the first week than those with uncomplicated *S. aureus* BSI. If these results can be confirmed in a larger cohort, HLA-DR measurement could possibly become an additional tool for early identification of patients who require further investigation to clear infectious foci and achieve source control.

In conclusion, PCR-based measurement of HLA-DR is a promising method for measurements of the immune state in BSI, but needs further evaluation in the intensive care unit setting to define the predictive and prognostic value for deleterious immunosuppression. The etiology of infection should be taken into consideration in future studies of translational immunology in sepsis.

Keywords: monocyte HLA-DR, sepsis, immunosuppression, bloodstream infection, HLA-DRA, CIITA, qRT-PCR

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LIST OF ORIGINAL PAPERS

This thesis is based on the following papers and manuscripts, which are referred to in the text by their Roman numericals:

I. Cajander S, Backman A, Tina E, Stralin K, Soderquist B, Kallman J. Preliminary results in quantitation of HLA-DRA by real-time PCR: a promising approach to identify immunosuppression in sepsis. Critical care (London, England). 2013;17(5):R223.

II. Cajander S, Tina E, Backman A, Magnuson A, Stralin K, Soderquist B, Källman J. Quantitative Real-Time Polymerase Chain Reaction Measurement of HLA-DRA Gene Expression in Whole Blood Is Highly Reproducible and Shows Changes That Reflect Dynamic Shifts in Monocyte Surface HLA-DR Expression during the Course of Sepsis. PloS one. 2016;11(5):e0154690.

III. Cajander S, Rasmussen G, Tina E, Magnuson A, Soderquist B, Källman J, Stralin K. Monocytic HLA–DR expression differs between bacterial etiologies and is inversely related to C-reactive protein and neutrophil count during the course of bloodstream infection. Submitted

IV. Rasmussen G, Cajander S, Backman A, Källman J, Soderquist B, Stralin K. Expression of *HLA-DRA* and *CD74* mRNA in whole blood during the course of complicated and uncomplicated *Staphylococcus aureus* bacteraemia. Submitted

ABBREVIATIONS

SIRS	Systemic inflammatory response syndrome
CARS	Compensatory anti-inflammatory response syndrome
ICU	Intensive care unit
SOFA	Sequential organ failure assessment score
BSI	Bloodstream infection
SAB	Staphylococcus aureus bacteremia
TNF	Tumour necrosis factor
TGF	Transforming growth factor
IL	Interleukin
PAMP	Pathogen-associated molecular pattern
DAMP	Damage-associated molecular pattern
PRR	Pathogen recognition receptor
TLR	Toll-like receptor
CLR	C-type lectin receptor
NOD	Nucleotide oligomerization domain
HMGB1	High mobility group box-1
HSP	Heat shock protein
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
miRNA	Micro-ribonucleic acid
HLA	Human leukocyte antigen
mHLA-DR	Monocytic human leukocyte antigen-D related
MHC	Major histocompatibility complex
APC	Antigen presenting cell
CD	Cluster of differentiation
Th	T helper cell
Treg	T regulatory cell
CMV	Cytomegalovirus
LPS	Lipopolysaccharide
IFN	Interferon
DC	Dendritic cell
NK	Natural killer
CTLA-4	Cytotoxic T lymphocyte antigen-4
BTLA	B and T lymphocyte attenuator

PD-1	Programmed cell death-1
PD-1L	Programmed cell death-1 ligand
MDSC	Myeloid derived suppressor cell
GM-CSF	Granulocyte macrophage-colony stimulating factor
G-CSF	Granulocyte-colony stimulating factor
PICS	Persistent inflammation-immunosuppression and catabolism syndrome
CIITA	Class II trans-activator
CRP	C-reactive protein
qRT-PCR	Quantitative real-time polymerase chain reaction
ROC	Receiver operating characteristic curve
AUC	Area under the curve

INTRODUCTION

Sepsis incidence and mortality

Sepsis is a life-threatening illness caused by a dysregulated host response to infection, affecting millions of people around the world [1]. It accounts for approximately 15-20% of all deaths in the developing world [2] and epidemiological data have shown a worrying trend of increasing incidence [3]. The true incidence of sepsis is, however, difficult to determine because there is wide variance, between data sets, in the diagnosis definition, and also in the methodology used for data extraction [3, 4]. During 2004-2009, the annual incidence of sepsis in the United States varied from 300 to 1031 per 100 000 persons, when using different definitions of sepsis in data extracted from one national data set. Regardless of the definition used, there was a similar time trend showing an annual increase of 13% [3].

Hospital mortality from sepsis ranges from 10% to 50% depending on the degree of severity [1, 5]. In septic shock, the case-fatality may be above 50%, but this is influenced by many factors [6-8]. The strongest predictor of death is the cumulative burden of organ dysfunction [6, 9, 10]. Age, comorbidity, sex, pathogen virulence, site of infection, time to antibiotic treatment [11, 12] and expertise of the treating center are also important for outcome [6, 13, 14]. Interestingly, there has been a declining trend in reported 28-day mortality rates from sepsis in high-income countries, without development of new sepsis-specific treatments [2, 15]. This could be due to successful treatment bundles including evidence-based recommendations for the initial management of supportive care [16-18] together with early administration of antibiotics [11, 12] and awareness of the disease [19]. On the other hand, several reports have shown a high burden of disease among survivors of sepsis and a high late mortality rate [20-23]. Recent studies have reported 2 and 3 year mortality rates of 45% and 71% respectively [24, 25]. As shown in Figure 1, advanced age and high comorbidity burden contribute to the high incidence of long-term deaths [26]. However, one in five who survive sepsis has a late death not explained by the health status before sepsis [27]. A recent study found that early deaths were mainly attributable to intractable multi-organ failure related to the primary infection or mesenteric ischemia, whereas late deaths were often were related to nosocomial infections [28]. Similarly,

Zhao et al. demonstrated that the risk of late death for septic shock patients who contracted secondary infections was 5.8 times higher than for patients who remained free of secondary infection [21]. Moreover, in an observational ICU study including over 1000 critically ill patients it was shown that late onset of shock or recurring shock had a significantly higher mortality compared to early onset of shock [5]. Long lasting defects in cellular and immune homeostasis after sepsis rendering the host vulnerable to secondary infections, have been identified as possible contributing factors to negative outcome and late mortality in sepsis [13, 20, 21, 23].

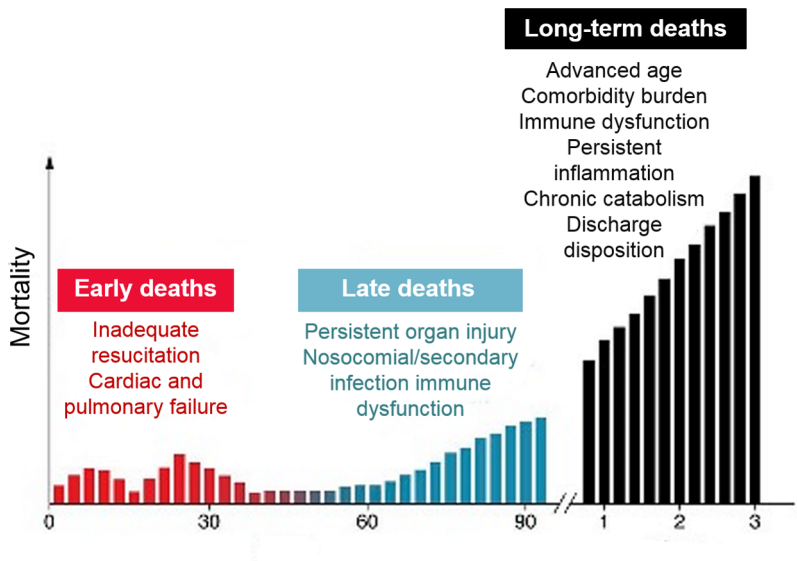

Figure 1. Modified from Delano and Ward, "Sepsis-induced immune dysfunction: can immune therapies reduce mortality?" Journal of Clinical Investigation, 2016 [23]. Two early peaks in mortality exist, albeit of low magnitude. A third upswing occurs approximately 60–90 days after sepsis and continues to soar as time progresses. This delay in sepsis mortality is thought to be the consequence of the more sophisticated intensive care unit (ICU) care that keeps elderly and comorbidly challenged patients alive longer despite ongoing immune, physiological, and biochemical aberrations.

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Sepsis history and pathophysiology

The understanding of sepsis pathogenesis is in constant progress. Historically, there have been different prevailing theories through the ages. The word "sepsis", was first encountered in Homer's "Iliad," where it was used as a derivative of the Greek word *sepo* [σηπω], which means "I rot"[29]. This word reflects the early understandings of sepsis, describing it as a process by which flesh rots, wounds fester, and similarly, swamps generate foul air [30]. The poisonous, foul air was thought to explain the endemic spread of diseases. This theory was later rejected by acceptance of the germ theory in the 19th century as Robert Koch and Louis Pasteur provided convincing evidence of microorganisms as causal factors for infectious diseases. In terms of this theory, sepsis was described as "blood poisoning," and a bacterial invasion into the bloodstream was thought to be the disease causative mechanism [30]. However, after the discovery of antimicrobial therapy, this theory of sepsis pathogenesis was proven to be insufficient because many patients who were treated with antibiotics and cleared the bacteremia still died from sepsis. It became clear that host responses in bacterial infection could be harmful to the host and therefore had a significant role in the pathogenesis [31]. In the 1980s, cytokines where discovered and identified as important mediators of a collateral endogenous tissue damage. Massive release of cytokines during sepsis, often referred to as a "cytokine storm," causing an overwhelming systemic inflammation with activation of complement and coagulation pathways, was thought to be the main cause of multi-organ failure and negative outcome in sepsis. Convincing animal experiments, such as the investigations by Tracey et al. using injections of tumor necrosis factor (TNF) [32] and TNF inhibitors [33], demonstrated a causal relation between pro-inflammatory cytokines and lethal sepsis. As a consequence, the first internationally recommended definitions for the sepsis diagnosis were based on a theory of systemic inflammatory response [34].

On the other hand, sepsis trials aiming to block different pathways that are associated with exaggerated inflammation have had repeated failures [35-39]. To date, over 30 interventional sepsis trials have demonstrated disappointing results [40, 41]. In addition, the only specific treatment (activated protein C) for sepsis was withdrawn from the market after failure to prove efficacy in a multicenter, post-marketing study [42]. This said, the former pathophysiology of sepsis based on excessive inflammation has been questioned and a need for a paradigmatic shift has been

proposed. In support of this, anti-inflammatory (immunosuppressive) responses with overexpression of interleukin (IL)-10 and signs of immunosuppression have been associated with a negative outcome in sepsis [43]. It has become evident that both pro- and anti-inflammatory responses are shown to be simultaneous events during sepsis [13]. Some researchers propose that the dominating inflammatory profile might be differently expressed in subpopulations of sepsis [41]. During septic shock for instance, it is still believed that the unbalanced response in terms of an overshooting pro-inflammatory reaction mediates the circulatory impairments, leading to unfavorable outcome, as described previously. Moreover, alterations in cellular metabolism and neuroendocrine signaling are found to be relevant for the development of organ failure and immunosuppression [44]. Importantly, no clear culprit mechanism has been found to explain the pathophysiology in all septic patients. Rather, sepsis pathophysiology is believed to be heterogeneous, with variation in the degree of impaired mechanisms [1]. Patients who are unable to recover from sepsis are thought to have homeostatic imbalance in the systems regulating these important mechanisms [1].

Sepsis definitions

Sepsis-1

The first operationalized consensus definition of sepsis was based on the theory of systemic inflammatory response syndrome (SIRS), as shown in Table 1. After a conference in 1991 this definition became internationally accepted. Sepsis was defined as a systemic inflammatory response to infection with increasing degrees of severity identified as severe sepsis and septic shock [34]. According to this definition, microbiological confirmation is not required, but infection should at least be suspected. This original definition is now referred to as "Sepsis-1".

Table 1. Criteria for the systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis, and septic shock, according to the 1991 American College of Chest Physicians (ACCP)/Society of Critical Care Medicine (SCCM) Consensus Conference.

TERM	CRITERIA
SIRS	Two out of the following four criteria: Temperature >38 °C or <36 °C Heart rate >90/min Hyperventilation evidenced by respiratory rate >20/min or arterial CO_2 lower than 32 mmHg White blood cell count >12 cells/L or lower than 4 cells/L
Sepsis	SIRS criteria with presumed or proven infection
Severe sepsis	Sepsis with organ dysfunction
Septic shock	Sepsis with hypotension despite adequate fluid resuscitation

Sepsis-2

The definition of sepsis was reevaluated in 2001 [45] without major changes to the Sepsis-1 definition except the suggested clinical signs and laboratory tests indicative of organ dysfunction and impaired tissue perfusion and the addition of general and inflammatory parameters, as shown in Table 2.

Table 2. Criteria for sepsis, based on the 2001 Society of Critical Care Medicine (SCCM)/American College of Chest Physicians (ACCP)/American Thoracic Society (ATS)/European Society of Intensive Care Medicine (ESCIM)/Surgical Infection Society (SIS) Consensus Conference.

PARAMETERS	CLINICAL SIGNS AND LABORATORY TESTS
General parameters	Fever, hypothermia, tachycardia, tachypnea, altered mental status, arterial hypotension, decreased urine output, significant peripheral edema, or positive fluid balance
Inflammatory parameters	Leukocytosis, leukopenia, hyperglycemia, increased C-reactive protein, procalcitonin, or creatinine, coagulation abnormalities, increased cardiac output, reduced mixed venous oxygen saturation
Hemodynamic parameters	Hypotension, elevated mixed venous oxygen saturation, elevated cardiac index
Organ dysfunction parameters	Arterial hypoxemia, acute oliguria, increase in creatinine level, elevated international normalized ratio or activated partial thromboplastin time, ileus, thrombocytopenia, hyperbilirubinemia
Tissue perfusion parameters	Hyperlactatemia, decreased capillary refill, or mottling

Sepsis-3

The Sepsis-1 and 2 definitions were found to be fairly unspecific. Signs of systemic inflammation were often present in other severe illnesses, such as burns, pancreatitis and trauma, that could be falsely diagnosed as sepsis [46]. Moreover, in 2014 Kaukonen et al. presented that the SIRS criteria excludes one in eight patients with severe infections treated in the ICU [15].

