CYTOKINE PROFILES IN INFLAMMATION

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TABLE OF CONTENTS

SCIENTIFIC ENVIRONMENT

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ABBREVIATIONS

α	alpha
β	beta
γ	gamma
κ	kappa
ω	omega
>	greater than
<	less than
=	equal to
~	approximately
%	per cent
°C	degree Celsius
ACCP	American College of Chest Physicians
ADAM	A Disintegrin and Metalloprotease
ADAMT	A Disintegrin and Metalloprotease with Thrombospondin Motifs
AHC	agglomerative hierarchical clustering
APC	antigen-presenting cell
ATS	American Thoracic Society
AUROC	area under receiver operating characteristic
bpm	beat per minute
CLR	C-type lectin receptor
COX-2	cyclo-oxygenase type 2
CV	coefficient of variation
DAMP	damage-associated molecular pattern
DC	dendritic cell
dl	decilitre
DVT	deep vein thrombosis
ECM	extracellular matrix
ED	emergency department
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ELR	Glu-Leu-Arg molecular motif
ESICM	European Society of Intensive Care Medicine
ESL1	E-selectin ligand
FiO_2	Fraction of Inspired Oxygen
G-CSF	granulocyte-colony stimulating factor
GlyCAM-1	Glycosylation-dependent cell adhesion molecule-1
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GVHD	graft-versus-host disease
h	hour
HGF	hepatocyte growth factor
HLA	human leukocyte antigen
ICAM-1	intercellular adhesion molecule-1
ICU	intensive care unit
IFN	interferon
Ig	immunoglobulin
IgA	immunoglobulin A
IGF	insulin-like growth factor
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
IL-6R	interleukin-6 receptor
ILC	innate lymphoid cell
ISS	International Staging System for multiple myeloma
JAK	Janus kinase
kDa	kilodalton
kg	kilogram
kPa	kilopascal
l	litre
LFA	lymphocyte function-associated antigen
LPS	lipopolysaccharide
m	metre
MAdCAM-1	mucosal vascular addressin cell adhesion molecule-1

MAP	mean arterial pressure
mg	milligram
MGUS	monoclonal gammopathy of undetermined significance
MHC	major histocompatibility complex
min	minute
miRNA	microRNA
µl	microlitre
mmHg	millimetre of mercury
µmol	micromole
mmol	millimole
MMP	matrix metalloprotease
MODS	multiple organ dysfunction syndrome
mRNA	messenger ribonucleic acid
NE	neutrophil elastase
NETs	neutrophil extracellular trap
NF-κB	nuclear factor kappa B
NK cell	natural killer cell
NKT cell	natural killer T-cell
NLR	NOD-like receptor
NO	nitric oxide
OPLSDA	orthogonal partial least squares discriminant analysis
PAF	platelet-activating factor
PAMP	pathogen-associated pattern
PaO_2	partial pressure of O2 in arterial blood
PCA	principal component analysis
pCO_2	partial pressure of carbon dioxide
PCT	procalcitonin
PE	pulmonary embolism; phycoerythrin
PRR	pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
PTS	post thrombotic syndrome
RIPK1	receptor-interacting serine/threonine protein kinase 1
R-ISS	revised International Staging System for multiple myeloma
RLR	RIG-I-like receptor
ROC	receiver operating characteristic
ROS	reactive oxygen species
s	second
SCCM	Society of Critical Care Medicine
SD	standard deviation
SE	staphylococcal enterotoxin
SIRS	systemic inflammatory response syndrome
SIS	Surgical Infection Society
SmeZ	streptococcal mitogenic exotoxin Z
SNP	single nucleotide polymorphism
SOFA	sequential (sepsis-related) organ failure assessment
SpeA	streptococcal pyrogenic exotoxin A
SpeC	streptococcal pyrogenic exotoxin C
STAT	signal transducer and activator of transcription proteins
TACE	TNF-α-converting enzyme
TCR	T-cell receptor
TEM	transendothelial migration
TF	tissue factor
TGF-β	transforming growth factor beta
Th-cell	T-helper cell
TIMP	tissue inhibitor of metalloproteases
TKR	tyrosine kinase receptor
TLR	Toll-like receptor
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TPO	thrombopoietin
TSLP	Thymic stromal lymphopoietin
TSST-1	toxic shock syndrome toxin-1
VCAM-1	vascular cell adhesion molecule-1
VGEF	vascular endothelial growth factor
VLA-4	very late antigen-4
VTE	venous thromboembolism
vWF	von Willebrand factor

SUMMARY

Inflammation is a complex tissue response to harmful stimuli such as microorganisms, damaged or dead cells, or irritants. The inflammatory process can be initiated by many different diseases, including thromboses, neoplastic diseases, and infections. In the present studies, the serum profiles of inflammation-associated soluble mediators were analysed in patients with multiple myeloma undergoing stem cell mobilization, patients with venous thromboses, as well as patients with severe bacterial infections, all treated at Haukeland University Hospital. The serum and plasma levels of cytokines, adhesion molecules, and matrix metalloproteases were measured, and the possible diagnostic or prognostic use of these mediators as broad mediator profiles was explored. Cytokines are essential in all stages of inflammation, from initiation to resolution. Adhesion molecules, including the soluble, as well as cell-bound, forms, are essential for cell-to-cell communication as well as for leukocyte migration, whereas matrix metalloproteases are important modulators of other soluble mediators. The recent development of multiplex technology has made it possible to determine the systemic (serum or plasma) levels of several mediators in small sample volumes. Up to 40 different inflammatory mediators were assessed, and using multiplex technology, broad cytokine profiles during inflammatory responses elicited by different clinical causes were investigated. Unsupervised hierarchical clustering was used to study the combined effect of several mediators in inflammatory networks.

Autologous stem cell transplantation is the initial treatment of patients younger than 65 years with multiple myeloma, and the broad inflammatory response during stem cell mobilization has not been studied previously. Results here showed that the cytokine network was altered during stem cell mobilization and harvesting. Unsupervised hierarchical clustering suggested that patients with relatively low chemokine levels in stem cell grafts had a poorer prognosis. In addition, patients with deep venous thrombosis (DVT) demonstrated a broad inflammatory response during the acute phase of the condition, compared to healthy controls. When investigating patients with suspected thrombosis, the levels of only four inflammatory mediators showed statistically significant differences in patients with and without thrombosis. Unsupervised hierarchical clustering revealed that the combined use of these inflammatory mediators could identify patient subsets that differed significantly in DVT frequency. The cohort of patients with sepsis also showed a broad inflammatory response. The levels of 16 inflammatory mediators were found to differ significantly during severe bacterial infections in sepsis patients with bacteraemia, compared with those without bacteraemia, with vascular cell adhesion molecule-1 (VCAM-1) as the mediator showing the most robust difference in a multivariate regression model. Unsupervised hierarchical clustering with six mediators differentiated 98% of patients with bacteraemia.

Taken together, these study findings suggest that systemic mediator profiles should be further investigated as possible diagnostic and prognostic tools in routine clinical practice.

LIST OF PAPERS

Paper I

Stem cell mobilization and harvesting by leukapheresis alters systemic cytokine levels in patients with multiple myeloma.

Mosevoll KA, Akkök ÇA, Hervig T, Melve GK, Bruserud Ø, Reikvam H.

Cytotherapy. 2013 Jul; 15(7):850-60.

Paper II

Systemic levels of the endothelium-derived soluble adhesion molecules endocan and E-selectin in patients with suspected deep vein thrombosis.

Mosevoll KA, Lindås R, Wendelbo Ø, Bruserud Ø, Reikvam H.

Springer plus. 2014 Sep 30; 3:571.

Paper III

Altered plasma levels of cytokines, soluble adhesion molecules and matrix metalloproteases in venous thrombosis.

Mosevoll KA, Lindås R, Tvedt TH, Bruserud Ø, Reikvam H.

Thrombosis Research. 2015 Jul; 136 (1):30-9.

Paper IV

Cytokine profiles can differentiate bacteraemia in patients with serious infections.

Mosevoll KA, Skrede S, Lunde Markussen D, Fanebust HR, Flaatten H, Aßmus J, Reikvam H, Bruserud Ø.

Manuscript

1 INTRODUCTION

1.1 INFLAMMATION

1.1.1 Definition

The term inflammation, derived from the Latin word *inflammatio*, is defined as a complex biological response of body tissues to harmful stimuli such as pathogens, damaged cells, or irritants (1-4). It is regarded as a protective response that involves immunocompetent cells, blood vessels, and a wide range of molecular mediators derived from the various cells involved in the inflammatory process. The main role of inflammation is to eliminate the initial cause of cell injury, to mediate clearance of necrotic cells and damaged tissues from the original insult and inflammatory process, and to initiate tissue repair. The classical local signs of inflammation are redness, heat, swelling, pain, and loss of function. In addition, severe inflammation also elicits systemic effects, probably mediated, at least in part, by circulating soluble mediators originating from the local inflammatory process.