Misclassification of the level of severity, based on the Sepsis-2 definition was also found to be a problem leading to miscoding of the diagnosis and biased reported mortality [47]. Whittaker et al. found substantial underreporting of severe sepsis, leading to falsely high mortality data in patients with sepsis [48]. In 2016, new consensus criteria for sepsis (Sepsis-3) were established based on advances in sepsis research and a new understanding of the pathophysiology involved. Sepsis is today defined as a "dysregulated host response to infection, leading to life-threatening organ dysfunction". In order to limit the diagnosis to patients with life-threatening disease, definitions were selected to define patients with hospital mortality of at least 10%[1]. Large databases were used to find suitable cutoff levels in

scoring systems for organ dysfunction. According to the Sepsis-3 definition, sepsis is therefore defined as an increase in the Sequential (sepsis-related) organ failure assessment score (SOFA), of ≥2 from baseline (Table 3). Patients with profound circulatory, cellular, and metabolic abnormalities are defined as having septic shock. This subset of patients is clinically identified based on requirement of vasoactive treatment to maintain mean arterial pressure ≥65 mmHg, and with lactate levels >2 mmol/L. In comparison to previous definitions, markers indicating inflammation, such as leukocyte count alterations or temperature, were removed. Also, the term "severe sepsis" was removed. The definition of septic shock was modified to only include patients treated with vasopressors. Collectively, patients diagnosed with sepsis according to this definition should be more severely ill in comparison to patients diagnosed with sepsis according to the previous definitions. As with previous definitions, infection should be suspected when diagnosing sepsis, but microbiological confirmation of the pathogen responsible for the infection is not required.

Table 3. Sequential (sepsis-related) organ failure assessment score, adapted from Singer et.al [1].

Organ system	SOFA Score				
	0	1	2	3	4
Respiration: PaO2/FIO2, kPA	≥53.3	<53.3	<40	<26.7 with respiratory support	<13.3 with respiratory support
Coagulation: thrombocytes x10^3	≥150	<150	<100	<50	<20
Liver: bilirubin, µmol/L	<20	20-32	33-101	102-204	204
Cardiovascular: mean arterial pressure, mm Hg	≥70	<70	Dobutamine (any dose) or <5	Epinephrine ≤0.1 or norepinephrine ≤0.1 or Dopamine 5.1-15	Epinephrine ≤0.1 or norepinephrine ≤0.1 or Dopamine >15
Central nervous system: Glasgow coma scale	15	13-14	10-12	6-9	<6
Renal: Creatinine	<110	110-170	171-299	300-440	>440

Catecholamine doses are given as µg/kg/min for at least 1 hour

Etiology and site of infection

The type of pathogen and site of infection are important determinants of sepsis related outcome [6, 14]. However, bacterial etiology is not always documented in patients with sepsis. Based on a prospective study from 1995, the frequency of blood culture positive sepsis varied with the disease severity, and was 17%, 25% and 69% respectively for sepsis, severe sepsis, and septic shock [49].

Contemporary data have also demonstrated that presence of bacteremia, independent of infectious site, is associated with high mortality (34%) in patients with severe sepsis [6]. In a meta-analysis of 510 published sepsis studies, *Acinetobacter* and *Candida* species were associated with the highest mortality rate [14]. Respiratory tract infections are the most prevalent site of infection [50] and are often associated with the highest mortality in sepsis [14, 51]. Among Gram-positive pulmonary infections *Staphylococcus aureus* etiology demonstrated higher mortality than *Streptococcus pneumoniae* etiology. In patients with severe sepsis, endocarditis is often associated with a high mortality despite its low prevalence. By contrast, genitourinary infections are associated with low attributable mortality despite their high frequency as a primary infection in sepsis [6, 14].

The reported mortality differences related to pathogen and source of infection in sepsis are also related to the clinical situation in which they occur. For instance, *Candida* and *Acinetobacter* species often occur as secondary infections, which are associated with worse outcomes than primary infections [52].

Considering the etiology and site-related differences in sepsis outcome, it is noteworthy that immunology studies in sepsis rarely account for these. In a recent meta-analysis of 57 immunology studies in intensive care medicine, only one specified the infection site and/or a specific pathogen [53].

Bloodstream infection

Blood culture-positive infections are today often referred to as bacteremic infections or bloodstream infections (BSIs). Bloodstream infections are found to be a major healthcare problem associated with burden of illness comparable to major trauma, acute stroke, and myocardial infarction [54-56]. Population based epidemiological studies have demonstrated that the most prevalent pathogens of community-onset BSI are: i) *Escherichia coli* ii) *S. aureus;* and iii) *S. pnemoniae* [57]. Contemporary data of the BSI incidence in Europe have demonstrated an increasing trend [58, 59], which might be related to changes in longevity with higher grade of comorbidities in the population. In Sweden, both incidence and mortality rates of BSI have demonstrated a gradual increase over time (2000-2013) [60].

Bloodstream infections caused by *S. aureus* etiology (*S. aureus* bacteremia, (SAB)) are associated with a particularly high mortality, estimated at 20–30% [55, 61]. However, the prognosis of SAB differs according to disease manifestation. It is therefore suggested to categorize SAB as either "complicated" or "uncomplicated" [62, 63]. Complicated SAB is often defined as a site of infection remote from the primary focus, caused by hematogenous seeding (e.g., endocarditis or osteomyelitis), or extension of infection beyond the primary focus (e.g., septic thrombophlebitis, abscess), or recurring infection [64].

Key immune responses in bacterial infection

Early immune responses to invasive pathogens include initiation of an inflammatory response in order to eliminate the pathogens and repair damaged tissue. Identification, capture, degradation, and presentation of microbial antigens are important key innate immunological processes in an efficient host response.

Identification of microbes and tissue damage

One of the key processes during the early response to bacterial invasion is identification of microbes. Pathogens express several evolutionarily conserved signature molecules known as "pathogen-associated molecular patterns (PAMPs)" [65]. Peptidoglycan and lipoteichoic acid are examples of common PAMPs found in the bacterial cell walls of Gram-positive bacteria. Lipopolysaccharide (LPS) is a well-studied PAMP found in the cell wall of Gram-negative bacteria [66]. When PAMPs are sensed by pathogen recognition receptors (PRRs) such as Toll like receptors (TLRs), C-type lectin receptors or Nod-like receptors, they induce an intracellular signaling cascade resulting in an array of anti-microbial immune responses [67]. Induction of gene transcription will generate secretion of various inflammatory cytokines, chemokines and type I interferons [68].

Additionally, endogenous molecules such as human deoxyribonucleic acid (DNA), adenosine triphosphate (ATP), heat shock proteins (HSPs) and high mobility group box (HMGB)-1, that are released following cell stress or cell damage, may also activate PRRs and the intracellular pathways leading to cytokine secretion [69]. These molecules are often referred to as "damage-associated molecular patterns (DAMPs) [67, 69]. Endogenous cellular damage, with release of DAMPS may be directly induced by toxic substances from activated immune cells or by bacterial toxins. For example, invasive *S. pneumoniae* produce the potent pore-forming cytotoxin pneumolysin and copious amounts of hydrogen peroxide, both of which kill host cells [70]. Intracellular activation of protein complexes called inflammasomes will further amplify the inflammatory response by inducing secretion of the very potent cytokines IL-1β and IL-18, leading to recruitment of more leukocytes to the site of infection [71]. Activated inflammasomes will also induce inflammatory cell death (pyroptosis) with further release of DAMPs, such as HMGB-1 [19, 72].

In brief, PAMPs and DAMPs that are sensed by PRRs and inflammasomes will activate intracellular signaling pathways leading to secretion

of cytokines and activation of immune cells [73]. During this acute phase response, pro-inflammatory cytokines will affect multiple cells in different organs, leading to the typical signs of infection, such as fever and malaise, and to production of acute phase proteins by the liver [71]. C-reactive protein (CRP), a commonly used marker of infection is an example of an acute phase protein induced by the pro-inflammatory cytokine IL-6.

Capture and killing

Another important step in the early response to microbes involves their capture and ingestion. To enable ingestion of bacteria, the first step involves binding to PRRs [66]. The cytoplasm of the immune cell then surrounds the pathogen and engulfs it within membrane-bound vesicle (phagosome) in the cytoplasm. This process is called "phagocytosis" [74]. The phagosome then fuses with a lysosome containing proteolytic enzymes. This leads to intracellular destruction of the microbe. Neutrophils and monocytes are important phagocytes circulating in the blood. Dendritic cells and macrophages are important tissue-residing phagocytosing cells [66].

Macrophages with phagocytosed microbes secrete IL-12, which in turn activates natural killer (NK) cells to kill host cells infected by intracellular pathogens.

In order to eliminate extracellular microbes, neutrophils, NK cells and cytotoxic T cells may release cytotoxic substances. Moreover, neutrophil cells may also inhibit bacterial proliferation by extruding their DNA with formation of so-called "neutrophil extracellular traps (NETs)" that trap bacteria and activate local coagulation mechanisms [75]. The release of toxic enzymes in neutrophils is strongly triggered by activated complement C5a, which is highly abundant in sepsis.

Antigen presentation by human leukocyte antigen class II

In a process called "antigen presentation," antigen peptides from phagocytosed pathogens are presented to CD4 Th cells by the human leucocyte antigen (HLA) class II molecule [76]. The HLA system is synonymous with the major histocompatibility complex (MHC) system [74]. The HLA class II molecules are present in monocytes, macrophages, dendritic cells and B-cells.

Antigen-dependent T cell activation requires three signals. Signal 1 is the antigen presented by a HLA class II molecule. Signal 2 is a co-stimulatory signal with interaction between (CD80/86) on the antigen-presenting cell (APC) and CD28 on the T cell (Figure 2). The third required signal is cytokine stimulation of the T cells [66]. Patients with the rare primary immunodeficiency disease "Bare lymphocyte syndrome" lack expression of HLA-class II [77]. These patients suffer from severe susceptibility to bacterial and viral infections [78].

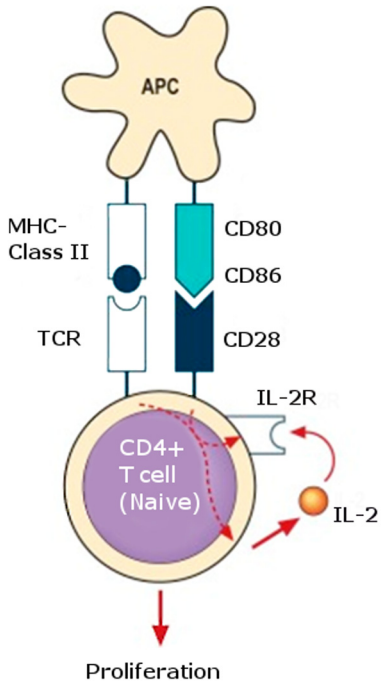


Figure 2. The major histocompatibility complex (MHC) class II heterodimer is a glycosylated cell-surface transmembrane protein expressed on antigen-presenting cells (APCs). A three-signal mechanism is required for CD4 T cell activation. An APC takes up a protein antigen and processes it into peptide fragments that are presented by class II MHC molecules. The first signal required for CD4 T cell activation is recognition by the T cell antigen receptor (TCR) of the class II MHC–peptide complex. The second, costimulatory signal is an interaction between CD28 on the T cell and CD80 or CD86 on the APC. These signals stimulate cytokine production and induce CD4 T cell proliferation.

Lymphocyte activation and differentiation

The activation of adaptive immunity is important for development of specific immune responses by inducing antigenic memory and amplifying the effect of innate immune cells [66]. In the host response to microorganisms, CD4 T helper (Th) cells are activated by MHC class II restricted antigen presentation and CD8 cytotoxic cells are activated by MHC class I restricted antigen presentation. The fate of naïve Th cell differentiation depends on the type of cytokine secreted by the infected immune cell, previously described as signal 3. Different pathogen types elicit different cytokine profiles. This leads to development of effector cells with distinct functions in response to certain types of pathogens [66, 79]. Some might recruit more neutrophils to the site of infection (e.g., Th17 cells stimulated by IL-17 and IL-22) or activate macrophages to kill ingested microbes (e.g., Th1 cells stimulated by IL-12 and interferon γ (IFN-γ), whereas others (e.g., Th2 cells stimulated by IL-4) promote mast cell and eosinophil activation. T helper-1 cells typically develop in response to pathogens activating NK cells and dendritic cells, such as intracellular bacteria, while Th2 cells typically develop in response to parasitic activation of mast cells and eosinophil activation. T helper-17 cells typically develop in response to extracellular pathogens [66].

The Th2 cytokine signaling (IL-4, IL-13) may further skew the differentiation of macrophages from classical "microbicidal" M1 macrophages into "tissue repair" M2 macrophages [80].

Accordingly, the typical Th1, Th2, or Th17 cytokine responses may be indicative of the type of infection, but many of these pathogen-related cytokine responses may also be overlapping [81]. The magnitude of the cytokine response is also related to the degree of endogenous tissue damage, or to the immune evasion strategies of the pathogen [67]. Furthermore, host factors such as genetic variations may also influence the nature of host responses [82].

Sepsis-induced immunosuppression

A suppressed immune response during the course of sepsis is today recognized as an important pathogenic mechanism contributing to the high late death rate and burden of this disease [21]. During the past 2 decades, several observational studies have found the paradoxical evidence of immunosuppression in sepsis patients who had previously been believed to succumb due to an overactive pro-inflammatory immune response [13, 83-86].

Evidence for immunosuppression in sepsis

Important observations that underlie this theory of acquired immunosuppression are based on the occurrence of deleterious secondary infections in previously immunocompetent patients treated for sepsis [41, 52, 87-89]. Also, the pathogen types responsible for secondary infections are indicative of a defective immune system. As an example, reactivation of latent viruses such as cytomegalovirus (CMV) [90, 91] and infections caused by bacteria common among immunocompromised individuals, such as *Stenotrophomonas species* or *Burkholderia cepacia* [21], are found to occur as secondary infections in sepsis [88]. Illustratively, over 40% of septic patients were shown to have reactivation of latent herpes viruses in a recent study [91]. Moreover, secondary infections are associated with unfavorable outcome [21].

There are three well-performed autopsy studies supporting the theory of sepsis-induced immunosuppression [83, 92, 93]. One of these [93] demonstrated a high proportion of unresolved infectious foci in patients who had died following sepsis. Remarkably, more than 80% of the deceased patients had signs of continuous infections even after being adequately treated for 7 days in the ICU. Another study showed extensive tissue lymphocyte apoptosis in critically ill patients who died from septic causes [92]. The last example is a well-sited study by Boomer and colleagues that demonstrated signs of profound immunosuppression in immune effector cells and tissue biopsies from multiple organs in patients who died following sepsis [83]. Importantly, a control group of patients who died of non-septic causes did not express signs of immunosuppression. In that study, sepsis patients also had functional signs of immunosuppression, demonstrated by significant reduction of cytokine secretion in response to LPS stimulation. Moreover, their HLA-D-related (HLA-DR) expression was diminished and lymphocytes were depleted from spleen tissue [83]. The

inability to mount efficient host responses upon secondary stimuli, such as LPS stimulation of monocytes, is called endotoxin tolerance and is described as a hallmark of severe immunosuppression or immunoparalysis [94]. Several studies have demonstrated an association between immunoparalysis and detrimental sepsis outcome [95-97].

Cells affected during sepsis-induced immunosuppression

An array of different alterations of the immune system has been identified during sepsis-induced immunosuppression. The hallmarks of immunosuppression following sepsis are: (i) endotoxin tolerance; (ii) reduction in antigen presentation and lymphocyte activation; and (iii) apoptosis of immune effector cells with a remaining Th2 dominance [13, 80, 98]. In this section, alterations in some of the important cells during immunosuppression will be described. Figure 3 summarizes the effects on innate and adaptive immune cells during sepsis-induced immunosupression.

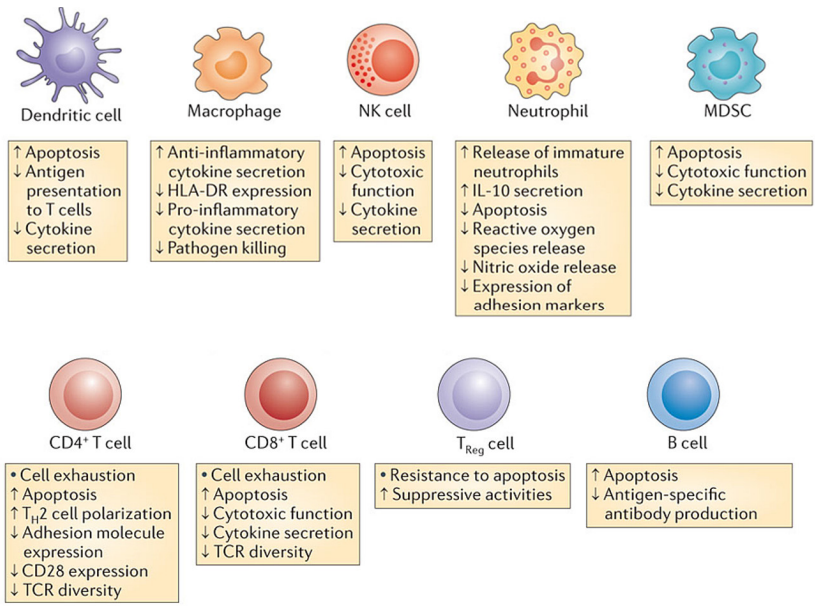


Figure 3. Alterations of innate immune cells (dendritic cells, macrophages, natural killer (NK) cells, neutrophils, myeloid-derived supressor cells (MDSCs)) and adaptive immune cells (CD4-T cells, CD8-T cells, T-regulatory cells (T-regs), B-cells) during sepsis-induced immunosuppression. Modified and adapted from Hotchkiss et al. [99]

Monocytes and macrophages

Monocytes have a crucial role in the immune response during sepsis, even if they only contribute to a maximum of 10 % of all blood leukocytes. Similar to neutrophils, they are important cells to combat invading pathogens by phagocytosis [66]. However, in contrast to neutrophils they circulate in the blood for several days before they pass into tissues and mature into macrophages or dendritic cells [80]. Additionally, monocytes also have a prolonged survival during sepsis [100]. Macrophages comprise subpopulations of cells (M1 and M2) that promote either pro- or anti-inflammatory activity [101, 102]. In sepsis-induced immunosuppression, macrophages are skewed towards a dominating M2 phenotype.

The direct antimicrobial killing capacity by monocytes does not seem to be altered during sepsis. More importantly, they possess a key regulating function by the ability to orchestrate both innate and adaptive immunity to be less effective in response to secondary stimuli [80, 103]. This regulatory function is mediated by reduced antigen-presenting capacity and altered cytokine signaling [86]. Monocytes have demonstrated a reduced capacity to secrete the pro-inflammatory cytokines TNF, IL-1, IL-6, and IL-12 after challenge by TLR2 or TLR4 agonists in septic patients [104]. The mechanisms behind monocyte tolerance are not fully understood, but analysis of monocyte mRNA-patterns suggests that epigenetic programming seem to play a pivotal role for the development of this anergy [105]. Many investigators agree that down regulation of HLA-DR on monocytes acts as a surrogate marker of this anergy [106-108].

Dendritic cells

Dendritic cells (DCs) are important as APCs. They also act as key regulators of the immune system by ability to orchestrate the immune response to be either stimulatory or inhibitory [79]. In response to pro-inflammatory signals they secrete stimulatory cytokines (IL-12, IFN-γ, TNF-α, IFN- α and IL-6) to optimize bacterial clearance. They are also important in viral infections trough activation of cytotoxic T cells and NK cells [66]. In a situation of anti-inflammatory signals (TGF-β, IL-10) in the surroundings of DCs, they act regulatory and will produce more signals to induce immune cell anergy. This also inhibits proliferation of conventional T cells. Instead generation of T-regulatory cells (T-regs) will take place [99]. Similar to monocytes, DCs also down regulate HLA-DR during sepsis-induced immunosuppression [109].

T cells

Lymphocytes are important cells in the adaptive immunity. In sepsis, the T cell population is shown to be deeply altered [110-113]. Several studies in septic patients have showed an association between the intensity and duration of lymphocyte alterations and risk of death or secondary infections in sepsis [113, 114].

Different alterations of the T cell population have been linked to sepsis-induced immunosuppression. First, they have been shown to be numerically reduced due to massive apoptosis (programmed cell death) [83, 111, 113]. Second, the remaining T cells are found to be exhausted upon secondary stimulation by LPS or recall antigens such as tetanus toxins [85]. Third, the T cell diversity is skewed towards a Th2 response [115] with increased proportion of regulatory T cells [103], which have been shown to suppress other effector T cell subsets [109].

A recent experimental study demonstrated that direct in-vitro LPS stimulation of isolated T cells did not induce T cell exhaustion. Instead, T cell exhaustion was shown to be dependent on the presence of monocyte cells with reduced HLA-DR expression [116]. This monocyte-dependent negative regulation of lymphocytes could be restored by stimulation with IFN-γ [116]. T cell exhaustion has also been described to be induced by stimulation with the typical Gram-positive TLR ligand peptidoglycan [117].

Exhausted T cells in sepsis may express a phenotype characterized by lower levels of CD3+ cells and co-stimulatory molecules, together with up-regulation of co-inhibitory molecules, such as programmed cell death receptor-1 (PD-1) [110, 118], or cytotoxic T lymphocyte associated protein 4 (CTLA-4) [119-121]. These characteristics have similarities with the alterations described in chronic viral infections and cancer [122]. Specific therapies targeting these alterations has been successful in cancer patients and are therefore suggested as promising treatments of immunosuppression in sepsis [13].

Neutrophils

Neutrophils are the most prevalent cell type of the innate immunity [66]. They are rapidly released into the circulation in response to cytokine signaling in the acute phase reaction following pathogen invasion. Once released into the circulation they normally undergo apoptosis within hours [123]. However, during sepsis the normal function leading to apoptosis in neutrophils has been shown to be inhibited and apoptosis delayed [124], leading to ongoing neutrophil dysfunction [125]. Up-regulation of pro-

grammed death ligand (PD-L) on septic neutrophils will instead induce apoptosis of other important immune cells, such as CD4 T cells [126].

Neutrophil dysfunction in sepsis may involve decreased recruitment to sites of infection (chemotaxis) and defects in oxidative burst [80, 127]. This is further compounded by release of immature neutrophils, with reduced ability to activate the complement-system. Decreased neutrophil functions in sepsis have been associated with an increased risk of nosocomial infections [128, 129] and death [84, 127].

Myeloid derived suppressor cells

Myeloid derived suppressor cells (MDSC) are also identified as important players during sepsis-induced immunosuppression. The cells are normally not present in peripheral blood from healthy patients but are shown to be present during cancer and in sepsis. Recent studies have demonstrated that these cells are functionally immunosuppressive and therefore suggested as possible inducers of sustained immunosuppression [130, 131]. A sustained high proportion of these cells during the course of sepsis are also associated with the prolonged stay in the ICUs and prevalence of nosocomial infections [130]. Of note, MDSCs in sepsis patients have demonstrated suppressed HLA-DR expression at the gene level, shown by diminished HLA-DRA messenger ribonucleic acid (mRNA) [130].

B cells

Reduced B cell counts in humans correlate with the incidence of nosocomial infections, but data regarding sepsis-induced alterations are conflicting [80]. Some researches argue that they are not generally reduced, while others have found significant reductions in absolute numbers during septic shock [132]. However, a recent study demonstrated that patients with sepsis had dysfunctional B cells in terms of impaired IgM production upon CpG (TLR9 ligand) stimulation [133]. Additionally, animal data suggest that B-cells have functions in sepsis, beyond antibody production. In particular, B-cells were shown to improve cytokine production and contribute to reduction in bacterial load (198).

Natural killer cells

NK cells are important for the clearance of infection. First, they inhibit bacterial growth by their direct capacity to kill infected cells [109, 134]. Second, they are a major endogenous source of the immunostimulatory cytokines IFN-**γ**, and granulocyte-macrophage colony stimulating factor

(GM-CSF) [135]. Similar to monocytes, they may also develop endotoxin tolerance with reduced IFN-γ production during sepsis [109]. In critically ill patients, NK cell exhaustion is shown to precede reactivation of CMV infections [136].

Mechanisms underlying immunosuppression in sepsis

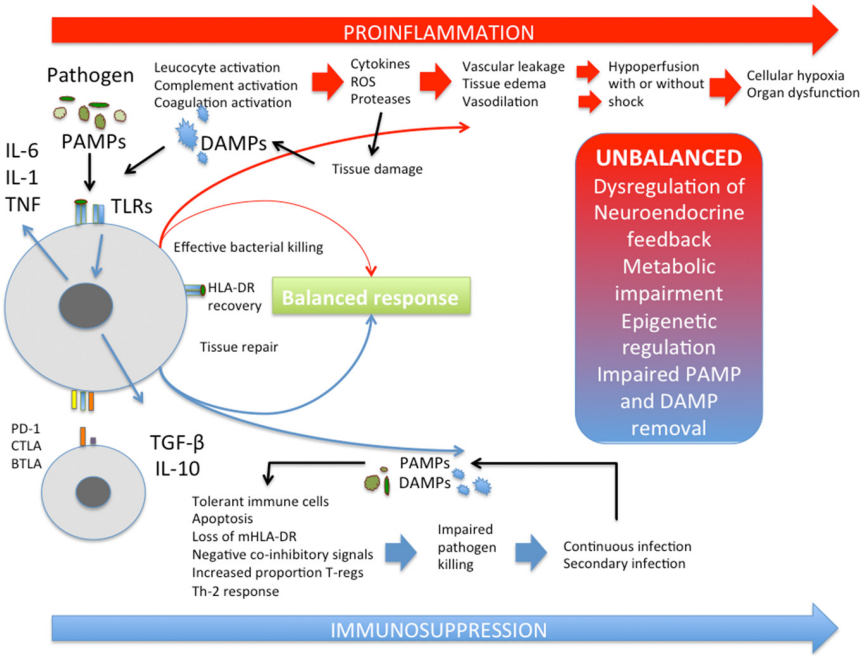


Figure 4. Schematic presentation of balanced and unbalanced host responses in sepsis.

Overshooting pro-inflammatory reaction following the initial steps of pathogen recognition leads to collateral host damage and organ dysfunction. Patients with balanced responses have simultaneous pro- and anti-inflammation with effective elimination of pathogens, and tissue repair. During sepsis-induced immunosuppression, immune cells have sustained anti-inflammatory responses with downregulated antigen presentation (reduced human leukocyte antigen-D related (HLA-DR)), and reduced cytokine production upon secondary Toll-like receptor (TLR) stimulation (endotoxin tolerance). Several important immune cells undergo changes with upregulation of negative co-stimulatory molecules such as PD-1, CTLA-4, B and T lymphocyte attenuator (BTLA) and also undergo apoptosis with a remaining Th2 regulatory T cell phenotype. These alterations leave the host vulnerable to secondary infection. Possible mechanisms

leading to unbalanced response include impaired neuroendocrine feedback regulation of immune cells, continuous DAMP and PAMP exposure, epigenetic regulation, and metabolic and autophagy disturbance.

In this section, some of the possible mechanisms leading to unbalanced immunosuppressed response, will be described.

Compensatory anti-inflammatory response syndrome

The mechanisms leading to harmful immunosuppression during sepsis are not fully understood although several studies have identified important regulatory cells and cytokines [41]. A syndrome called "compensatory inflammatory response syndrome (CARS)," which involves high levels of anti-inflammatory cytokine secretion (IL-10, TGF-β), was thought to follow the initial SIRS phase, as an important mechanism to shut off the exaggerated pro-inflammation [86, 137, 138]. However, this SIRS-CARS theory as a biphasic event has been questioned after it was found that pro- and antiinflammatory responses can occur simultaneously during trauma and sepsis [139-141].

Neurological and immunological feedback systems

Even if the compensatory immune response is not biphasic, the regulatory mechanisms identified in this context have been proposed to be relevant for development of immunosuppression [142]. In particular, neurological and immunological feedback systems have been identified as possible regulators in the progression to immunosuppression [67]. The regulatory links between these systems comprise two components: an afferent (sensory) and an efferent (regulatory) arm. It has been demonstrated in several studies that this system can modulate the magnitude of TNF-response to LPS, which is one of the hallmarks of sepsis-induced immunosuppression [44]. Experimental studies have shown that activation of the efferent vagus nerve can induce a switch from pro-inflammatory to an anti-inflammatory immune response, which is mediated by immune effector cells in the spleen [44]. The vagus nerve activation operates via cholinergic anti-inflammatory signaling by acetylcholine receptors (7nAChR) expressed on non-neuronal cytokine-producing cells. According to this inflammatory reflex mechanism, the presence of PAMPs and DAMPs also seems to play an important role, because they act as triggers of the afferent arm in the feedback loop [44].

The adrenergic system has been shown to be another modulator of the immune system. It is suggested to modulate cell death, mitochondrial func-

tion and inflammatory signaling. Both hematopoietic and lymphopoietic tissues are innervated by sympathetic neurons. The majority of lymphoid cells also express beta-adrenergic receptors on their cell surface [109]. In patients with trauma or heart failure, beta blockers are shown to modulate immune responses, but this has not yet been demonstrated in sepsis patients [109]. However, use of esmolol, a short-acting beta blocker, has been associated with a better outcome in septic shock [143].

Sustained PAMP and DAMP exposure

Prolonged sepsis trajectories are shown to be associated with risk of sepsis-induced immunosuppression. Recently, the term "persistent inflammation-immunosuppression catabolism syndrome (PICS)" was coined to identify patients at risk of contracting sepsis-induced immunosuppression [144, 145]. According to the PICS theory, prolonged inflammation, possibly induced by DAMPs and PAMPs from continuous infectious foci, precedes immunosuppression. Persistent inflammation-immunosuppression catabolism syndrome is suggested to be identified by measurement of sustained CRP elevation and neutrophils, along with lymphocytopenia and low albumin levels.