Inflammation can be caused by a wide range of biologically very different conditions. Thus, understanding the inflammatory response and the immune mechanisms underlying the inflammatory process is essential in determining the detailed pathogenesis of human disease such as microbial infections, cancer, vascular disorders, and autoimmune reactions (2, 4-6). The inflammatory response can be studied at different levels—from clinical examination, including macroscopic description of the disease, to describing the cellular components at a microscopic level and characterizing the intra- and intercellular signalling at a molecular level. The magnitude of the inflammatory response depends on the biology of the initiating events and ranges from minor inflammatory responses (as seen during physical irritation), via more prominent responses (as seen in sterile inflammation, e.g. thromboses), to the inflammatory storm seen in septic shock (2, 3).

1.1.2 The cytokine network in inflammation

The cytokine network consists of soluble mediators, i.e. cytokines, which are essential to the communication network between key cellular players in an inflammatory response. The term cytokine is derived from the Greek words *kyttaro* (cell) and *kines* (movement) and represents a broad and loose category of small proteins (~5–20 kDa) which play an important role in the communication between various immunocompetent cells and between immunocompetent cells and other cell types (e.g. endothelial cells) involved in inflammation (1). Cytokines act via their specific receptors and regulate maturation, growth, and responsiveness of different cell populations. They may affect the cytokine-releasing cell directly (i.e. autocrine effects) or neighbouring cells in the common microenvironment, thereby creating a paracrine signalling loop. There is a terminology overlap between cytokines and growth factors, but less so between cytokines and hormones. It is possible for a given cytokine to be

released by many different cells, whereas hormones tend to be secreted by specific cells and act on distant organs and effector cells. However, cytokines can also be detected outside the microenvironment of their releasing cells (e.g. serum or plasma) and hence can also exert distant effects.

Specialized immunocompetent cells express pattern recognition receptors (PRRs) which recognize different harmful injuries known as damage-associated molecular patterns (DAMPs) or pathogen-associated patterns (PAMPs), thereby initiating a cascade of immune reactions (7, 8). The main effector molecules in these immune reactions are pro-inflammatory cytokines that are activated and released through the inflammasome (a multiprotein oligomer including specialized innate immune system receptors and sensors) (7, 8). The biological effects of pro-inflammatory cytokines are further modulated through the interplay between the various pro-inflammatory mediators and their interactions with anti-inflammatory cytokines and other soluble mediators like soluble adhesion molecules, proteases, and protease inhibitors. All these soluble mediators, together with a wide range of immunocompetent and tissue cells, form a highly dynamic network, which often renders it difficult to predict and explain in detail the pathogenesis of inflammatory diseases in clinical models (9).

Cytokines, adhesion molecules, and matrix metalloproteases (MMPs) are key components involved in inflammation (1, 10, 11). Cytokines are important for cell-cell communication during inflammation, and they are highly heterogeneous and can be classified based on their function or structure (1). Adhesion molecules are important mediators of cellular adhesion between leucocytes and endothelial cells and can exist in the membrane-bound as well as the biologically active soluble forms (11). Finally, MMPs have emerged not only as molecules involved in modelling extracellular tissue, but also as important regulators of inflammatory responses, e.g. through their activation and modulation of pro-inflammatory cytokines (10).

In addition to its roles in autoimmune and infectious diseases, as mentioned in Section 1.1.1, p. 1, inflammation is also involved in the pathogenesis of several disorders. Thromboembolic disease has been shown to be associated with local inflammatory response (5), and inflammation has been recognized as an important player in the pathogenesis of both venous and arterial thromboses (12-14). Furthermore, sepsis is in itself and as a major complication of surgical interventions and intensive anti-cancer treatment, associated with a high mortality rate, and thus septic response to severe infection is probably the most extensively studied inflammatory process. Recent studies of biomarker profiles have suggested that treatment targeting combinations of pro-inflammatory mediators should be investigated further in patients with sepsis (15, 16). Treatment specifically targeting individual cytokines, e.g. tumour necrosis factor (TNF)-α inhibitors targeting TNF-α, is now a powerful therapy option, especially in autoimmune inflammatory diseases (17), whereas as yet cytokine-directed therapy has not become part of routine treatment for patients with severe infections, e.g. sepsis (18). In

note, biomarker studies have suggested that diagnostic or therapeutic procedures, e.g. peripheral blood stem cell apheresis, may also have pro-inflammatory consequences (19).

Section 1.4, p. 10 will describe in detail individual cytokines, as well as other soluble mediators (i.e. soluble adhesion molecules, MMPs, and their inhibitors) involved in inflammation and discuss cytokine classification. As will be described in Section 2.5, p. 39 in the Methods section, the recent development of multiplex technology and new bioinformatic tools has enabled the assessment of profiles of soluble mediators in serum or plasma as part of studies of inflammatory responses (20, 21).

1.2 ENDOTHELIAL CELLS, INFLAMMATION, AND COAGULATION

1.2.1 The coagulation cascade and role of endothelial cells in inflammation

Endothelial cells are key regulators of the inflammatory response, as they: (i) form a physical barrier for blood cells and regulate the vascular permeability for immune cells, soluble proteins, electrolytes, and water; (ii) regulate the intravascular coagulation; (iii) regulate the vascular tone and blood pressure through initiation of vasoconstriction/vasodilatation; and finally (iv) release hormones and other soluble mediators, such as cytokines, that initiate and regulate the inflammatory process (22).

Endothelial cells activate, control, and direct leukocytes mainly through their cell surface expression of adhesion molecules and the release of chemotactic chemokines after activation. This enables immunocompetent cells to adhere to the endothelial cells and consequently cross the vessel wall by transendothelial migration (TEM), thus resulting in accumulation of immunocompetent cells at the inflammation site (11). Rapid endothelial activation (i.e. within minutes) is induced by stimuli like histamine and platelet-activating factor (PAF), initiating the expression of preformed adhesion molecules. In contrast, pro-inflammatory cytokines, such as interleukin (IL)-1β and TNF-α, induce a slower endothelial activation (i.e. within hours), involving transcriptional activation of adhering molecules and chemoattractants (11).

Following endothelial wall damage, the coagulation cascade is initiated, which leads to the formation of a haemostatic clot sealing the wound. The subendothelial layer activates the coagulation cascade via exposure of tissue factor (TF) and platelets via exposure of von Willebrand factor (vWF) and collagen. Cross-activation between the coagulation cascade and activated platelets (14) subsequently takes place. Endothelial wall damage with subsequent exposure of the subendothelial layer and clot formation represents a critical step in the pathogenesis of arterial disease and thrombosis, e.g. in plaque formation in atherosclerosis (14).

1.2.2 Endothelial cells in the regulation of coagulation in venous thrombosis

The pathogenesis of deep vein thrombosis (DVT) differs from that of arterial thrombosis, as venous thrombosis is initiated by intravascular events without exposure of the subendothelial layer. A study using a mouse model of DVT demonstrated that an inflamed endothelium increases the expression of a wide range of adhesion molecules that attach neutrophils and monocytes to the vessel wall, as an initial step in the formation of a venous thrombus, and that early venous thromboses mainly consist of leukocytes and relatively few platelets (12). A complex interplay between monocytes, neutrophils, platelets, and the coagulation cascade leads to the formation of a thrombin-rich thrombus. Activated monocytes express TF that initiates the extrinsic pathway of the coagulation cascade. Thus, findings from the study suggest that TF expression by monocytes appears to be more important than endothelial expression of TF in triggering the coagulation cascade in DVT. Moreover, the same study showed that neutropenia, genetic knockout of factor XII, and NET disintegration protected against the formation of DVT, indicating that neutrophil activation is a prerequisite for DVT formation, since neutrophils bind coagulation factor XII and release neutrophil extracellular traps (NETs) which, together with platelets, activate the coagulation cascade both through the intrinsic and extrinsic pathways (12).

1.2.3 Immunothrombosis

Several inflammatory diseases, such as infections and cancer, predispose to an increased risk for DVT, likely due to the close interplay between various leukocytes and the coagulation system, as described in the previous two sections (3, 23, 24). The innate complement system can also directly activate the coagulation cascade. This close interplay has given rise to the concept of immunothrombosis as an effector function of the immune system whereby intravascular thromboses act as part of the innate immune system and help to recognize, trap, and break down invading pathogens (23, 25). Intravascular thromboses have been shown to have antimicrobial properties. For example, fibrin exerts direct antimicrobial effects; activated monocytes and neutrophil granulocytes accumulate in the thrombus and enhance microbial clearance; NETs from neutrophils can bind several antibacterial peptides; and platelets also exert binding properties for pathogens. Furthermore, mice with a defective coagulation system, e.g. fibrin or TF expression, have been shown to be predisposed to severe infections (3). Certain microbes have developed defence mechanisms to avoid the antimicrobial effects of immunothrombosis. For instance, *Streptococcus pyogenes* and *Streptococcus pneumoniae* express DNase that degrades prothrombotic NETs, whereas several streptococcal species use streptokinase to degrade fibrin (3). On the other hand, *Staphylococcus aureus* can activate the coagulation system through various molecular mechanisms, thereby establishing a multilayered barrier against invading immune cells. This procoagulant effect may, at least in part, explain the observations seen in large clinical studies where patients with *S. aureus* infections have an increased risk for venous thromboses

(26). Taken together, these findings strongly highlight the clinical relevance of the relationship between inflammation and coagulation.