It has been shown that the dose of added PAMPs in in vitro experiments corresponds to the level of HLA-DR downregulation [146, 147]. Moreover, major surgery with higher levels of DAMPs generates more pronounced HLA-DR downregulation compared to less advanced surgery [148]. Interestingly, continuous exposure to live bacteria has shown remarkably different host responses compared to sustained exposure to killed bacterial components [70, 147], suggesting that pathogen-related factors of live bacteria may actively influence repression of host response pathways. Accordingly, pathogen-related factors may be important in this context. Bacterial pathogens express widely different virulence factors and may therefore give rise to different clinical trajectories. For example, some pathogens, such as invasive *S. pneumoniae* [70] and *Neisseria meningitidis*, are known to induce a high bacterial load but are fairly easy to eradicate with antibiotic treatment. On the other hand, other pathogens, such as *S. aureus [149]*, *Mycobacterium tuberculosis,* or *Burkholderia pseudomallei [150]*, are known to cause chronic and recurrent infections. Interestingly, *S. aureus*, a common pathogen of BSIs, is known to be difficult to eradicate even when bacterial strains are susceptible to standard antibiotic therapy [149]. This said, pathogens with propensity to cause chronic infec-

tions could impact on the risk for progression into immunosuppression, but this has not been well studied.

The secondary infection itself might additionally contribute to sustained immunosuppression. This could be mediated either by additional PAMP and DAMP presence or by specific mechanisms attributable to the pathogen. For instance, CMV and Epstein-Barr virus (EBV) infections, which have been shown to reactivate in ICU-treated patients, are known to downregulate HLA-DR specifically via production of viral IL-10 [151, 152]. The presence of common entry routes for infection, such as intravenous catheters and mechanical ventilation, is another additional risk factor for second hits and, consequently, also development of immunosuppression.

Epigenetic regulation

Another explanatory theory of immunosuppression in sepsis includes epigenetic regulation of genes encoding pro and anti-inflammatory factors [105]. Epigenetics is a general term involving mechanisms that control gene expression patterns without modifying the underlying DNA sequence of an organism [105]. This results in changed accessibility of the DNA to transcription factors. Additionally, post-transcriptional regulation of mRNA can be achieved by complementary gene interference driven by micro-RNAs (miRNAs), resulting in the downregulation of protein expression through targeted degradation of specific mRNAs. Upregulation and downregulation of specific miRNAs have been demonstrated in both the early and the late phases of sepsis [153]. They play a central role in sepsis induced immunosuppression [101, 154]. For example, they can disrupt the synthesis of pro-inflammatory cytokines in innate immunotolerance [155-157]. Specifically, microRNA-146a has been shown to play a key role in endotoxin tolerance by downregulating IL-1 receptor-associated kinase 1. When specific miRNAs have been blocked, they showed decreased production of suppressive myeloid cells and increased bacterial clearance [158]. Epigenetic regulation has also been identified as a key mechanism in suppression of adaptive immunity by promoting Th2 skewing [153]. Moreover, epigenetic regulation by chromatin modifications has been proposed as a mechanism explaining downregulation of class II transactivator (CIITA), the master regulator of HLA-DR gene expression, in monocytes of septic patients [159].

Davenport et al. investigated the genomic landscape of the individual host response during sepsis and found two distinct transcriptional signa-

tures early during sepsis [160]. The response signature with features of immunosuppression (down-regulation of genes involved in HLA-DR expression, T-cell activation and endotoxin tolerance) were associated with higher mortality.

Autophagy

The discovery of autophagy was rewarded with the Nobel Prize in Physiology or Medicine in 2016. Autophagy provides a way to eliminate DAMPs and PAMPs in vesicles targeted for lysosomal degradation [161] and reduced inflammasome activation. It is therefore an important mechanism for the resolution of infection. Disturbances in T-cell autophagy have been suggested as a possible mechanism of sepsis induced immunosuppression [162, 163].

Alterations in immune cell metabolism

The diverse and integral functions of the immune system require precise control of cellular, metabolic and bioenergetics pathways. During sepsis-induced immunosuppression, metabolic pathways are altered with consequences of a failure to increase aerobic glycolysis [164]. It has been suggested that the failure to increase glycolysis impacts immune cell phenotype and function. Gene expression analysis in patients admitted to intensive care demonstrated reduced expression of genes involved in gluconeogenesis and glycolysis at onset of immunosuppression and secondary infection [165]. Interestingly, immunostimulation by interferon-γ could restore both cytokine production and the ability to induce glycolytic responses [164].

Regulation of human leukocyte antigen-D related expression

Based on genetic mutations found in MHC-class II deficiency disease, CIITA has been reported to be an important protein for the transcriptional regulation of MHC class II genes [78, 166].

During sepsis, the regulation of HLA-DR expression has been shown to be predominantly under transcriptional control [167]. Surface mHLA-DR expression in sepsis appears to correlate with mRNA levels of several CIITA-controlled HLA-DR gene transcripts including HLA-DR alpha chain (HLA-DRA) and CD74 [167, 168]. Transcription of CIITA furthermore appears to be inhibited by several factors, such as anti-inflammatory cytokines (transforming growth factor beta (TGF-ß) and IL-10) and nitric

oxide [169]. Additionally, CMV and EBV infections have been reported to inhibit CIITA [170].

Interestingly, IFN-γ is shown to be a potent inducer of CIITA mRNA and HLA-DRA mRNA [171, 172] with ability to restore downregulated HLA-DR in blood from septic patients [168]. In addition to the CIITA-dependent regulation of HLA-DR, there might also be parallel mechanisms contributing to the loss of surface HLA-DR in bacterial infections. Perry et al. demonstrated that the mechanisms of HLA-DR regulation involves gene transcription, impaired posttranslational processing, and shedding from cell surface [173]. Interestingly, GM-CSF stimulation restored HLA-DR expression at all levels [173]. Additionally, in vitro studies have reported intracellular sequestration of mHLA-DR in response to stimulation with *S. aureus* bacteria [146] and to IL-10 [174].

Each HLA-DR molecule consists of two transmembrane chains, alpha and beta, as shown in Figure 5. The alpha-chain, encoded by *HLA-DRA*, is essentially invariant, while the beta-chain carries the extreme polymorphism characteristic of these antigens. The alpha-1 and beta-1 domains together form a peptide-binding cleft presenting the antigen peptide to CD4 T cells. The alpha-2 domain is an important binding site for the CD4 T cell Co-receptor [169].

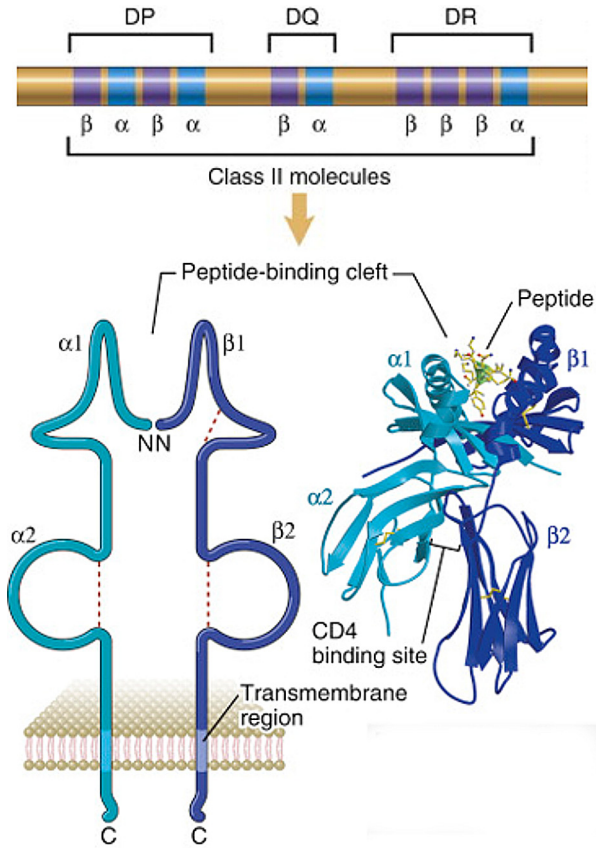


Figure 5. The human leukocyte antigen (HLA) complex, and structure of the HLA dimer. The location of genes in the HLA complex is shown. The class II region contains the genes encoding the HLA class II molecules (HLA-DP, HLA-DQ and HLA-DR). Also shown is the crystal structure of a class II molecule. (Crystal structure, courtesy of Dr. P. Bjorkman, California Institute of Technology, Pasadena, CA, USA.) Image modified from Kumar, Vinay "Diseases of the Immune System," Robbins Basic Pathology, Chapter 4, 99-159.e1

Human leukocyte antigen-D related expression in immunosuppression

The HLA-DR heterodimer acts as an important immunological synapse in the antigen dependent lymphocyte activation and its loss of expression on monocytes (mHLA-DR) has been associated with decreased responsiveness to LPS exposure in vitro [176]. Several investigators have found that loss of HLA-DR expression is predictive of adverse outcome in terms of mortality [177, 178] and secondary infections [179, 180]. Downregulation of HLA-DR is also demonstrated to be predictive of negative outcome and secondary infections in different clinical situations of severe illness, such as trauma [108, 181, 182], major surgery [183] [184], burns [185], pancreatitis [186] and brain lesions [187].

In healthy volunteers, mHLA-DR expression has shown high reproducibility between individuals, independent of age, sex, or diurnal variations [188].

Accordingly, mHLA-DR has been suggested as a suitable marker for immunoparalysis and to guide initiation of immunostimulating therapy [144]. However, there are some conflicting data regarding the predictive value of HLA-DR for unfavorable outcome. These discrepancies may be influenced by different analytical procedures when analyzing mHLA-DR values. In particular, differences in pre-analytical handling can widely influence results [189]. Due to this discrepancy, Docke et al. have developed recommendations for standardized measurements of mHLA-DR by flow cytometry (FCM) [189]. Although standardization has improved the reproducibility and enabled interlaboratory comparisons of results [190], these recommendations still have several drawbacks, limiting their clinical use. This in turn hampers possibilities to perform large scale studies. In particular, immediate handling with transport on ice and antibody staining within 4 hours is required, according to Docke et al. This limits study inclusion outside of laboratory opening hours and inclusion of patients from hospitals without flow cytometers. In polymerase chain reaction (PCR)-based measurements of HLA-DR, samples may be collected at any time point and kept frozen until later analysis.

There is still no consensus on cutoff levels to indicate clinically relevant immunosuppression. Some investigators have suggested that mHLA-DR levels between 5000 and 15 000 antibodies per cell (AB/c) [189] indicate immunosuppression, while others report cutoff values <2000 AB/c [106].

In a retrospective study including 413 critically ill patients in whom mHLA-DR was measured using the standardized FCM protocol every third day during their ICU stay, no clear cutoff values could discriminate non-survivors from survivors, due to overlapping values [191]. The lowest mean mHLA-DR value was 14 611 AB/c among non-survivors and 19 611 AB/c in survivors. The authors speculated that the etiology of the illness could have influenced the results since a significant association was found for the different diagnoses within the groups of survivors and non-survivors. However, this could not be further addressed due to limitations in patient numbers. It is noteworthy that no studies evaluating the prognostic value of mHLA-DR in sepsis have addressed the question whether bacterial etiology impacts the results. Considering the differences in virulence and variations in host immune responses attributable to different pathogens, this should be relevant.

Levels of mHLA-DR are variable during the course of infection, which further complicates general recommendations for interventions based on a single value. Several reports indicate that a negative slope or failure to restore mHLA-DR over time would be more predictive of negative outcome, compared to a single value [97, 108, 192, 193].

Immunotherapy

Interventions aiming to restore homeostasis in sepsis by boosting a suppressed immune response have received considerable attention in recent years. Promising preclinical data has been generated on therapeutic blockers of lymphocyte apoptosis and restoration of lymphocyte function [194-196]. Monoclonal antibodies targeting the co-inhibitory molecules PD-1, PD-1L and CTLA-4 on T cells have shown promising results in animal models of primary and secondary fungal sepsis [197]. Interestingly, PD-1 blockade also restores MHC-class II expression [197]. Stimulation with the pleiotropic cytokine IL-7 is suggested as a promising future immunostimulation therapy due to its potential to induce T cell survival and proliferation and its ability to help exhausted cells to recover [13]. Moreover, IFN-γ and GM-CSF have been described as potent inducers of HLA-DR with ability to restore immune responsiveness in endotoxin-tolerant cells [115, 144, 198]. Granulocyte macrophage colony-stimulating factor is one of the best studied immunostimulant in humans, but has not yet been proven in large scale studies [104, 198-201]. One sepsis randomized controlled trial (RCT) used mHLA-DR as a biomarker to initiate immunostimulation [104]. In that study, mHLA-DR <8000 AB/c was used on two occasions for detecting immunosuppression. The results demonstrated efficacy in terms of shorter hospital stay and also shorter duration of mechanical ventilation. Furthermore, mHLA-DR as well as monocyte tolerance was significantly restored in the GM-CSF treated patients in comparison to controls [104]. In another RCT, mHLA-DR <10 000AB/C was used to initiate GM-CSF treatment on the first day after surgery [202]. In that study, GM-CSF-treated patients had a shorter duration of infection compared to controls and the therapy was well tolerated.

Interferon-γ therapy has also been shown to be beneficial as adjunctive immunotherapy in patients with persistent *S. aureus* sepsis or invasive fungal infection [172, 203]. According to a published case series in leukemia patients, combined therapy with IFN-γ and G-CSF resulted in clinical response and was well tolerated when given as adjuvant therapy for months in patients with refractory invasive fungal infections [204]. Table 4 summarizes selected potential immunomodulating agents for treatment of sepsis.

Table 4. Potential immunomodulating agents in sepsis.

Agent	Function	Proven effects
GM-CSF	• Increases myelopoesis. • Activates monocytic or macrophage population to produce cytokines. • Increases HLA-DR on antigen-presenting cells. • Increases neutrophil phagocytosis and killing in combination with IFN-γ.	• Reversed immunoparalysis [104, 198]. • Decreased rate of infectious complications [199, 201] • Decreased mechanical ventilation time [104]. • Decreased the number of patient ICU days [104]. • Decreased the APACHE II score [104].
IFN-γ	• Increases monocyte expression of inflammatory cytokines. • Increases HLA-DR expression and antigen presentation. • Increases macrophage and neutrophil bactericidal activity.	• Reversed immunoparalysis [205]. • Trend towards improved survival [206].
IL-7	• Induces T cell survival and proliferation • Protects from apoptosis. • Rejuvenates T cell exhaustion. • Increases T cell activation and adhesion molecule expression. • Increases IL-17 dependent neutrophil recruitment.	• Reversed key immunological defects in animal models of sepsis [207] [208].
IL-15	• Improves the development, function and homeostasis of memory CD 8 T cells, NK cells, and intestinal epithelial cells. • Induces proliferation of memory and naïve CD 8 T cells and CD4 T cells. • Increases the production of pro-inflammatory cytokines when combined with IL-12. • Increases DC activation.	• Improved survival in animal models of sepsis [209].
PD-L1-antibody	• Releases checkpoint inhibition. • Prevents T cell exhaustion or T cells anergy. • Reduces T cell apoptosis. • Modulates myeloid cell interactions with the endothelium.	• Improved survival in animal models of sepsis [210].
CTLA4-antibody	• Suppresses T reg cell suppression. • Reduces T cell apoptosis. • Releases checkpoint inhibition. • Prevents T cell exhaustion or T cell anergy.	• CTLA-4 specific antibodies improved outcome to sepsis in rodent models [211].
Thymosin-α	• Increases CD 4 T cell and NK cell numbers. • Increases HLA-DR expression on APCs. • Enhances antiviral activity.	• Possible trend towards better survival [212].