1.2.4 Endothelial cell communication in inflammation

Endothelial cells express adhesion molecules, which are formed either by proteolytic cleavage or by alternative splicing. Many endothelial adhesion markers can be detected in their biologically active soluble forms, and vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-), and E-selectin (27) are the most commonly used molecular markers of endothelial cell damage in studies. Interestingly, inflammation-induced changes in soluble adhesion molecule levels depend on the pathological context. For example, the serum level of E-selectin is decreased in febrile neutropenia but increased in meningococcal infections (27, 28).

Several other soluble mediators have been considered as markers of endothelial cell damage or activation, including thrombomodulin and vWF (29). One soluble mediator is endocan, a 50-kDa large, soluble proteoglycan, consisting of 165 amino acids and a single dermatan sulfate chain. It is expressed by endothelial cells and can be detected in serum and plasma samples (30). Endocan expression can be increased by pro-inflammatory cytokines and plays an important role in cell adhesion in inflammation. Previous studies have also demonstrated increased endocan levels in infectious diseases, as well as in sterile inflammation like pulmonary embolism (PE) and acute graft-versus-host disease (GVHD) in patients undergoing allogeneic stem cell transplantation (30, 31).However, use of these markers has been limited, as their levels depend on a variety of host factors, including renal function, hepatic metabolism, drug interactions, or other diseases like hypertension and diabetes (29, 32).

Another mechanism for cell-to-cell communication is the release of exosomes by cells. These are microparticles that act as efficient messengers in cell-to-cell communication whereby their cargos (lipids, proteins, messenger ribonucleic acids (mRNAs), and microRNAs (miRNAs) can be functionally delivered between different cell types; they also act as regulators in carcinogenesis and leukaemogenesis (33-35).

Finally, another marker of conditioning-induced endothelial damage is the number of circulating endothelial cells, e.g. during inflammation (29, 32). It is feasible to implement their use as a marker in routine clinical practice through the use of flow cytometry. However, the biological consequences of elevated numbers of circulating endothelial cells need to be characterized further.

1.3 IMMUNOCOMPETENT CELLS: A BRIEF OVERVIEW

A brief overview of the functional characteristics of various immunocompetent cells and their importance in the cytokine network is presented in Table 1 and Figure 1. The first step in inflammation is carried out by the innate immune system, while the second step involves the adaptive immune system which more specifically targets pathogens in the inflammatory immune response (36).

The first step of an inflammatory response involves monocytes, which are known to influence the coagulation cascade and play a role in thrombus formation and in sterile inflammation associated with thromboses (3). Circulating monocytes in peripheral blood migrate to tissues and differentiate into macrophages or dendritic cells (DCs) (37), which recognize tissue damage or pathogens through PRRs, as well as being antigen-presenting cells (APCs). Macrophages play an important role in the regulation of tissue inflammation following activation by PRRs and phagocytosis during the initial step of an inflammatory response, and may be polarized to either the pro-inflammatory M1 phenotype or the anti-inflammatory M2 phenotype (38-40). DCs reside mainly near epithelial surfaces and their main function is in phagocytosis and subsequently as APCs presenting antigenic peptides derived from the phagocytosed material in secondary lymphoid tissues. The secondary adaptive immune response is thereby initiated (36).

Granulocytes constitute a heterogeneous group of circulating immunocompetent cells, including neutrophils, eosinophils, basophils, and mast cells, of which neutrophils are the most abundant. Following activation, neutrophils have important functions both intravascularly and in tissues during inflammation. They are important in defence against bacteria through phagocytosis and in sterile inflammation, and promote thrombosis formation via their NETs (3, 41). Eosinophils, basophils, and mast cells are important players in defence against parasites and have important roles in sterile inflammation associated with allergic reactions (42).

Moreover, platelets are also involved in inflammatory immune responses. They participate in the development of inflammation both directly, through their release of a wide range of immunoregulatory cytokines, and indirectly through activation of complement and the coagulation cascade (43).

The second step of inflammation (i.e. the adaptive immune response) is initiated through interactions between APCs and T-helper (Th)-cells, cytotoxic T-cells, and B-cells in secondary lymphoid tissues like lymph nodes and the spleen. Stimulation, activation, and expansion of antigen-specific cells through their recognition of antigenic peptides presented in the context of self-human leukocyte antigen (HLA) reinforce the antigen-specific part of the immune response (44). The T-cell antigenic repertoire is initially established in the thymus where cells that recognize self-antigens are deleted and/or cells not recognizing self-antigens are selected. Th-cells have important functions in

facilitating immune responses and, depending on the stimulation, they may polarize to different phenotypes (i.e. Th1, Th2, or Th17) that stimulate different parts of the inflammatory immune responses (Table 1, Figure 1) (42). Following their stimulation, antigen-specific B-lymphocytes differentiate into plasma cells that produce specific immunoglobulins, typically immunoglobulin M (IgM) during the initial response and subsequently immunoglobulin G (IgG) and immunoglobulin A (IgA) antibodies. The expanded cytotoxic T-cell clones recognize specific non-self-molecules and induces cell death through their ligation of death receptors and release of soluble pro-apoptotic mediators such as perforins (42).

Natural killer (NK) cells use the missing self-antigen presented by major histocompatibility complex (MHC) class I as activation signal and induce cell death through molecular mechanisms similar to cytotoxic T-cells (45). The natural killer T (NKT) cells constitute a different cell type that recognizes missing lipid self-antigens through a different receptor (CD1d), although they exert similar functions as NK cells (46). The recently identified innate lymphoid cells (ILCs) are currently classified into three different subsets (ILC1, 2, and 3) and participate in the regulation of immune responses through their release of Th1, Th2, or Th17 cytokines (47).

The cytokine profile in peripheral blood (i.e. plasma or serum levels) can be referred to as the systemic cytokine network. A variety of immunocompetent cells contribute to this network, along with changes in cytokine levels observed during inflammation, as illustrated in Tables 1 and 2. Thus, the cytokine profile reflects the events occurring as part of a local inflammatory response or the development of a systemic inflammatory acute phase response. However, circulating cytokine levels are not determined solely by their altered release in inflammation. Cytokines are also important in the differentiation or expansion of immunocompetent cells during an inflammatory response, and they undergo cellular binding, as well as consumption, that will also influence their systemic circulating levels.

Table 1. Immune cells and their main functions

	Main functions	Stimulators of proliferation and activation	Factors secreted by immune cell
Innate immune system			
Monocytes	Recognize PAMP/DAMP through PRRs Phagocytosis, APC, differentiation to macrophages or DCs Initiation of thrombosis (TF)	IL-1β, IL-3, CCL2, GM-CSF, , activated by PAMP/DAMP	Wide range of pro-inflammatory cytokines
Macrophages	Recognize PAMP/DAMP through PRR Phagocytosis, APC, regulation of innate immune response	M-CSF, GM-CSF	Dependent on polarization
Macrophages: M1 polarized	Facilitate pro-inflammatory responses	IFN-γ, TNF-α	IL-12, IL-23, TNF-α, IL-1b, IL-6, CXCL9/10
Macrophages: M2 polarized	Inhibit immune response	IL-4, IL-10, IL-13, CCL2, CXCL4	IL-10, TGF-β, IL-1ra, CCL17/22/24
Dendritic cells	Recognize PAMP/DAMP through PRR Phagocytosis, APC Polarization abilities suggested	GM-CSF, IL-4	IFN-α, IL-1, IL-6, IL-10, IL-12, IL-23, IL-27, TNF-α
Neutrophils	Phagocytosis, initiation of thrombosis (NETs) Polarization abilities suggested	G-CSF, GM-CSF, IL-3, IL-11, IL-23	Wide range of pro-inflammatory cytokines
Eosinophils	Defence against helminth parasites and allergic reactions. Support plasma cell viability	GM-CSF, IL-3, IL-5	IL-6, IL-10
Basophils	Defence against parasites, bacteria, and viruses, allergic reaction Support plasma cell viability	IL-3, IL-18, IL-33	IL-4, IL-13, IL-6
Mast cells	Defence against parasites, bacteria, and viruses, allergic reaction	IL-4, IL-9, IL-3, IL-33	IL-4, IL-13
Both innate and adaptive immune systems			
NK cell	Defence against cancer and infectious agents through recognizing (missing) self-peptides by MHC class I	IL-2, IL-7, IL-12, IL,15, IL-21	IFN-γ, TNF, GM-CSF, IL-5, IL-10, IL-13
NKT cells	Defence against cancer and infectious agents through recognizing self-lipids by CD1d molecules	TCR–CD1d interaction, pro-inflammatory cytokines	IFN-γ, TNF, IL-2, IL-4, IL-10, IL-13, IL-17, IL-21, IL-22, GM-CSF
Other ILCs—several subtypes (ILC1/ILC2/IL17/ILC22)	Directs Th-cell polarization	General: IL-2, IL-4, IL-7, IL-15, IL-21 Dependent on subtype: IL-12, IL-15/IL-7/IL-7Rα	Dependent on subtype IFN-γ/IL-4, IL-5, IL-9, IL-13/IL-17, IFN-γ/IL-22
Adaptive immune system			
Th-cells ($CD4^+$)	Matured in response to antigen and polarized according to local cytokine environment. Recognize specific non-self MHC class II antigens	See subgroups below	See subgroups below
Th1 polarized	Facilitate macrophage activation, cell-mediated immunity, phagocytosis, and defence against intracellular pathogens	IL-12, IL-18, IFN-γ	IL-2, IFN-γ, TNF-α
Th2 polarized	Facilitate antibody production, eosinophil activation, macrophage inhibition, and defence against parasites	IL-4, IL-33, IL-25	IL-4, IL-5, IL-10, IL-13
Th17 polarized	Pro-inflammatory	IL-6, TGF-β	IL-17, IL-22
Th9 polarized	Allergic reactions?	IL-4, TGF-β	IL-9
Th22 polarized	Allergic reactions?	IL-6, TNF-α	IL-22, IL-13, TNF-α
Treg-cells	Regulation of immune response	IL-10, TGF-β, IL-35	IL-10, TGF-β, IL-35
Cytotoxic T-cells ($CD8^+$)	Killer T-cells Recognize specific non-self MHC class I antigens	IFN-γ, CCL3/CCL4, IL-12, IL-18, antigen presentation	IFN-γ, TNF
B-cells	APC, formation of plasma cells and Memory B-cells	IL-4, IL-10, TNF-α	
Plasma cells	Immunoglobulin formation	IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-21, APRIL	IL-6, secreted immunoglobulins
Memory B-cells	Immunological memory	IL-4, , IL-6, absence of IL-10	

Cytokines that induce proliferation and are produced by the cells are listed in the two right columns respectively (40, 42, 46-52).
APC, antigen-presenting cell; DAMP, damage-associated molecular pattern; DC, dendritic cell; G-CSF, granulocyte-colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; ILC, innate lymphoid cell; M-CSF, macrophage colony-stimulating factor; MHC, major histocompatibility complex; NET, neutrophil extracellular trap; NK, natural killer; NKT, natural killer T-cell; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor;; TCR, T-cell receptor; TF, tissue factor; TGF, transforming growth factor; Th, T-helper; TNF, tumour necrosis factor; Treg, T-regulatory.

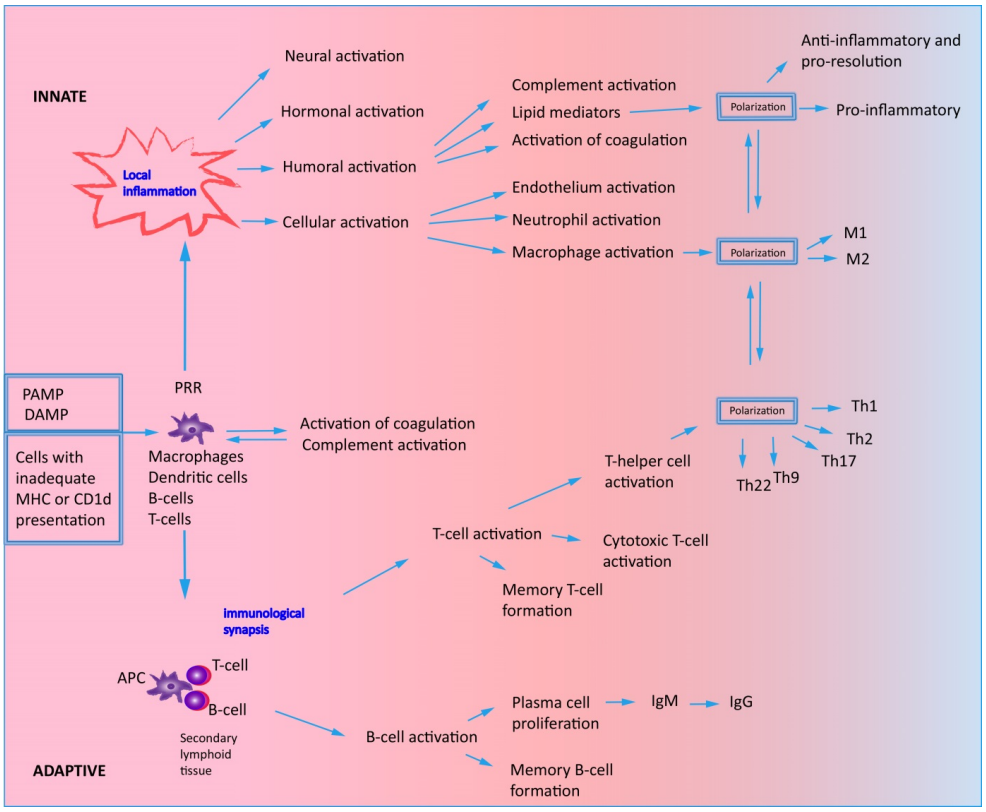


Figure 1. An overview of the inflammatory response.
The figure shows a selection of different cellular and non-cellular components of the inflammatory response. The immune response is normally initiated following expression of DAMP/PAMP or inadequate MHC/CD1d presentation. Endothelial components and coagulation are vital for the initiation, maintenance, and regulation of inflammation (p. 3). The main immunocompetent cells are described in the main text (p. 6–8), and relevant cytokines (p. 10), MMPs (p. 14), adhesion molecules (p. 15), and other non-cellular inflammatory mediators (p. 16) are described separately. The time course of the inflammatory process is dependent on the net stimuli from DAMPs/PAMPs, and local and systemic cellular and non-cellular components whereby macrophage polarization (M1 and M2), Th-cells (Th1, Th2, Th17), and lipid mediators (pro-inflammatory and anti-inflammatory/pro-resolution) determine and regulate the inflammatory environment. APC, antigen-presenting cell; DAMP, damage-associated molecular pattern; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor.

1.4 CYTOKINES INVOLVED IN INFLAMMATION

1.4.1 The cytokine system: classification and description

Cytokines can be classified based on their mechanism of action in the immune system, i.e. pro-inflammatory, anti-inflammatory, or adaptive, and can be subdivided into families based on their receptor types (Table 2) (1). Chemokines represent a separate subset of cytokines and are classified into four different families according to their structure (53-56). Hormones, however, are not easily distinguished from cytokines, as several hormones bind to interferon-type receptors and some are also classified as cytokines. As illustrated in Table 2, many cytokines have additional functions that are not reflected in their categorization of pro-inflammatory, anti-inflammatory, or adaptive. For this reason, their immunoregulatory functions are only referred to as 'a main function' or 'an important function', but not as 'the main function' or 'the most important function'. Similarly, publications included in this thesis have not used this strict cytokine classification of 'pro-inflammatory', 'anti-inflammatory', and 'adaptive'.

The main pro-inflammatory cytokines belong to the IL-1, IL-6, IL-17, interferon, and TNF families. The IL-1 family is essential for initiation of the inflammatory cascade (57, 58), and cytokines in the IL-6 family have both immunoregulatory, as well as other systemic, effects (59). The IL-17-family comprises six members that are pro-inflammatory (8). The three interferon families have anti-viral properties (60, 61), and the TNF family is essential for inflammatory activation (62). The main anti-inflammatory cytokines include those in the IL-10 (63) and IL-12 families (64-67), and there are four cytokines families classified as adaptive (Common γ-chain receptor ligand family, Common B-chain receptor ligand family, Shared IL-2β chain family, Shared receptors family) (68, 69).

Cytokines bind to their specific cell surface receptors, although several cytokine receptors also exist in soluble form and can be detected in serum and plasma (59). Soluble receptors are biologically active with inhibitory effects and thus compete with membrane-bound receptors for cytokine binding. Use of recombinant TNF-α receptor in the treatment of inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (62, 70) provides one example where such competition between soluble and membrane-bound receptors for cytokine binding is exploited therapeutically.