Table modified from Hotchkiss et al.[13]

AIMS

The aims of this thesis were,

- To assess if the expression of HLA-DRA and CIITA mRNA, measured by qRT-PCR, is downregulated in patients with sepsis and to evaluate how HLA-DRA correlates with monocyte surface expression of HLA-DR (Paper I).

- To evaluate if the dynamic expression of HLA-DR in sepsis could be robustly measured by qRT- PCR as an alternative approach to flow cytometry based measurement (Paper II).

- To study how etiology of bloodstream infection and sepsis influences expression levels of mHLA-DR during the course of infection (Paper III).

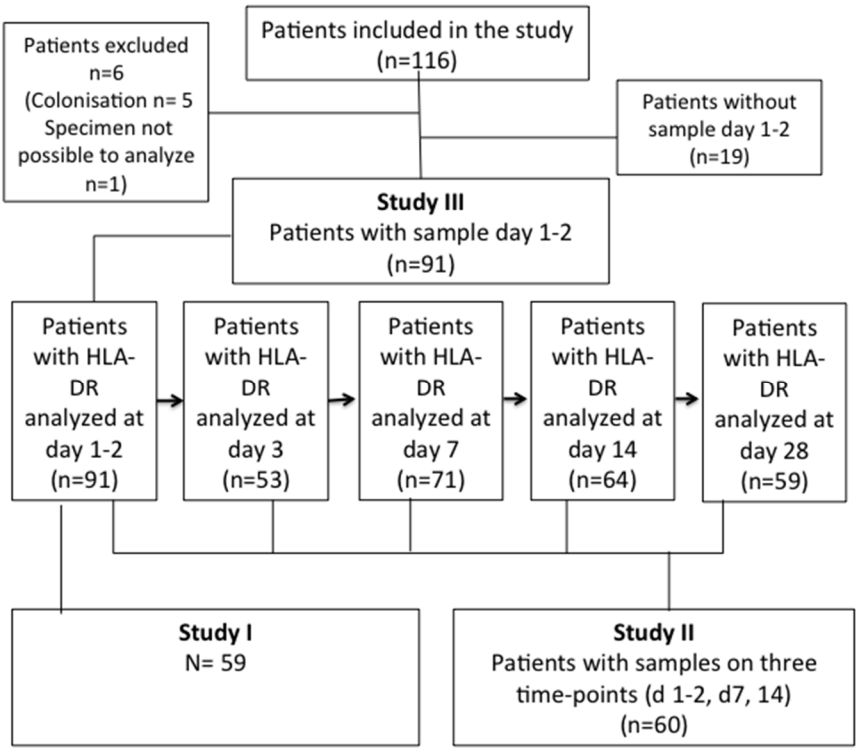
- To describe the expression of mHLA-DR in relation to CRP and white blood cell counts in patients with and without development of secondary bloodstream infection or death 3-60 days post-admission (Paper III).

- To evaluate if the HLA-DRA and CD74 mRNA expression is differently expressed in patients with complicated and uncomplicated *S. aureus* bacteremia (SAB) (Paper IV).

MATERIALS AND METHODS

Patients and sepsis definitions

The study entitled "Dynamics of sepsis" was a prospective study of patients hospitalized due to bloodstream infection during February 2011 and June 2014, at Örebro University Hospital, Sweden. This study had several aims including evaluation of techniques for bacterial detection and assessment of immunologic host responses during the course of bacteremic sepsis.



Flow chart of patients in the "Dynamics of sepsis" study and selection to study I, II and III.

Paper I.

The study population in paper I consisted of patients enrolled during the first 19 months of the "Dynamics of sepsis" (DOS) study. At the time when PCR-measurements where first evaluated, the DOS-study was ongoing. Consequently, we chose to include all samplings available from day 1-2 at that time (n=62). Patients without growth of pathogenic bacteria (n=2) were excluded and additionally one patient was removed due to wrong coding of the initial sampling time-point. All patients had sepsis according to Sepsis-2 definitions based on SIRS criteria. Severe sepsis was defined by evidence of hypoperfusion, organfailure or acute hypotension (systolic pressure ≤90 mmHg). We used the Swedish recommendations of clinical signs and laboratory tests with suggested cutoff levels indicating organ dysfunction and decreased tissue perfusion [213]. Septic shock was defined as persisting hypotension despite adequate fluid resuscitation in patients with severe sepsis.

Healthy blood donors > 40 years of age (n=30) were used as controls.

Paper II.

This study population was selected from all patients included in the "Dynamics of sepsis" study. Inclusion criteria were met if patients with bloodstream infection caused by pathogenic bacteria participated in samplings on all the following days; Day1-2, day 7±1 and day 14±2. All patients had sepsis according to Sepsis-2 definition. Severity was additionally assessed by acute change in SOFA score on admission in patients defined to have severe sepsis or septic shock (n=20) [214].

Blood donors from study 1 were used as controls for PCR-based measurements. Blood donors recruited for the "Dynamics of sepsis" study (n=31) and mHLA-DR values in controls from study I (n=30) were used as a control group (n=61) for mHLA-DR in non-septic patients.

Thirty-five sepsis patients who were included in study I were also included in study II. mHLA-DR results from day 1-2 in these patients were also used in this study cohort. Separate qRT-PCR measurements of these patients were performed in paper II.

Paper III.

Ninety-one patients were selected from the "Dynamics of sepsis study". Inclusion criteria were patients with pathogenic bacteremia and mHLA-DR values sampled on day 1-2. Sepsis was defined by the Sepsis-3 definitions.

Paper IV.

Twenty patients were included in a separate study aiming to assess dynamic changes of immune responses in *S. aureus* bacteremia. Patients were prospectively included from July 2012 to June 2014 at Örebro University Hospital, Sweden. Sampling was performed on five occasions during the course of bacteremia (day 1, day 2, day 3, day 5 and day 7). Patients with symptoms and signs of acute infection were enrolled on the day of blood culture positivity for *S. aureus* in ≥1 blood culture bottle (day 1). Sepsis was defined by the Sepsis-3 definitions. Since this study was performed in an overlapping time-period as the "Dynamics of sepsis" study, eleven patients were included in both studies. However, completely separated sample collections and blood analyses were performed in these studies.

Blood cultures

Venous blood, 15–20 ml, was collected in two sets of blood culture bottles, two aerobic and two anaerobic, from each patient in different blood draws and incubated using the BACTEC (Becton Dickinson, Franklin Lakes, NJ, USA) system. The bacteria were identified to the species level by routine diagnostic laboratory procedures. Identification of bacteria was confirmed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MicroflexLT, Biotyper 3.1; Bruker Daltonics, Bremen, Germany).

Flow cytometry

Blood was sampled in ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes that were immediately placed on ice and transported to the laboratory for flow cytometry analysis of HLA–DR expression on monocytes ($CD14^+$ cells), according to the protocol of Docke et al.[189]. The samples were prepared within 4 hours of collection. Antibody staining was performed using QuantiBRITE™ Anti-HLA–DR PE*/Anti-Monocyte PerCP-Cy5.5 (BD Biosciences, San Jose, CA, USA) and QuantiBRITE™ PE* (BD Biosciences), in accordance with the instructions of the manufacturer. An FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with an argon laser (488 nm) and HeNe laser (633 nm) and EXPO 32 software was used for flow cytometry analysis. Kaluza v. 1.2 software (Beckman Coulter) was used for data analysis, and results are expressed as number of antibodies bound per cell (AB/c).

RNA extraction, reverse transcription, and quantitative PCR

The blood cell mRNA expressions of *HLA-DRA*, *CIITA* (paper I and II) and *CD74* (paper IV) were studied by using quantitative real-time PCR.

The amount of each target gene mRNA was calculated in relation to the mRNA expression of a reference gene. Peptidylpropylisomerase B (*PPIB*) was chosen as a reference gene based on previously reported stability in inflammatory conditions (132), and due to similar efficiency in qPCR amplification as the targeted genes (*HLA-DRA, CIITA* and *CD 74*).

Whole blood samples were drawn, preserved in PAXgene tubes and stored frozen until analysis. The methods of isolation of total RNA, complementary DNA (cDNA) preparation from RNA and quantitative PCR (qPCR) have been described in Paper II and I. The basic steps of qRT-PCR can be described as below.

A specific amount of purified RNA is transcribed into cDNA. A defined small amount of this cDNA is thereafter examined by qPCR using gene specific primer-probe master mixes. These primers will ensure amplification of the sequences and the probes will specifically identify, as well as signal, the production of copies of target mRNA during the PCR-analysis. The amount of target cDNA and thus mRNA is inversely proportional to the time the qPCR takes to reach an exponential amplification of mRNA i.e. the cycle threshold (Ct) value. This is visualized by the increase in fluorescence intensity. The amount of target mRNA is therefore referred to as a time in the qPCR, and measured by numbers of cycles required to reach the Ct. The assays were run in triplicate reactions, on a qPCR-reaction plate, to get a controlled measurement that makes it easier to control for errors in the qPCR-process. In case of high variation in triplicates, the sample (Paper1) or the whole series of dynamic samples for a patient (Paper 2) were re-run on a new qPCR-reaction plate. Re-runs were made with stored cDNA if possible or new cDNA-preparation, so the samples to be compared had been treated equally prior to qPCR. A number of correctly analyzed samples were in that way run twice or more, and were used in calculating inter-assay variations for a sample. An average Ct-value for the triplicate samples was used in the calculations. A negative control (NegC) consisted of triplicate samples for the respective gene target-reaction without any cDNA.

The mRNA levels of *HLA-DRA, CIITA* and *CD74* were calculated as ratios in relation to PPIB by the ΔΔCt-method ($2^{-((CtTarget-CtNegC)-(CtPPIB-CtNegC))}$).

Statistics

Descriptive statistics were presented as medians with interquartile ranges (IQRs; 25th, 50th, and 75th percentiles). Normality was assessed with the Shapiro-Wilk test. For comparison between groups, an unpaired t-test or Mann-Whitney U test was used, depending on data distribution. Lognormal data were log transformed prior to statistic calculations. The Chi-2 test or Fisher's exact test was used for comparison of proportions. The non-parametric Spearman's rho test was used to assess correlations between two variables. A p-value of <0.05 was considered significant. All statistical analyses were performed with version 22 of the SPSS software package (IBM Corp., Armonk; NY, USA).

Linear mixed models for repeated measurements were used to evaluate the dynamic variation in HLA–DR at different time points. A heterogeneous, first-order autoregressive correlation structure was chosen due to best model fit, evaluated using Akaike information criteria (AIC). Time was modeled on a continuous scale to evaluate whether the slope of the geometric mean of mHLA–DR over time showed significant interaction with bacterial etiology, indicating different dynamics. The slopes of the mean mHLA–DR changes over time were estimated for different severity of sepsis (Paper II) and etiologies (Paper III) stratified for presence/absence of sepsis and preexisting immunosuppression (Paper III). With pairwise after tests, the geometric mean differences in mHLA–DR between subpopulations of different factors were assessed at each time point.

Ethics

The studies were conducted in accordance with the Declaration of Helsinki, and were approved by the Regional Ethical Review Board in Uppsala, Sweden (ref: 2012/018 and 2009/024 respectively). A written informed consent was obtained in all cases.

RESULTS AND DISCUSSION

mRNA expression of HLA-DRA and CIITA in sepsis (I, II)

Monitoring of mHLA-DR by flow cytometry (FCM) has been suggested to identify patients with sepsis-induced immunosuppression. However, this approach has disadvantages due to specific laboratory requirements. Messenger RNA-based HLA-DR monitoring by quantitative real-time PCR (qRT-PCR) technique would improve the clinical use and facilitate conduction of large multicenter studies. In Papers I and II, an mRNA-based HLA-DR monitoring method based on qRT-PCR was evaluated as an alternative method to traditional FCM.

In Paper I, HLA-DR expression, measured by qRT-PCR and FCM on days 1-2 after admission, was significantly lower in the total number of septic patients compared to controls. The mHLA-DR expression was found to correlate with the mRNA levels of HLA-DR, demonstrating the highest correlation to HLA-DRA (r=0.84, p<0.001), as shown in Figure 6.

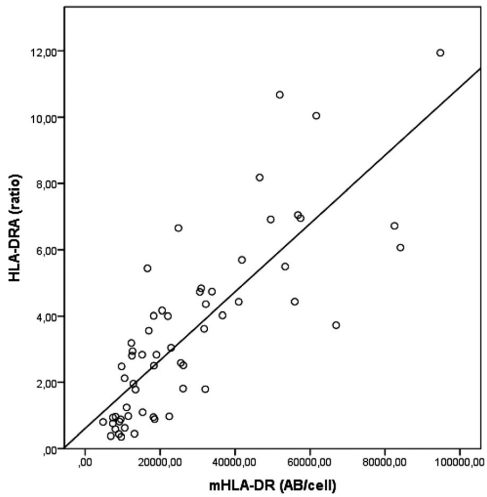


Figure 6. Correlation between human leukocyte antigen D-related (HLA-DR) alpha chain (HLA-DRA) messenger ribonucleic acid (mRNA), measured by quantitative real-time polymerase chain reaction (qRT-PCR), and monocyte surface HLA-DR, measured by flow cytometry (FCM), in 59 sepsis patients on days 1–2 post-admission (r=0.84, p<0.001).

A low expression level of mRNA encoding CIITA, the major transactivator of HLA-DR gene expression, supported previous findings of a transcriptional downregulation of HLA-DR genes in patients with sepsis [167]. Additionally, patients with severe sepsis or septic shock (n=11) expressed lower levels of both mHLA-DR and HLA-DRA mRNA compared to patients with non-severe sepsis ($p<0.05$), suggesting a possible association with severity.

Moreover, patients with non-severe sepsis caused by Gram-positive bacteria (n=21) had lower expression of HLA-DR expression compared to patients with Gram-negative bacteria (n=24).

Dynamics of HLA-DRA and CIITA in relation to mHLA-DR (II)

There are sparse data regarding the dynamic expression of HLA-DRA and CIITA mRNA in sepsis. One previous study in burn patients studied the dynamic expressions of these markers in a very limited set of patients (n=9). The authors concluded that these markers had similar expression over time as mHLA-DR [185].

In Paper II, HLA-DR was assessed dynamically during the course of non-severe and severe sepsis to evaluate if the recovery pattern over time was similarly expressed in the different markers (mHLA-DR, HLA-DRA mRNA, and CIITA mRNA). In patients with severe sepsis, all markers were initially depressed and recovered gradually over time in a similar pattern, as shown for mHLA-DR and HLA-DRA in Figure 7 (1A-1B). However, an important difference was observed between the monocyte surface expression and mRNA level expression of HLA-DR. The mean difference in HLA-DR expression between non-severe and severe sepsis was found to be greater when measuring the HLA-DRA mRNA and CIITA mRNA, than when measuring mHLA-DR, as shown in Table 5. Moreover, HLA-DRA, but not mHLA-DR, was significantly lower on days 1-2 in patients with higher severity according to the SOFA score increase on admission, as shown in Figure 7. The reason for these differences could be related to the fact that mRNA transcripts were measured in whole blood and not only on monocytes. As previously described, HLA-DR variations are known to be present in many important blood immune cells, including dendritic cells and myeloid-derived suppressor cells [130].