Table 2. Important cytokine families and their functions

	Pro-inflammatory							**Pro- and anti-inflammatory**	**Anti-inflammatory**	**Adaptive immunity**			
Family (1)	IL-1 (57, 58)	IL-6 (59)	TNF (62)	IL-17 (71, 72)	Type I IFN (60, 61)	Type II IFN (60, 61)	Type III IFN (60, 61)	IL-12 (63)	IL-10 (64-67)	Common γ-chain receptor ligand (68, 69)	Common B-chain receptor ligand (1)	Shared IL-2β chain (1)	Shared receptors (69)
Receptor superfamily	IL-1/TLR	Type I cytokine receptor	TNF receptor family	Type II cytokine receptor	Type II cytokine receptor	Type II cytokine receptor	Type II cytokine receptor	Type I cytokine receptor	Type II cytokine receptor	Type I cytokine receptor	Type I cytokine receptor	Type I cytokine receptor	Type I cytokine receptor
Important cytokine members	IL-1α IL-1β IL-1ra IL-18 IL-33 IL-36α IL-36 γ IL-36γ IL-36ra IL-37 IL-1Hy2	IL-6 IL-11 IL-27 IL-30 IL-31 CNTF CT-1 LIF OPN OSM	TNF-α TNF-β BAFF APRIL CD40L	IL-17A-F IL-25 (IL-17E)	IFN-α IFN-β IFN-ω IFN-κ Limitin	IFN-γ	IFN-λ1 (IL-29) IFN-λ2 (IL-28) IFN-λ3 (IL-28B)	IL-12 IL-23 IL-27 IL-35	IL-10 IL-19 IL-20 IL-22 IL-24 IL-26 IL-28 IL-29	IL-2 IL-4 IL-7 IL-9 IL-15 IL-21	IL-3 IL-5 GM-CSF	IL-2 IL-15	IL-13 TSLP
Important effects on immune cells (examples)	IL-1: activated following inflammasome activation. Pyrogenic; pro-inflammatory; stimulates macrophages, B-, T-, and NK cells	IL-6: broad inflammatory activation (acute phase); plasma cell differentiation; IgG production; Th17 differentiation	TNF-α: phagocyte cell activation; endotoxic shock. Activates neutrophils, eosinophils, and macrophages	T-cell proliferation; neutrophil activation; Th2 and Th17 chemokine production	IFN-γ: anti-viral effects. Increases MHC I and II expression; enhances Th1 differentiation			IL-12: activates T-cells, NK cell, Th1; pro-inflammatory IL-23: pro-inflammatory IL-27: mainly anti-inflammatory IL-35: anti-inflammatory	IL-10: inhibits cytokine production and mononuclear cell function; anti-inflammatory; promotes IgA	IL-2: proliferation and activation of T-, B-, and NK cells, Treg homeostasis IL-4: B- and mast-cell proliferation; Th2 responses	IL-5: B-cell and eosinophil proliferation	IL-2: T-, NK-, and B-cell proliferation	IL-13: mediate Th2 responses
Important effects on endothelial cells (examples)	Activates endothelium; induces expression of adhesion molecules	Activation	Activation		Activation; induces chemokine production (macrophages and T-cells attracted). Promotes CD4 adhesion; upregulation of MHC I and II presentation					IL-4: activates VCAM-1 expression			IL-13: activates VCAM-1 expression
Intracellular signalling (73)	MyD88, TIRAP/MAL, TRIF, TRAM, and SARM	JAK1, JAK2, TYK2, STAT3	FADD, TRADD, TRAF	JAK/STAT	JAK1, TYK2, STAT1, STAT2, STAT4	JAK1, JAK2, STAT1	STAT 1	JAK2, TYK2, STAT4	JAK1, STAT1	JAK1, JAK3, STAT5A/B	JAK2, STAT5A/B	STAT1, STAT2	JAK1, JAK2, TYK2, STAT6

Classification according to cytokine structure and pro- and anti-inflammatory properties is shown. Categorization based on pro- and anti-inflammatory properties reflects the main cytokine effects. A more detailed description of cytokine activation of different immune cells is given in Table 1. Chemokines are presented in Table 3. The cytokine superfamilies transforming growth factor beta (TGF-β) receptor and receptor tyrosine kinases are not shown. Most hormones also act via cytokine receptors, and a larger group of hormone receptors belong to the type I interferon receptor group. See text for further description.
GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; NK, natural killer; TSLP, Thymic stromal lymphopoietin; VCAM-1, vascular cell adhesion molecule-1.

1.4.2 The chemokine cytokine family

Chemokines were originally referred to as chemoattractants or chemotactic cytokines, as leukocyte recruitment and cellular migration towards an inflammatory focus relies on chemotactic concentration gradients (25, 53-56). As with many other cytokines, the exact function and overall biological effects of many chemokines highly depend on the biological context, e.g. the local cytokine network, as well as the microenvironment, and ultimately the activation status, of different communicating cells. Chemokines are involved in various disease processes, including infections and the development of cancer or vascular diseases.

Chemokines, together with their corresponding receptors, can be grouped into four classes, based on their shared structure and the number and position of conserved cysteine residues (i.e. CCL, CL, CXC, and CX3C chemokines and receptors) (74), as shown in Table 3. Most chemokines were originally named according to their functional characteristics, and although a new standardized nomenclature has been developed, many of these old names are still in use, some of which are given in parentheses alongside their corresponding new chemokine name in Table 3 (56). At the time when they were first described, chemokines were also categorized according to their functional properties, i.e. inflammatory, homeostatic, or dual-function chemokines (75, 76). Furthermore, a subgroup of inflammatory chemokines can be classified as either ELR+ (i.e. angiogenic) or ELR− (i.e. angiostatic) chemokines, based on the presence or absence, respectively, of the Glu-Leu-Arg motif (76). The ELR+ chemokines mediate proangiogenic effects through the CXCR1 and CXCR2 receptors expressed by endothelial cells. In contrast, the ELR− chemokines are antiangiogenic, thereby blocking new microvessel formation (76).

Table 3. Chemokines and their receptors

CC chemokine/receptor family

Chemokine	Function	Receptor
CCL1 (I-309)	I	CCR8, CCR11
CCL2 (MCP-1, MCAF)	I	CCR2
CCL3 (MIP-1α/LD78α)	I	CCR1, CCR5
CCL3L1 (LD78β)	I	CCR5
CCL4 (MIP-1β)	I	CCR5
CCL4L	I	CCR5
CCL4L1	I	CCR5
CCL5 (RANTES)	I, Pt	CCR1, CCR3, CCR4, CCR5
CCL6 (C-10)		CCR1, CCR2, CCR3
CCL7 (MCP-3)	I	CCR1, CCR2, CCR3
CCL8 (MCP-2)	I	CCR1, CCR2,CCR5,CCR11
CCL9 (MRP-2/MIP-1γ)		CCR1
CCL10 (MRP-2/MIP-1γ)		CCR1
CCL11 (eotaxin)	D	CCR3
CCL12 (MCP-5)	I	CCR2
CCL13 (MCP-4)	I	CCR1, CCR2, CCR3,CCR11
CCL14 (HCC-1)	Pt	CCR1
CCL15 (HCC-2, Lkn-1)	Pt	CCR1, CCR3
CCL16 (HCC-4, LEC)	U	CCR1
CCL17 (TARC)	D	CCR4
CCL18 (DC-CK1, PARC)	H	Unknown
CCL19 (MIP-3β, ELC)	H	CCR7, CCR11
CCL20 (MIP-3α, LARC)	D	CCR6
CCL21 (SLC)	H	CCR7, CCR11
CCL22 (MDC, STCP-1)	D	CCR4
CCL23 (MPIF-1)	P	CCR1
CCL24 (MPIF-2, eotaxin2)	H	CCR3
CCL25 (TECK)	H	CCR9, CCR11
CCL26 (eotaxin-3)	I	CCR3
CCL27 (CTACK, ILC)	H	CCR2, CCR3, CCR10
CCL28 (MEC)	H	CCR3, CCR10

CXC chemokine/receptor family

Chemokine	Function	Receptor
CXCL1 (GROα, MGSA-α)	I, ELR+	CXCR2N, CXCR1
CXCL2 (GROβ, MGSAβ)	I, ELR+	CXCR2
CXCL3 (GROγ, MGSAγ)	I, ELR+	CXCR2
CXCL4 (PF4)	Pt, ELR−	CXCR3
CXCL4L1 (PF4V1)	Pt, ELR−	CRCR3
CXCL5 (ENA-78)	I, ELR+	CXCR1, CXCR2
CXCL6 (GCP-2)	I, ELR+	CXCR1, CXCR2
CXCL7 (NAP-2)	Pt, I, ELR−	CXCR2
CXCL8 (IL-8)	I, ELR+	CXCR1, CXCR2
CXCL9 (Mig)	I, ELR−	CXCR3
CXCL10 (IP-10)	I, ELR−	CXCR3
CXCL11 (I-TAC)	I, ELR−	CXCR3
CXCL12 (SDF-1α/β)	H, ELR−	CXCR4, CXCR7
CXCL13 (BLC, BCA-1)	H, ELR−	CXCR3, CXCR5
CXCL14 (BRAK, bolekine)	H, ELR−	Unknown
CXCL15	U, ELR−	Unknown
CXCL16 (SR-PSOX)	I	CXCR6
CXCL17 (VCC1, DMC)	U	Unknown

C chemokine/receptor family

XCL1 (lymphotactin)	D	XCR1
XCL2 (SCM1-b)	D	XCR1

CX3C chemokine/receptor family

CX3CL1 (fractalkine)	D	CX3CR1

Chemokines are divided into four main classes (adapted from references (1, 76). Individual chemokines are presented according to the recent nomenclature (former names shown in parentheses), together with their corresponding receptors and function.
D, dual; ELR+, angiogenic; ELR−, angiostatic; I, inflammatory; H, homeostatic; Pt, platelet; U, unknown.

1.5 OTHER SOLUBLE MEDIATORS INVOLVED IN INFLAMMATION

1.5.1 Matrix metalloproteases and protease inhibitors

Matrix metalloproteases (MMPs) and protease inhibitors both interact with cytokine and adhesion molecule systems at various levels. Hence, one may argue that these mediators should be regarded as functional parts of the cytokine network. This point will be described in more detail below, but this functional crosstalk between proteases and cytokines supports the scientific strategy that one should investigate soluble mediator profiles involving various mediators, and not only at the level of individual mediators.

MMPs are zinc-dependent enzymes belonging to the metzincin superfamily of zinc endopeptidases that, to date, comprise 24 mammalian proteases (10, 77, 78). A common feature of MMPs is that they display broad and overlapping substrate specificity. MMPs are traditionally classified partly according to their substrate specificity and partly according to their cellular localization (Table 4), while there also exists an alternative classification based on their structure (77).

1.5.1.1 Proteases as part of the inflammatory response

MMPs are important in inflammatory responses through their regulation of inflammatory mediators, as well as in maintenance of the function and integrity of physical barriers (10). At a transcriptional level, there are several cytokines and chemokines sharing common regulators, and one example of this transcriptional crosstalk is the regulatory action of nuclear factor kappa B (NF-κB), which induces MMPs, as well as the chemokines CCL2–4, CXCL1, and CXCL8 (79-81). When activated, MMPs exert direct effects on cytokines and chemokines. Cytokine activation, modulation, and inactivation by MMPs are summarized in Table 4. MMPs activate various cytokines, such as TNF-α, IL-1β, CXCL8, CXCL1, and TGF-β, through cleavage of cytokine pro-enzymes. Other mediators, such as CXCL5, insulin-like growth factor (IGF), and vascular endothelial growth factor (VGEF), are released from the extracellular matrix (ECM) through matrix degradation by MMPs. Inactivation of cytokines, such as IL-1β and CXCL5/6/12, by MMPs occurs through cleavage, while cleavage of other chemokines, such as CCL2/7/8/13, by MMPs results in the formation of the corresponding chemokine receptor antagonists.

1.5.1.2 ADAMs (A Disintegrin and A Metalloproteases)

ADAMs are proteases closely related to MMPs. Over 40 family members have been characterized, approximately half of which with zinc-dependent activity (82). ADAM17, formerly known as TNF-α-converting enzyme (TACE), is one of the most important and most closely regulated ADAMs. It is a major contributor to cleavage of membrane-bound cytokines, chemokines, growth factors, and their

receptors, thus playing an important role in the inflammatory process (83, 84). In addition, both MMPs and ADAM17 are important in the shedding and formation of soluble adhesion molecules (85).

Table 4. Effects of matrix metalloproteases on cytokines

	Activates/increases latent cytokine cleaved or released from ECM	Inactivates/forms receptor antagonist
Collagenases		
MMP-1	TNF-α, IGF, VEGF	IL-1β^{+}, CCL2^{++}, CCL7^{++}, CCL8^{++}, CCL13^{++}, CXCL12^{+}
MMP-8	CXCL5, CXCL8	
MMP-13	TNF-α, TGF-β	CCL7^{++}, CXCL12^{+}
Gelatinases		
MMP-2	TNF-α, TNF-β, IL-1β, IGF, VEGF	IL-1β^{+}, CCL7^{++}, CXCL12^{+}
MMP-9	TNF-α, IL-1β, CXCL8*, TGF-β, IGF, VEGF	IL-1β^{+}, IL-2Rα^{+}, CXCL5^{+}, CXCL6^{+}, CXCL12^{+}
Stromelysins[1]		
MMP-3	TNF-α, IL-1β, IGF, TGF-β, EGF, VEGF, osteopontin	IL-1β^{+}, CCL2^{++}, CCL7^{++}, CCL8^{++}, CCL13^{++}, CXCL12^{+}
Stromelysin-like		
MMP-11	IGF	
MMP-12	TNF-α	
Matrilysins[1]		
MMP-7	TNF-α, EGF, CXCL1, osteopontin, VEGF	
Transmembrane[1]		
MMP-14		CCL7^{++}, CXCL12^{+}
MMP-16	VEGF	
MMP-17	TNF-α	
MMP-19-like		
MMP-19	VEGF	
ADAM17		
	TNF-α, CX3CL1, RANKL	

MMPs are arranged based on the traditional classification, and the important cytokines affected and their mechanisms are shown. See text for further description of the mechanisms of cytokine activation, modulation, or inactivation. The table is based on references (10, 77, 78).
[1]MMPs not included in the table: stromelysins (MMP-10), matrilysins (MMP-26), transmembrane (MMP-15, MMP-24, MMP-25), GPI-like (MMP-17, MMP-25), MMP-19-like (MMP-28), and other (MMP-18/20/23).
*Potentiated/increased bioactivity. $^{+}$Cytokine cleaved and inactivated. $^{++}$Cytokine cleaved and receptor antagonist formed.
ADAM, A Disintegrin and A Metalloproteases; ECM, extracellular matrix; EGF, epidermal growth factor; IGF, insulin-like growth factor; IL, interleukin; RANKL, receptor activator of nuclear factor kappa-B ligand; TGF, transforming growth factor; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

1.5.2 Soluble adhesion molecules

Adhesion molecules are important for cell-to-cell contact and intercellular communication. They exist as soluble isoforms as a result of proteolytic enzyme activity in the process of shedding, although soluble adhesion molecules can also be directly encoded as their distinct isoforms, independent of shedding (85). The adhesion molecule family include selectins (L-, E-, and P-selectins) and various immunoglobulin (Ig) family members (e.g. VCAM-1, ICAM-1/2) (Table 5). E-selectin is expressed by endothelial cells, L-selectin by leukocytes, and P-selectin by endothelial cells and platelets, and they are all induced by pro-inflammatory mediators. Selectins are important for leukocyte rolling, and P-selectin is also involved in thrombus formation and intravascular coagulation during infection. Ig family members are of particular importance in firm adhesion and trans-endothelial migration of leukocytes, and both ICAM-1 and VCAM-1 are expressed by endothelial cells.

1.5.2.1 Importance of shedding of adhesion molecules in balancing the inflammatory reaction

During an inflammatory response, rapid shedding of adhesion molecules gives rise to their soluble isoforms which act as competitive inhibitors, reducing leukocyte adhesion and the inflammatory response. Several sheddases have been identified (e.g. caspases, ADAM17, MMPs, neutrophil elastase

(NE)), as shown in Table 5. The shedding response is required for regulating inflammatory responses, and there needs to be a balance between the anti-inflammatory effects of shedding and pro-inflammatory mechanisms, as aberrant shedding can lead to sustained inflammation.

Table 5. Adhesion molecules

Adhesion molecule	Cellular expression	Ligands	Induction by inflammatory mediators	Expression	Biological function	Sheddases
E-selectin	Endothelial cells	ESL-1, PSGL-1	TNF-α, LPS, IL-1	Inducible	Rolling	Caspase
L-selectin	Leukocytes	GlyCAM-1, MAdCAM-1	TNF-α, LPS, IL-1, IL-6	Constitutive, inducible	Rolling	ADAM17
P-selectin	Platelets and endothelial cells	PSGL-1	TNF-α, IL-4, IL-13, histamine, thrombin	Constitutive	Rolling	MMP
ICAM-1	Endothelial cells	Mac-1, LFA-1	TNF-α, LPS, IL-1	Constitutive, inducible	Firm adhesion, TEM	ADAM17, NE
VCAM-1	Endothelial cells	VLA-4	TNF-α, LPS, IL-1	Constitutive, inducible	Firm adhesion, TEM	ADAM17, NE

ADAM, A Disintegrin and Metalloprotease; ESLG-1, E-selectin ligand; GlyCAM-1, Glycosylation-dependent cell adhesion molecule-1; IL, interleukin; LFA, lymphocyte function-associated antigen; LPS, lipopolysaccharide; MAdCAM-1, mucosal vascular addressin cell adhesion molecule-1; MMP, matrix metalloprotease; NE, neutrophil elastase; PSGL-1, P-selectin glycoprotein ligand-1; TEM, trans-endothelial migration; TNF, tumour necrosis factor, VLA-4, very late antigen-4.
Adapted from reference (85).

1.5.3 Other non-cellular inflammatory mediators

The complement system is an important component of the innate immune system and is distributed both in blood and intestinal fluids. It recognizes specific PAMPs, initiates a proteolytic cascade reaction that targets microorganisms, and activates pro-inflammatory responses that help to initiate the cellular innate immune response (86). **Lipid metabolites** play a key role in inflammation and are capable of driving the inflammatory process in either a pro-inflammatory or an anti-inflammatory (i.e. resolution) direction, depending on which metabolites are activated, although all are derived from phospholipids–arachidonic acid (87). Serum **miRNAs** are soluble mediators that help regulate the inflammatory response. miRNAs regulate gene expression through their effects on translation and transcription of target mRNAs. Several studies have suggested that differences in miRNA expression are associated with disease stage and prognosis in sepsis as well as in other diseases, e.g. rheumatoid arthritis, Crohn's disease, and various cancers (88-91). Another non-cellular inflammatory mediator is the **exosome** which is a cell-derived microvesicle and involved in cell-to-cell communication (92).

1.6 INTERACTION BETWEEN DIFFERENT SIGNALLING CASCADES

Different signalling cascades, as well as different mediators, can and will interact with each other. Therefore, at a cellular level, it might be difficult to predict the end-response of a single cytokine from a given cell. To emphasize the complexity of these interactions, some important interactions between different signalling cascades will be presented. The complexity of these interactions is a background for the rationale of combining different mediators, thereby presenting a clearer picture of the inflammatory response.