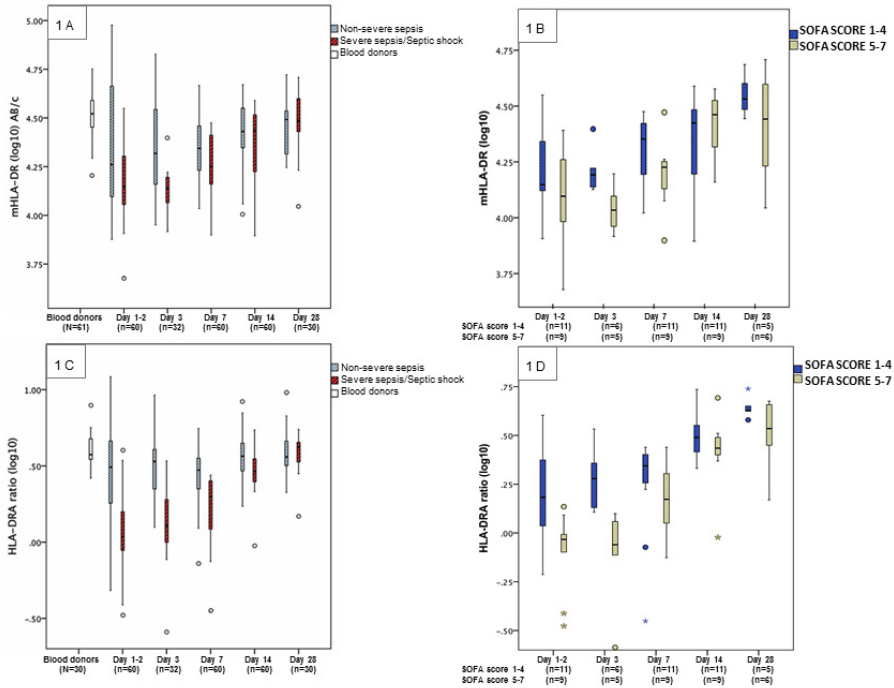


Figure 7. (1A) Monocyte human leukocyte antigen D-related (mHLA-DR), and (1C) HLA-DR alpha chain (HLA-DRA) messenger ribonucleic acid (mRNA) expression in the whole sepsis cohort (n=60), categorized by sepsis severity according to Sepsis-2 definitions. (1B) mHLA-DR, and (1D) HLA-DRA mRNA expression in patients with severe sepsis or septic shock (n=20), categorized by Sequential Organ Failure Assessment (SOFA) score on admission. In (1B), significant differences between SOFA score groups were shown on day 3 (p=0.024). In (1D), significant differences between SOFA score groups were demonstrated on days 1–2 (p=0.009) and day 3 (p=0.014).

Table 5. The mean difference in monocytic human leukocyte antigen D-related (mHLA-DR), HLA-DR alpha chain (HLA-DRA), and Class II transactivator (CIITA) expression between severity groups (severe sepsis and septic shock versus non-severe sepsis), calculated on a logarithmic scale, expressed as ratios, and presented at each time point.

	Days	Mean difference (95% CI)	P-value unadjusted	P-value adjusted
mHLA-DR	1-2	**0.63 (0.45-1.00)**	**0.008**	**0.04**
	3	**0.58 (0.42-0.81)**	**0.002**	**0.01**
	7	0.81 (0.66-1.00)	0.06	0.28
	14	0.84 (0.68-1.04)	0.11	0.57
	28	0.52 (0.73-1.21)	0.62	1
HLA-DRA	1-2	**0.40 (0.28-0.59)**	**<0.001**	**<0.001**
	3	**0.44 (0.30-0.64)**	**<0.001**	**<0.001**
	7	**0.59 (0.46-0.77)**	**<0.001**	**<0.001**
	14	0.79 (0.64-0.97)	**0.026**	0.13
	28	1.00 (0.78-1.29)	0.988	1
CIITA	1-2	**0.48 (0.32-0.72)**	**0.001**	**0.005**
	3	**0.41 (0.27-0.62)**	**<0.001**	**<0.001**
	7	**0.56 (0.41-0.76)**	**<0.001**	**<0.001**
	14	**0.64 (0.51-0.81)**	**<0.001**	**<0.001**
	28	0.74 (0.52-1.03)	0.069	0.35

In **Paper II**, PCR method validation was performed by repeated measurements. We demonstrated a robust method with high reproducibility. There were low threshold variations of the qRT–PCR system, low intraassay variations of Ct values within triplicates, and low interassay variations of the calculated target gene ratios. We were able to show that the reference gene *PPIB*, during the course of bacteremic sepsis, was as stable as in the blood donor controls without sepsis. This supports previous data demonstrating stability of *PPIB* in inflammatory conditions [215]. Accordingly, dynamic variations in *HLA–DRA* and *CIITA* gene expression were shown to be reliably detected during the course of sepsis. The coefficient of variation of the HLA-DRA ratio between different samples analyzed twice was found to be 12% (n=38) in this cohort. When summarizing all samples from Papers I, II, and IV that were analyzed twice (n=72), the coefficient of variation of the HLA-DRA ratio was found to be similar, 14%. Nevertheless, this means that changes in expression should be greater than this when interpreting significant individual differences over time.

The results of Papers I and II confirmed results presented by another research group who demonstrated a strong correlation between HLA-DR mRNA expression and mHLA-DR levels in patients with sepsis and an association between the extent of downregulation and the clinical course [167]. However, follow-up studies performed in the ICU setting will be required to validate the diagnostic and predictive value of mRNA-based HLA-DR assessment in sepsis-induced immunosuppression.

We suggest that our described method for detection of HLA-DRA mRNA in whole blood could be used to identify patients with increased risk of secondary infection following trauma, as described in a study by Timmermans et al. [182]. In that study, a negative slope of HLA-DRA between admission and day 3 was predictive of development of secondary infections, independent of age and severity. Furthermore, in the study by Timmermans et al. it was shown that ex vivo production of TNF-α and IL-6 upon secondary stimulation with LPS correlated to the HLA-DRA expression. The authors suggested that the release of the DAMP HSP-70 and free nuclear DNA following trauma was involved in the mechanism of HLA-DRA downregulation (153).

Monocyte HLA-DR and bacterial etiology (III)

Monocyte HLA-DR expression has been suggested as a useful diagnostic biomarker to identify sepsis-induced immunosuppression. However, the levels and dynamics of mHLA-DR are poorly studied in relation to the pathogen responsible for the infection and to common markers of inflammation. In this work, mHLA was measured during the course of infection in patients with BSIs caused by different bacterial pathogens. The three most prevalent pathogens were *S. pneumoniae* (n=27), *S. aureus* (n=22), and *E. coli/K. pneumoniae* (n=23) /other etiologies (n=19).

Escherichia coli/K. pneumoniae etiologies had high levels of mHLA-DR throughout the course of infection, in contrast to *S. aureus* and *S. pneumoniae* etiologies where levels were initially decreased and became gradually elevated over time, as shown in Figure 8. Interestingly, there were differences in recovery slopes within the group of Gram-positive infections, i.e., *S. pneumoniae* was associated with quick recovery after day 3, whereas *S. aureus* demonstrated a sustained low mHLA-DR level during the study period.

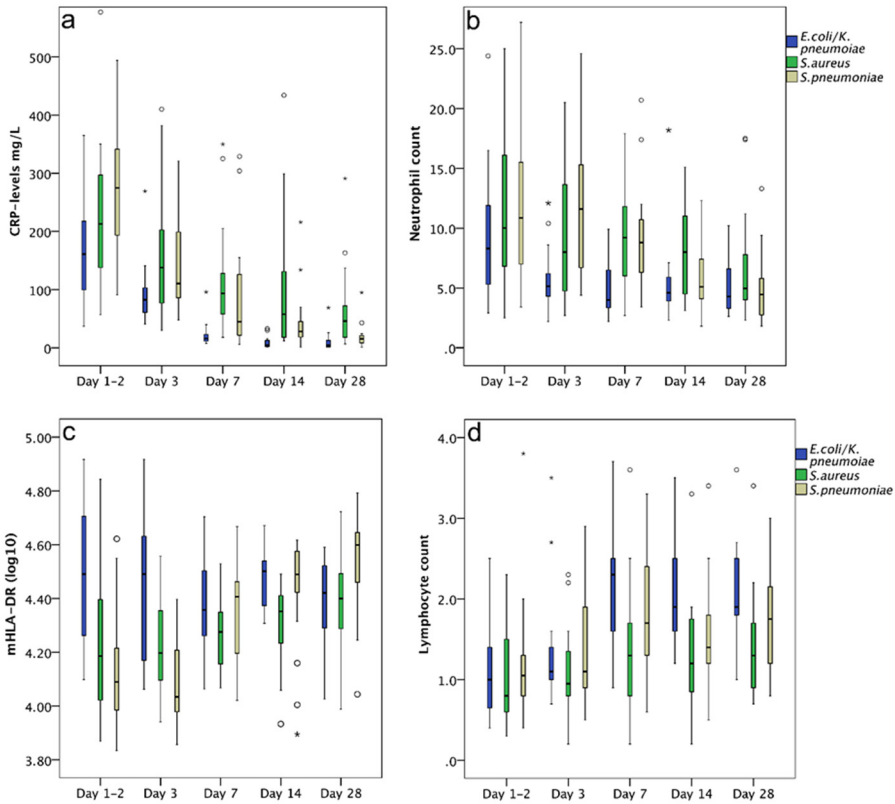


Figure 8 a–d. Dynamic variation of (a) C-reactive protein (CRP); (b) neutrophil count; (c) monocyte human leukocyte antigen-D-related (mHLA–DR); and (d) lymphocyte count, presented in groups defined by bacterial etiology of bloodstream infection. The x-axis presents sampling time points, in days after hospital admission.

Forty-seven of the 91 BSI patients had sepsis, according to the updated Sepsis-3 criteria. These patients had significantly lower mHLA-DR values on days 1–2 and 3. Considering the results presented in Papers I and II, it was expected to find lower mHLA-DR levels in patients with higher degree of organ failure. However, the suggested SOFA score cutoff level of ≥2 (Sepsis-3) had not been evaluated previously. With this established, it was also important to consider whether the etiology-related differences remained after adjustments for presence or absence of sepsis. Hence, unad-

justed and adjusted multivariate analysis of potential confounders was performed. Results showed that severity (SOFA score), bacterial etiology, and preexisting immunosuppression were all important for the expression levels. In the mixed model analysis of dynamic expression, etiology-related differences demonstrated significantly different slopes over time, independent of Sepsis-3 or preexisting immunosuppression.

This suggests that the mHLA-DR expression in sepsis depends on the bacterial etiology, sepsis severity, and time point for sampling in sepsis. Where immunostimulation therapy is guided by mHLA-DR levels it is therefore important to consider whether the decrease in mHLA-DR is transient or will continue.

The highest levels of mHLA-DR were demonstrated in patients with *E-coli/ K. pneumoniae* etiologies. This supports similar results presented by Janols et al., demonstrating that mHLA-DR was higher in sepsis caused by Gram negative etiology than Gram positive etiology [216] . HLA-DR was also shown to be up-regulated in gram negative infections in a study of patients with infective enterocolitis caused by *Salmonella*, *Campylobacter* and *Shigella* [217]. In that study, mHLA-DR was higher in infected patients than in healthy controls.

However, there are some conflicting data regarding HLA-DR expression in Gram-negative infections. In a study by Gogos et al., the lowest mHLA-DR levels were found in sepsis caused by *K. pneumoniae* and *Acinetobacter baumanii* [218]. These between-study discrepancies may be related to different study settings. The study by Gogos et al. included patients with nosocomial infections who were admitted to ICUs in Greece. Since Greece is one of the countries in Europe with the highest incidence of drug-resistant, Gram-negative bacteria [219, 220], it might be suspected that ineffective antimicrobial treatment could have influenced the possibility to resolve infection in these cases. Moreover, they had a low rate of microbiologically documented infections and assessed mHLA-DR only at one time point in the early phase of infection [218].

In our study population, the majority of patients with *E. coli/K. pneumon*ia BSI had urinary tract infections, which is reported as the most common cause of *E-coli* BSI but is an uncommon cause of sepsis in patients who develop protracted trajectories and secondary infections. It is therefore possible that community onset BSI with this etiology and site of infection are less likely to cause down regulated mHLA-DR levels and subsequent immunosuppression.

Staphylococcus aureus etiology was associated with longer duration of depressed mHLA-DR compared to other etiologies in this cohort. This is an interesting finding, as *S. aureus* is also associated with a high mortality from community-onset BSI [55, 61]. Possibly, this mortality difference could be related to development of immunosuppression, resulting in reduced bacterial clearance and chronic infection. Further studies are warranted to address this relationship.

Streptococcus pneumoniae is the most common cause of community-acquired pneumonia. Our data suggest that this etiology of BSI often induces low initial mHLA-DR levels that are quickly restored. This may be related to activation of NK cells, which are known to expand and become active during *S. pneumoniae* infections [218]. As described previously, activated NK cells are an important source of both IFN-γ and GM-CSF production [135]. Possibly, this etiology is associated with a lower risk for immunosuppression in sepsis. In support of this theory, patients with pneumococcal meningitis [221] and community-acquired pneumonia [222] have been shown to benefit from immunosuppressive cortisone therapy, which is inefficient in sepsis patients in general [38, 223].

Monocyte HLA-DR in patients with negative outcome (III)

Altogether eleven of the 91 patients who were eligible for dynamic evaluation had a negative outcome, either secondary BSI or death 3–60 days from admission. Patients with negative outcome had more severe sepsis, were older, and were hospitalized for longer than patients without negative outcome. Median mHLA-DR levels were lower in patients with negative outcome, but there were overlapping values in early measurements on days 1–2 and 3. Consequently, in this setting, HLA-DR could not discern a negative outcome by a single measurement before day 3. Regarding later time points, patient numbers were too small to perform statistical tests on predictive values adjusted for confounders. Besides, measurements performed later than day 7 would not be as useful for predicting the events. However, when studying the expression levels over time, patients with a negative outcome did not express a significant recovery during the course of infection in contrast to patients without a negative outcome. This supports results from previous studies, demonstrating that a lack of recovery is associated to a negative outcome [97, 193].