1.6.1 Chemokines: biased signalling and functional selectivity

Inflammatory chemokines are promiscuous, so blocking of a single chemokine receptor will inhibit the actions of several chemokine, and similarly neutralization of a single chemokine will affect the signalling of several receptors. Binding to a chemokine receptor can lead to the activation of several downstream intracellular signalling pathways. However, the relative strength of this activation may differ among the various downstream pathways, depending on the ligand, receptor, or tissue type involved. This functional selectivity relating to the activation of downstream signalling pathways is termed biased signalling (93). Ligand bias describes the process whereby different chemokines, i.e. ligands, bind to the same receptor, with the activated intracellular signalling downstream to the receptor depending on the ligand. Receptor bias is the process whereby the same ligand binds to different receptors, with the ligand-initiated intracellular signalling depending on the receptor activated. Tissue bias refers to the process whereby the same receptor–ligand complex is activated, with the activated signalling pathway depending on the tissue type. Although highly complex, the concept of biased signalling is important in helping us to understand chemokine and receptor interactions. It is important to keep in mind that biased signalling is not necessarily absolute. To reiterate the above-mentioned point, several intracellular pathways may be activated at the same time by chemokine receptor binding, but not to the same relative strength of activation.

1.6.2 Cross-communication between different signalling systems

G protein-coupled receptors (GPCRs) and tyrosine kinase receptors (TKRs) are two major classes of cell surface transmembrane proteins. Crosstalk between these receptors helps to modulate their downstream intracellular receptor signalling (76, 94). Their transactivation can be bidirectional, i.e. TKRs and GPCRs form complexes that are the structural basis for the crosstalk. Several forms of transactivation have been described. Transactivation of TKRs by GPCRs is mediated both via ligand-dependent (through MMPs and ADAMs) and ligand-independent mechanisms (through reactive oxygen species (ROS) or intracellular tyrosine kinases). Functional transactivation of TKRs through ROS represents a possible pharmacological target to alter cell growth and proliferation in cancer (76,

94). TKRs may also mediate GPCR transactivation through complex formation. These TKR–GPCR complexes become internalized and subsequently initiate downstream intracellular signalling cascades. Cross-communication is also important between GPCRs and Toll-like receptors (TLRs) in macrophages in PAMP recognition whereby crosstalk modifies the signalling cascade that regulates the expression of chemokines and chemokine receptors, as well as other cytokines.

1.6.3 Trans-signalling

IL-6 is expressed in a wide range of immunocompetent cells, as well as other cell types, including mononuclear phagocytes, B- and T-cells, fibroblasts, endothelial cells, keratinocytes, hepatocytes, and various bone marrow cells (59). IL-6 binds to its specific receptor, an IL-6-binding chain (termed IL-6 receptor (IL-6R) which exists either as a membrane-bound receptor in very few cell types only, hepatocytes, neutrophils, monocytes and CD4 + T-cells, or in a circulating soluble form. IL-6 binding to the membrane-bound IL-6R with subsequent initiation of downstream intracellular signalling is termed classical signalling. IL-6 can also bind to the soluble IL-6R, forming a receptor–ligand complex which, in turn, binds to membrane-bound gp130 protein, which is widely expressed by all cell types, thereby initiating transactivation or trans-signalling (59). Thus, in contrast to many other soluble cytokine receptors, the soluble IL-6R has signal-initiating, and not inhibitory, effects. Traditional activation is particularly important in inflammation and haematopoiesis, whereas trans-signalling is important in several tissue types in inflammation and tissue repair (59). Therapeutic blockade of IL-6 is now used in the treatment of chronic inflammatory diseases, although (to date) not in the treatment of cancer or sepsis (95).

1.6.4 Downstream intracellular signalling and pathway crosstalk

Receptor ligation can initiate downstream signalling through intracellular signal transduction pathway(s), resulting in target gene activation. In this section, a brief overview of some important signal transduction pathways for the different receptor superfamilies listed in Table 2 is given. Downstream signalling following receptor activation can be modulated by signalling cascades initiated by ligation of other receptors. It is therefore often more correct to refer to signalling networks of highly interconnected signalling pathways that may exert crosstalk both at the receptor level (receptor transactivation; see above) and between downstream signalling pathways, as illustrated by the examples described below.

JAK/STAT pathways. The Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) pathways are activated by over 50 cytokines and hormones, mainly by types I and II cytokine receptors (Table 2). In mammals, there exist three JAK (JAK1–3) and seven STAT proteins (STAT1–4, STAT5a, STAT5b, and STAT6) (96). Upon binding to their transmembrane receptors, JAKs are activated, which triggers the activation of downstream STAT pathways. Although this relatively small number of different ‘building blocks’ can initiate apparently similar signal transduction pathways,

these activated pathways can lead to fundamentally different biological responses through activation of different genes. Several important processes underlie this wide difference in biological responses. Even though different cytokines activate the same STAT pathways, there may be lineage-dependent differences relating to gene activation. Similar cytokines and receptors may activate the same pathway in the same cell and yet still lead to different outcomes, possibly due to differences in signal intensity and/or duration. Many cytokines often give heterogeneous STAT activation whereby several STAT pathways are activated, in addition to the main pathway. This opens for crosstalk between cytokines and signalling pathways, as different cytokine/receptor combinations and cytokine gradients can influence the same pathway(s). An example of the dichotomous outcome resulting from the activation of one same signalling pathway is the case of IL-6 (family) and IL-10. Both activate the STAT3 pathway whereby IL-6 affects immunocompetent cells and induces a pro-inflammatory response, whereas IL-10 produces an anti-inflammatory response (96).

TLR and IL-1 signalling. IL-1 receptors share structural homology with TLRs. TLRs play an important role in innate immunity through their recognition of exogenous PAMPs and DAMPs, as well as a wide range of endogenous ligands (97). Upon receptor activation by a ligand, the intracellular signalling pathway is activated via a downstream cascade, resulting in pro-inflammatory signalling through effects on downstream signalling and induced transcription of pro-inflammatory genes. These pro-inflammatory effects include induction of adhesion molecules, cyclo-oxygenase type 2 (COX-2), chemokines, cytokines, tissue-degrading enzymes, and synthesis of nitric oxide (NO). In addition to the inflammatory response, the cascade also induces apoptosis of surrounding cells. This final step in the signalling pathway is considered a possible therapeutic target in cancer and inflammatory and autoimmune diseases (57).

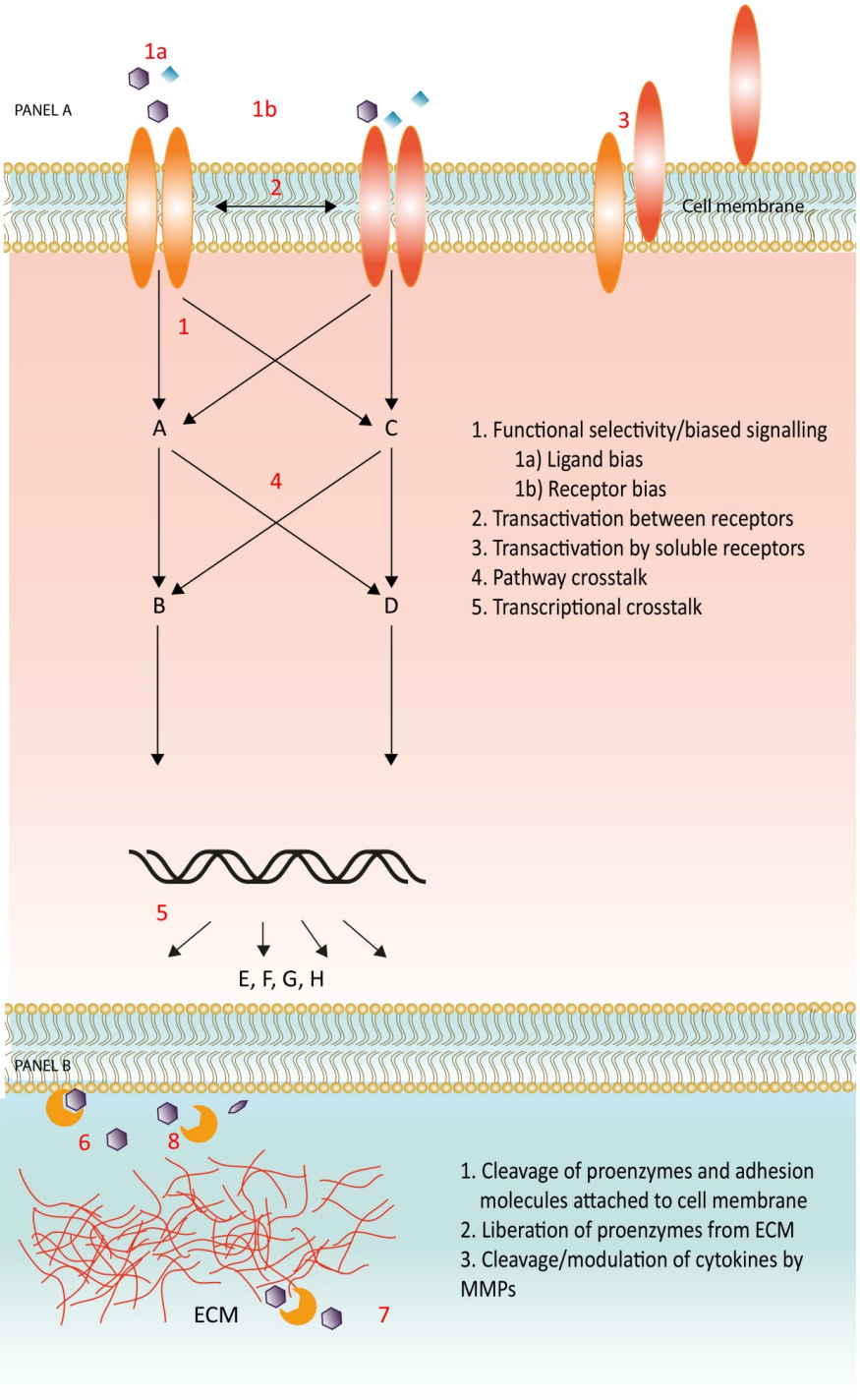
TNF receptor signalling. The TNF superfamily has 19 cytokine members, and 29 TNF receptors have been identified so far (70). TNF can act as a pro-inflammatory mediator, as well as inducing cell death either through classical or regulated apoptosis (necroptosis). The intracellular signalling pathways are complex, with the downstream pathways of the TNF receptors 1 and 2 (TNFR1 and 2) being the best characterized. The pro-inflammatory and anti-apoptotic signalling downstream to TNFR1 involves canonical nuclear factor kappa B (NF-κB) activation. Pro-apoptotic signalling requires certain active co-factors (receptor-interacting serine/threonine protein kinase 1 (RIPK1) and active caspases), whereas necroptosis requires RIPK1 and inactive caspases. This is an example of receptor activation with different end-results, depending on the type of downstream co-factors. Therapeutic TNF inhibitors are effective in treating chronic inflammatory diseases, although side effects are common. Therefore, specific targeting of downstream intracellular signalling is currently under investigation in clinical trials, to develop alternative therapeutic strategies to TNF neutralization (62, 70).