The mHLA-DR levels were not deeply depressed in all patients with negative outcome at the early time points of infection. It should be noted that two patients who were identified as having negative outcome and

who died at a later time point (days 58 and 60), never experienced secondary infection. These patients expressed high HLA-DR levels on days 1–2 (33 000 and 35 100 AB/C, respectively). Among the patients who developed secondary infections, median mHLA-DR was 8600 AB/C (range 7800–24 600 AB/C) on days 1–2. Patients without secondary infection had higher levels, expressing a median mHLA-DR value of 18 200 AB/C

On days 1–2, we had the highest numbers of sampled patients (n=91) and therefore performed receiver operating characteristic (ROC) testing to compare the discriminatory value of mHLA-DR and HLA-DRA for prediction of secondary infection. We found that unadjusted area under the curve (AUC) of mHLA-DR for prediction of secondary infection was 0.78, p=0.035 (95%CI 0.58–0.98). Interestingly, the results for HLA-DRA were very similar, with an AUC of 0.78, p=0.035 (95%CI 0.65–0.91) (unpublished data). Although firm conclusions could not be drawn regarding the predictive value in this limited cohort, it was relevant to perform ROC analysis to compare the performance of these different methods.

HLA-DR in relation to markers of inflammation (III)

Development of sepsis-induced immunosuppression is possibly linked to prolonged inflammation by sustained elevation of CRP, increased neutrophil counts, and lymphocytopenia [224]. As previously described, these parameters are included in the suggested diagnostics of PICS. However, no previous study has described the dynamic expression of these markers in relation to mHLA-DR. In Paper III, by assessing the expression of commonly used inflammatory markers in relation to mHLA-DR we found an association between sustained inflammation and immunosuppression. The correlation between mHLA-DR and the investigated markers at each time point varied from weak to moderate, as demonstrated in Figure 9, but showed a clear association in dynamic expression, as seen in Figure 10. In patients with negative outcome, CRP and neutrophil counts were continuously elevated after day 7. In contrast to this result, both lymphocyte counts and HLA-DR expression were found to be low from day 7 in patients with negative outcome. This finding supports the PICS theory of persisting inflammation and immunosuppression in patients with negative outcome. However, the PICS theory has important drawbacks due to its focus on late stages in sepsis (after 7–14 days). In particular, it is possible that the sustained CRP or neutrophil elevation that is seen during protracted clinical trajectories may actually reflect an already ongoing second hit. It would therefore be more useful to assess the predictive value of

early dynamics before day 7 or, alternatively, to assess the predictive value of a set of different early markers of immunosuppression. It has been shown that expression of mHLA-DR, regulatory T cells (T regs), and a marker of neutrophil deactivation combine additively to stratify risk of nosocomial infection in critically ill patients [225].

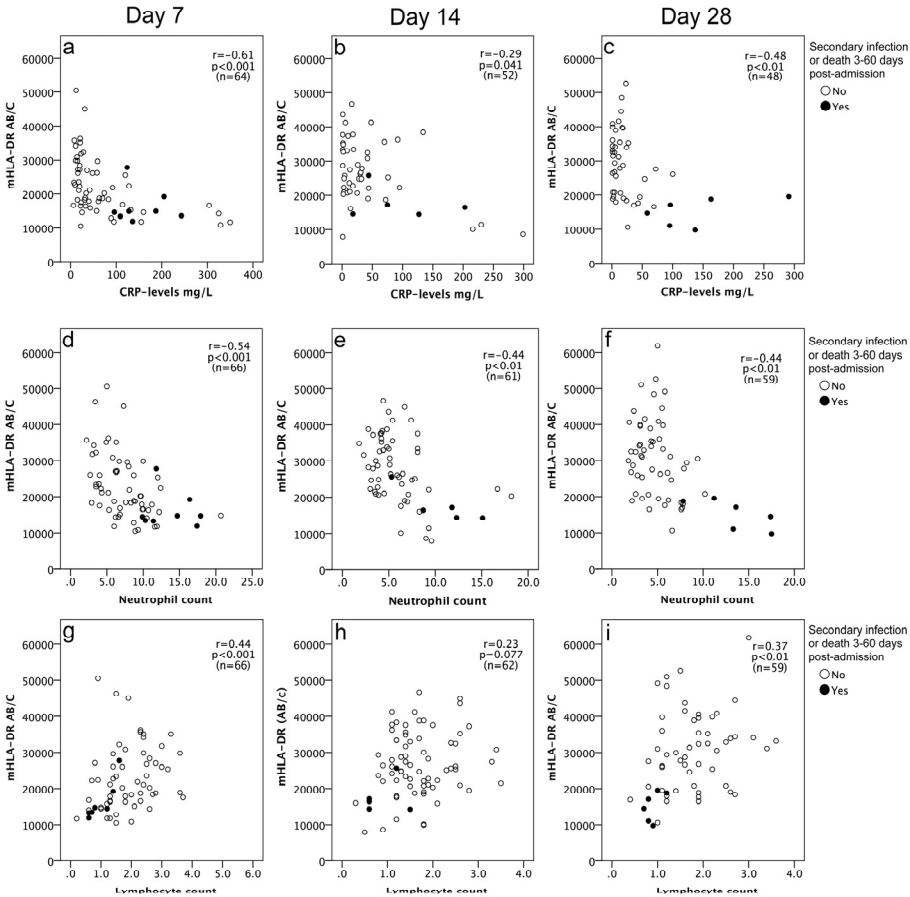


Figure 9. Monocyte human leukocyte antigen D-related (mHLA-DR) expression on post-admission days 7 (a, d, and g), 14 (b, e, and h), and 28 (c, f, and i), in relation to C-reactive protein (CRP) (a–c), neutrophil counts (d–f), and lymphocyte counts (g–i), in bloodstream infection (BSI) with and without negative outcome. Filled circles represent patients with negative outcome. Open circles represent patients without negative outcome.

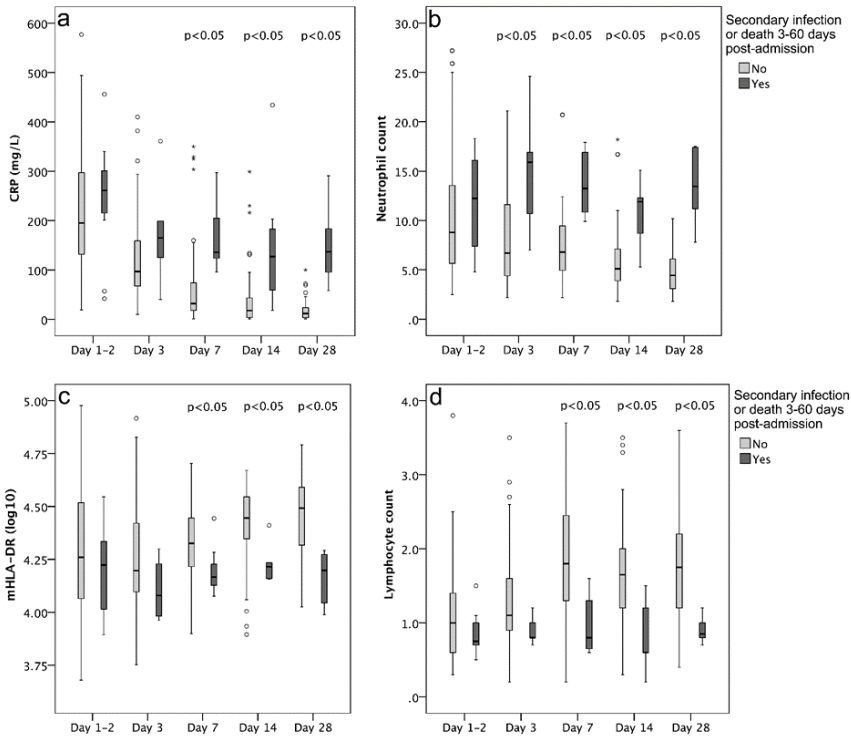


Figure 10. Dynamic variation in (a) C-reactive protein (CRP) levels; (b) neutrophil counts; (c) monocyte human leukocyte antigen D-related (mHLA-DR) expression; and (d) lymphocyte counts, in patients with negative outcome (i.e., secondary bloodstream infection (BSI) or death 3–60 days post-admission) and without negative outcome.

HLA-DR expression in complicated and uncomplicated *Staphylococcus aureus* bacteremia (IV)

Considering the high mortality from SAB and the importance of identifying patients with complicated infections, we aimed to assess whether HLA-DR expression could be discriminatory in this context.

In Paper IV, we therefore studied whether levels of HLA-DR were lower in complicated compared to uncomplicated SAB. "Complicated SAB" was defined as the presence of an episode with hematogenous seeding (e.g., infective endocarditis or osteoarticular infection), extension of infection beyond the primary focus (e.g., deep-seated abscesses), embolic stroke, or attributable mortality, according to the definition by Fowler et al. [62]. All other cases were defined as "uncomplicated SAB."

We found significant differences between these groups already in the early phase of SAB. Both HLA-DRA and CD74 were lower in patients with complicated SAB. Expression of HLA-DRA showed the greatest, and significant, differences between groups during the whole study period (7 days), as shown in Figure 11. These differences were not explained by the presence or absence of sepsis. Moreover, HLA-DRA on day 7 was lower in patients who died within 60 days. These results illustrate that patients without complicated SAB are able to upregulate HLA-DR transcription at the gene level. Since persisting bacteremia is a hallmark of complicated SAB, it is possible that sustained PAMP and DAMP exposure is associated with these differences in HLA-DR expression. However, due to limited patient numbers and novel findings in this context, these results will need additional confirmation in larger patient cohorts. In future studies measuring the temporal changes in HLA-DR during SAB, it would also be relevant to study the association to bacterial load and duration of bacteremia.

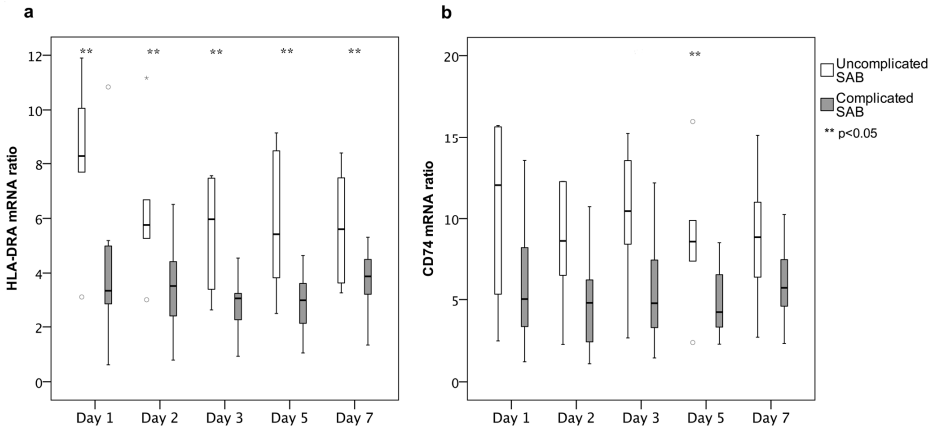


Figure 11. Expression of (a) human leukocyte antigen D-related alpha chain (HLA-DRA) messenger ribonucleic acid (mRNA) and (b) CD74 mRNA ratios in 20 patients with Staphylococcus aureus bacteremia (SAB), divided into complicated (n=14) and uncomplicated (n=6) SAB. Circles (○) represent outliers more than 1.5 box lengths from the edge of the box; single asterisks (*) represent outliers more than three box lengths from the edge of the box; and double asterisks (**) indicate significance (p<0.05).

Conclusions of the thesis

- HLA-DRA and CIITA mRNA measured by qRT-PCR were down-regulated in sepsis and HLA-DRA correlated well to HLA-DR surface expression on monocytes (mHLA-DR) in the early phase of sepsis.

- The dynamic expression of HLA-DRA mRNA in sepsis demonstrated initially low levels that gradually recovered over time, similar to mHLA-DR. HLA-DR markers measured by qRT-PCR demonstrated greater differences between non-severe and severe sepsis compared to mHLA-DR.

- Quantitative RT-PCR measurement of HLA-DRA was found to be robust during the course of sepsis and appears to be a promising method for monitoring the immune state in sepsis.

- The dynamic mHLA-DR expression varied according to the bacterial etiology of BSI independent of presence or absence of sepsis (Sepsis-3) or preexisting immunosuppression.

- The dynamics of mHLA-DR was inversely related to CRP and neutrophil count. In patients with unfavorable outcome, sustained CRP and neutrophil elevation was demonstrated along with low mHLA-DR and lymphocyte count. This indicates persisting inflammation in development of acquired immunosuppression in sepsis.

- Patients with complicated SAB had significantly lower HLA-DRA and lower CD74 than patients with uncomplicated *S. aureus* bacteremia (SAB). This demonstrates inhibited HLA-DR gene transcription in patients with complicated SAB.

General discussion and future perspectives

In the new understanding of sepsis pathophysiology, both overactive and suppressed immune responses are suggested to be important mediators of negative outcome. Poorly defined patients, especially regarding their immune status, may therefore in part explain why many sepsis trials targeting immune responses have failed. Some therapies could in fact have been efficient in certain subpopulations. For example, it has retrospectively been shown that patients expressing a specific cytokine signature responded better to adjuvant cortisone therapy [226]. Moreover, cortisone therapy has been shown to be beneficial when guided by CRP levels in sepsis patients with pneumonia [222]. In order to develop future therapies and thus reduce the deleterious effects of sepsis it is therefore mandatory to control for the substantial heterogeneity within the disease. Biomarkers characterizing the immune state are suggested to individualize treatments [227]. In this thesis we demonstrated substantial variations over time in suggested biomarkers of immune status.

Most importantly, we found differences related to the etiology of infection, a factor that is surprisingly rarely considered in immunology studies of sepsis [53].

Patients with *S. pneumoniae* infections demonstrated low initial mHLA-DR expression, with a fast recovery after day 3. By contrast, *S. aureus* patients had a delayed mHLA-DR recovery. Theoretically, these pathogens may elicit somewhat different cytokine responses, but this should not explain the continuous differences up to 28 days after admission. It is more likely that etiology-related differences in mHLA-DR expression are related to the clinical trajectory via differences in ability to cause continuous infection. This assumption is based on the following findings: (i) Respiratory infections were the major cause of pneumococcal infection, which are known to be easily treated by a short course of antibiotics. By contrast, *S. aureus* patients had a high percentage of osteoarthritis, stent graft infections, and endocarditis, which are known to be difficult to eradicate even with long-term antibiotic treatment; (ii) mHLA-DR dynamics were inversely related to CRP and neutrophil counts during the course of infection, with the slowest decline in *S. aureus* infections; and (iii) the results of the study reported in Paper IV showed that patients with complicated SAB had lower HLA-DR expression compared to patients with uncomplicated SAB. Moreover, unpublished data from an ongoing study (Ziegler et al. ECCMID 2017) evaluating quantitative measurements of bacterial DNA

by 16S PCR in the same patient cohort have demonstrated longer duration of DNA positivity in patients with *S. aureus* etiology. These findings support the theory of sustained PAMP and DAMP exposure in patients who develop immunosuppression. In future research it will be interesting to study the association between common DAMPs, such as HSP70 and HMGB-1, and development of sepsis-induced immunosuppression.

It is still not clear whether factors related to the different pathogens manipulate the host response differently, or whether differences are related to the unspecific burden of sustained DAMP and PAMP exposure that is associated to the typical type of infections they cause. As an example of this complexity, *S. aureus* bacteria may be equipped with virulence factors that impair the functions of important pathways of the immune response, such as HLA-DR expression [146] and factors leading to higher host tissue adhesion [228]. These factors together lead to disease progression and development of deep-seated foci, which in turn will harbor continuous bacterial exposure.

The mechanisms leading to HLA-DR down regulation in situations of high PAMP and DAMP exposure may involve different pathways. For example, disturbances in metabolism or mechanisms of cell autophagy also leave the host less effective in PAMP and DAMP removal, but has not studied in this thesis. This needs to be evaluated in sepsis studies addressing the causes of immunosuppression. Indeed, some of the proposed mechanisms for immunosuppression may also be consequences of physiological changes in immune function during the course of infection. However, independent of the mechanism leading to persistent inflammation with sustained DAMP and PAMP exposure it seems logical to be aggressive in the surgical removal of deep-seated foci to help reduce the bacterial burden. Achieving source control in the management of sepsis has been shown to be an important factor for better outcome [229] and should therefore be a routine procedure in the daily assessment of septic patients. Of interest, efforts to reach source control have been emphasized in the recently updated "Surviving Sepsis Campaign" guidelines [230].

Even if blood culture positivity in sepsis is related to severity of the disease [49], confirmation of the bacterial etiology in septic shock is associated with better prognosis. In a study by Daviaud et al. [28], undefined etiology of septic shock was an independent risk factor for both early and late deaths. Consequently, knowing the specific etiology is important, probably because of increased possibilities to tailor pathogen-specific treatment. In this context, antimicrobial susceptibility testing is pivotal for

correct antimicrobial coverage. Moreover, knowledge about disease manifestations for different pathogens can lead to faster achievement of source control. In fact, the risk of causing complicated infections varies between different pathogens. Physicians treating severe infections do not always account for this. For example, it has been shown that patients with SAB have a better prognosis if physicians specialized in infectious diseases are consulted [64, 231, 232]. In Paper IV, we demonstrated significant differences in the early HLA-DR expression for patients with complicated compared to uncomplicated SAB. If these results can be confirmed in a larger cohort, HLA-DR measurement could possibly become an additional tool for early identification of patients who will require further investigation to clear infectious foci and achieve source control.

In our study, patients who developed severe secondary infections had higher mHLA-DR levels than the cutoff levels previously used for initiation of immunostimulation (8000 AB/c). Consequently, this cut-off level probably has a low sensitivity for immunosuppression in this cohort. However, higher cut-off levels would probably generate lower specificity with risk for initiation of unnecessary treatments. Given the high degree of heterogeneous host responses in patients with sepsis, as shown in this thesis, it is more suitable to monitor immune responses by repeated measurements. In Paper III, mHLA-DR and common markers of inflammation were normalized earlier in patients without negative outcome. This is in line with results from other researchers [233]. However, we were also able to demonstrate how the temporal changes in mHLA-DR were related to commonly used laboratory tests, such as CRP, neutrophil, and lymphocyte counts. Interestingly, these markers have been suggested to be associated with immunosuppression by researchers supporting the PICS-theory, but they have not previously been evaluated in relation to mHLA-DR.

A question to be raised is whether mHLA-DR monitoring alone is optimal for identifying immunosuppression. In view of the increased knowledge in this field, a set of different markers reflecting the immunological alterations should reasonably be more precise. In a future perspective, it would be even better if a distinct disease-related pathological marker were identified to avoid misinterpretations related to physiological responses. In order to identify disease specific markers, we need more ICU studies using high-throughput transcription profiling techniques addressing novel gene expressions or mRNA patters during immunosuppression [234].

Considering promising preclinical data on immunostimulatory cytokines, such as IL-7 or IL-15, and immune checkpoint inhibitors such as PD-1- and PD-1L-inhibitor, to rejuvenate T cell exhaustion, it would be desirable to evaluate such treatments in sepsis. However, reliable predictive biomarkers have not yet been identified that define who will benefit from this method of treatment, and there is only a partial understanding of the mechanisms of sensitivity or resistance to this type of immunotherapy. Nonetheless, two recent reports surprisingly found that rescue of exhausted CD 8 T cells were dependent on expression of the costimulatory T cell receptor CD 28, and suggested that its expression may predict treatment responses to PD-1 blockade, when it is used in the cancer field [235, 236]. In a future perspective it would be desirable to identify the immune phenotypes that would benefit from immunostimulating treatments in sepsis, with high precision. This approach of so-called "precision medicine" [227] is successfully used in some types of cancer where treatments may be tailored according to the typical cell pathology [122, 237]. However, true precision medicine, which also refers to characterization and treatment based on individual genetics, may be difficult to implement in sepsis due to requirement of complicated diagnostics in a dynamic disease, not to mention the high costs of investigations and novel treatments. Nevertheless, a case report has shown that immunostimulation with PD-1 blockers in combination with IFN-γ has been used as a successful lifesaving treatment in intractable fungal infection [238]. Possibly, it is strategic to evaluate such therapies in certain etiologies known to be difficult to eradicate and to be associated with high mortality. As mentioned previously, fungal infections and pathogens associated with decreased anitibiotic susceptibility, such as *A. baumanii*, are shown to be independently associated with high mortality in sepsis [14].

Endotoxin tests demonstrating LPS responsiveness have historically often been considered as reliable test for immunoparalysis, but they have recently been shown to require standardization of the measurement technique in order to be useful [94]. As an alternative, mHLA-DR measured by FCM is considered as the method of choice because of fewer laboratory requirements and a standardized technique [107]. Today, mHLA-DR is still not implemented as a routine test even though suggested to be a reliable marker of immunosuppression. In a future perspective, it is more likely that immunosuppression will be identified by parameters that are easy to monitor and accessible to all clinicians. This could either be achieved by easily accessible diagnostic biomarkers with a short turnaround time for

the clinician or by a combination of parameters including different risk factors. A comprehensive study that characterizes patients who develop protracted infections and secondary infections based on easily accessible laboratory tests and clinical identifiers, as suggested in the PICS-definition [224] is therefore warranted. In contrast to previous studies, patients with pre-existing immunosuppression should be included. And again, measuring the immune status at the gene level is another option that would facilitate conduction of clinical studies.

In this thesis, we have shown that HLA-DRA measured by qRT-PCR correlated to mHLA-DR expression, which supports previous findings by Pachot [167], and Le Tulzo et al. [168]. Additionally, we found that HLA-DRA appeared to have a slightly higher discrimination for severe sepsis. According to our results and the results from the study by Timmermans et al. [182], HLA-DRA seems to be a promising marker for predicting immunosuppression in sepsis. In Paper IV, we also studied the immune marker CD74, which was lower in complicated SAB but was not significantly different from uncomplicated SAB, in this limited cohort. Considering the promising results of CD74 as a prognostic marker in septic shock patients demonstrated by Cazalis et al. [106], however, CD74 may still have merit as a biomarker of sepsis-induced immunosuppression.

In future research, it will be relevant to evaluate the usefulness of both CD74 and HLA-DRA in an ICU setting where patients have a higher risk of developing acquired immunosuppression. In an ongoing study including approximately 300 patients at the ICU of Karolinska University Hospital in Huddinge, Sweden, we aim to evaluate the predictive value of these markers in the context of secondary infection. If HLA-DRA or CD74 can be used to predict secondary infection in the ICU it will be relevant to design immunostimulation trials guided by HLA-DR mRNA markers. Previous studies have demonstrated that immunostimulation can restore downregulated mRNA levels as well as surface expression of HLA-DR. These findings, in combination with the results from Paper II, support the possibility to monitor the effect of immunostimulation by dynamic assessment of HLA-DR mRNA expression.

In future care of sepsis patients admitted to the ICU, I would like to see evidence-based guidelines for the management of patients beyond the early stages. The new challenge is how to manage the marathon and not the sprint. Ideally, future guidelines will include several treatment options and recommended investigations tailored to the clinical situation and the individual immune status. In order to reach this goal, it is important for both

translational and clinical researchers to consider dynamic and etiology-related differences when evaluating immune responses in septic patients. In the context of increasing sepsis incidence and emergence of resistant pathogens, we need to look back into the past to be able to move forward and development new interventions. Let us be inspired by the pioneers of successful treatment concepts in sepsis, such as the Surviving Sepsis Campaign, and let us continue to focus on immunology. This time, however, we need to get the timing right and pay more attention to the germs - again.

Svensk sammanfattning

Sepsis är ett allvarligt tillstånd som kan uppstå när kroppen reagerar kraftfullt på en bakteriell infektion. Under de senaste åren har kunskapen inom sepsisområdet ökat och det har visats att en stor andel av de sepsispatienter som idag överlever den akuta fasen i början av sjukdomsförloppet senare riskerar att utveckla ett försvagat immunsvar med förekomst av sekundära infektioner eller svårigheter att läka ut den primära infektionen. Detta definieras som sepsisinducerad immunsuppression. Ett sänkt uttryck av HLA-DR på monocyter (mHLA-DR) har föreslagits som markör för att identifiera patienter med sepsis-inducerad immunsuppression men även för att styra insättning av immunstimulerande behandling. Det saknas idag kunskap om uttrycket av mHLA-DR skiljer sig beroende på vilken sorts bakterie som ligger bakom infektionen.

Metoden som föreslås för mätning av HLA-DR idag sker via flödescytometri (FCM), som har vissa begränsningar i användbarhet. För att generera tillförlitliga resultat finns krav på omhändertagande av blodprov inom 4 timmar, vilket innebär att mHLA-DR inte kan analyseras under kvällstid eller helg.

Syftet med detta avhandlingsarbete var att öka kunskapen om immunmarkören HLA-DR hos patienter med blododlingspositiva infektioner och sepsis samt att studera om den kan mätas med en alternativ PCR-baserad metod.

I avhandlingsarbetet visas att uttrycket av HLA-DRA mRNA, mätt med så kallad PCR-teknik, samvarierar med uttrycket av mHLA-DR mätt med FCM. Detta stöder observationer från andra forskargrupper som har studerat en liknande metod. En konsekvens av detta är att vår beskrivna metod har använts i uppföljande studier där immunsvaret studerats över tid. En holländsk grupp har till exempel visat att återhämtningen av HLA-DRA mRNA nivåer efter trauma uttrycktes annorlunda för de som ådrog sig sekundära infektioner, jämfört med de som inte fick infektioner.

I avhandlingsarbetet studerades även hur mHLA-DR uttrycktes under det kliniska förloppet av blododlingspositiv infektion och sepsis. Det fanns stora skillnader i uttrycket av mHLA-DR över tid i de tre vanligaste bakteriegrupperna (*Staphylococcus aureus*, *Streptococcus pneumoniae* och *Escherichia coli/Klebsiella pneumoniae*). Patienter med infektioner orsakade av *S. aureus* och *S. pneumoniae* etiologi hade de lägsta initiala nivåerna. Patienter med *S. pneumoniae* infektioner hade däremot snabbt normaliserade nivåer efter dag 3, medan mHLA-DR nivåerna var kvarstående

låga under hela studieperioden hos patienter med *S. aureus* infektioner. *E. coli* och *K. pneumoniae* infektioner var förenade med de högsta nivåerna av mHLA-DR.

Vidare visas att patienter med blododlingspositiv infektion orsakad av *S. aureus*, uttrycker lägre nivåer HLA-DRA mRNA och CD74 mRNA vid komplicerade jämfört med okomplicerade infektioner. Detta är hypotesgenererande för framtida större studier med syfte att studera prediktiva värdet av HLA-DRA mRNA som diagnostisk biomarkör för komplicerad *S. aureus* infektion.

Sammanfattningsvis har resultaten från avhandlingsarbetet ökat kunskapen om hur HLA-DR uttrycks vid blododlingspositiva infektioner av varierande svårighetsgrad och bakteriell etiologi. Mätning av HLA-DRA på mRNA nivå har visat sig vara en lovande markör för att mäta immunsvaret över tid men behöver upprepas i studier utförda på intensivvårdade patienter för att kunna avgöra dess värde för identifiering av allvarlig sepsis-inducerad immunsuppression.

Acknowledgments

I would like to express my sincere gratitude to everyone who has helped and supported me in this work. I especially would like to thank:

Jan Källman, my supervisor, for his outstanding enthusiasm and valuable support during these years. Without him, this would not have been possible. He has been an important factor in making space for research in our hospital and clinic. I appreciate his wisdom and never ending faith in me, it has brought me strength and self-reliance.

Kristoffer Strålin, my co-supervisor and the principal investigator of the "Dynamics of sepsis study". He once inspired me to initiate this research and still tirelessly keeps challenging and inspiring me with his huge interest and deep knowledge in the understandings of sepsis. His humble approach and professional guidance (around the clock) have been extremely valuable for me in this work.

Bo Söderquist, my co-supervisor, who has a tremendous knowledge in the field of microbiology and infectious diseases. He always gives good advice and is supportive. I am also grateful for having the opportunity to teach medical students together with him. This inspires and triggers me to be updated in this field. I appreciate his wisdom and experience in research.

Anders Bäckman, my co-author, who is a hero in the laboratory. His knowledge and helpful attitude has been indispensable. It has always been enjoyable to work with him.

Elisabet Tina, my co-author, for her extremely valuable help in everything and especially her outstanding knowledge in flow cytometry. Her intelligence and experience in conducting studies have also been valuable. Every time we meet, I get in a good mood.

Gunlög Rasmussen, my co-author, for her deep knowledge about the Staphylococcal bacteria. I will always remember our joyful late evenings spent together, working with collaborating projects. As a bonus, we became good friends.

Anders Magnuson, my co-author, for important advice and professional education in the learning process of statistics.

Henrik Eliasson, head of the Department of Infectious Diseases in Örebro, who prioritizes time for research and contributes with a good sense of humor to the working environment.

All **my colleagues** at the clinic and at the Department of Microbiology. **Per Arneborn**, my role model and senior colleague, for stimulating discussions and good advice. My colleague **Anna Lange** "down under" for being such a supportive, amusing and enthusiastic friend and for sharing the same interests, including immunology. Also, I would like to bring a special thanks to **Ingrid Ziegler** for rewarding discussions regarding future collaborations and her reliably positive attitude with ability to solve problems, even faster than she runs. **Charlotte Lybeck** for being a wonderful roommate. **Martin Widlund** and **Simon Athlin** for nice efforts to schedule time for research.

My friends **Louise Klasson, Zlatko Savic, Patrik Danielson, Daniel Öhlund** and the members of **Kulturklubben** for spicing up my life outside the hospital.

My parents **Kerstin Cajander** and **Stefan Cajander** for always encouraging me and for giving valuable support to my family. My cheering **sisters**, reminding me to dance.

My husband **Per Cajander** for his enthusiasm, love and endless support, and by challenging me with his knowledge in intensive care medicine. Our children **Victor** and **Alfred** for giving us a meaningful life. I love you all!

Finally, I would like to thank the Region Örebro County's Research Committee, Nyckelfonden (Örebro University Hospital), and ALF research funding for financial support.

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