It is outside the scope of this Introduction section to describe in detail receptor-initiated downstream signalling for all cytokines investigated as part of the research work presented in this thesis. However, the previously described crosstalk at the receptor level (i.e. receptor transactivation), together with the few examples given above of common downstream signalling pathways for various cytokine receptors, and the crosstalk between various intracellular pathways, demonstrate the relevance of our scientific rationale for investigating soluble mediator profiles, rather than individual cytokines, in translational and clinical studies.

Figure 2. Cross-interaction between cytokines, adhesion molecules, and matrix metalloproteases.

Panel (A) shows trans-signalling between cytokines at different stages in the signalling cascade. Ligand bias (1a): a receptor can activate different signalling pathways (A, C), depending on the ligand attached (chemokines, p. 17). Receptor bias (1b): the same ligand can bind to different receptors and result in activation of different signalling pathways, depending on the receptor (A, C) (chemokines, p.17). Transactivation between receptors (2): different receptors interact and modulate the resulting signalling pathways (A, C) (e.g. GPCR/TKR/TLR interaction; p. 17). Transactivation by soluble receptors (3): soluble receptors can fuse with membrane-bound receptors, thus activating new signalling pathways (e.g. soluble IL-6R, p.18). Pathway crosstalk (4): different cytokines can affect the same downstream signalling pathway, with the end-result depending on the net impact from several cytokines (e.g. JAK/STAT pathways; p. 18). Transcriptional crosstalk (5): cytokines and chemokines share common transcriptional regulators, and activation of pro-inflammatory genes is often coupled, with several inflammatory mediators (E, F, G, and H) often activated simultaneously (e.g. NF-κB activation).

Panel (B) shows (1) activation of membrane-bound proenzymes and adhesion molecules by MMPs, (2) MMP-mediated liberation of ECM-bound proenzymes, and (3) inactivation or modulation of cytokines by MMPs (p. 14).


PANEL A
1a
1b
2
3
Cell membrane
1
A
C
4
B
D
5
E, F, G, H
1. Functional selectivity/biased signalling
1a) Ligand bias
1b) Receptor bias
2. Transactivation between receptors
3. Transactivation by soluble receptors
4. Pathway crosstalk
5. Transcriptional crosstalk
PANEL B
6
8
ECM
7
1. Cleavage of proenzymes and adhesion molecules attached to cell membrane
2. Liberation of proenzymes from ECM
3. Cleavage/modulation of cytokines by MMPs

1.7 MULTIPLE MYELOMA

1.7.1 Treatment of patients with multiple myeloma

Multiple myeloma is a plasma cell neoplasia that accounts for approximately 1% of all cancers and 13% of haematological malignancies (98-100), and its onset may be preceded by monoclonal gammopathy of undetermined significance (MGUS) or a solitary myeloma. The local bone marrow microenvironment and interactions with bone marrow stromal cells are important factors influencing the development of multiple myeloma (4, 99-103). The neoplastic cells show plasma cell or plasma blast morphology, and analysis of the Ig variable regions indicates that multiple myeloma clones do not undergo somatic hypermutation and thus originate from a late stage in plasma cell development, although precursor determinants for multiple myeloma are also found in pre-B-cell stages (4, 99, 100, 103).

Indications for treatment of multiple myeloma include symptomatic disease or the development of complications, e.g. pain, anaemia, hypercalcaemia, symptomatic local tumours, pathological fractures, or renal failure. Standard care for patients below 65 years of age (biologic age) with multiple myeloma and no comorbidity usually comprises initial induction chemotherapy to at least stabilize the disease and preferably to achieve partial or complete response (104). This is followed by mobilization and harvesting of peripheral blood stem cells, conditioning treatment, and finally autologous stem cell transplantation. It is generally accepted that autotransplantation should be undertaken relatively early in myeloma treatment, whereas there is no general agreement regarding optimal late treatment. Late treatment includes the use of immunomodulatory drugs (i.e. thalidomide, lenalidomide, and pomalidomide), proteasome inhibitors (i.e. bortezomib, carfilzomib), histone deacetylase inhibitors, or conventional cytotoxic drugs, either alone or in combination, and all of which often in combination with steroids (105-111). So far, conventional chemotherapy and autologous stem cell transplantation have not been shown to be curative; however, despite this, allogeneic stem cell transplantation is used in a minority of multiple myeloma patients (98, 103, 104, 112-114). Stem cell harvesting is a multistep process that can be undertaken by pre-treatment using cyclophosphamide plus granulocyte-colony stimulating factor (G-CSF) or alternatively G-CSF alone, whereas plerixafor (a CXCR4 inhibitor) can be added as an option for patients with insufficient mobilization of $CD34^+$ cells by chemotherapy and G-CSF treatment (104). Finally, it is also important to remember that irradiation therapy is usually effective for the treatment of local complications of multiple myeloma (105), as well as bisphosphonates which are also part of the treatment regime for many patients (115).

1.7.2 The cytokine network in multiple myeloma

Several adhesion molecules and cytokines play an important role in crosstalk between myeloma cells and their neighbouring stromal cells in the bone marrow microenvironment, thus helping to support

and maintain myeloma cell growth. Among the key cytokines involved in the cytokine network are IL-6, IL-15, IL-21, TNF-α, CCL3, VEGF, TGF-β, and IGF-1 (4, 103). Particularly important is IL-6 that functions both in paracrine and autocrine signalling loops, thereby contributing to myeloma cell growth, survival, and drug resistance. IL-6 is therefore considered as a possible pharmacological target in multiple myeloma (4). Moreover, studies have shown a correlation between TNF-α levels and disease severity, thus underlining the importance of TNF-α in the disease process (103). Several chemokine receptors (e.g. CCR1, CCR5, and CXCR3) are also expressed by myeloma cells and are involved in myeloma cell growth and survival, thereby contributing to disease progression through their functional effects on myeloma cells. CXCL12/CXCR4 is possibly the most important interaction that directs both bone marrow homing of myeloma cells and the interaction between myeloma cells and osteoclasts, thus contributing to clinical manifestation of the disease and osteoclast-mediated skeletal destruction (116-120). Several studies have investigated the systemic levels of cytokines in myeloma patients, and from their findings, it seems justified to make the general conclusion that high cytokine levels correlate with aggressive, extensive, and/or symptomatic disease. Disease progression in multiple myeloma is also likely associated with an immunocompromised status with decreased innate and adaptive immune responses due to functional impairment of monocytes and dendritic cells and consequently their release of immunoregulatory cytokines (121-123).

Furthermore, several studies have demonstrated that the systemic levels of soluble ECM molecules are also altered in multiple myeloma, which may have a prognostic impact in myeloma patients (124-128). These molecules also bind to various cytokines, thus serving as a local extracellular cytokine reservoir (129). However, the functional importance of this crosstalk between cytokines and the ECM molecules in the bone marrow in multiple myeloma has not been elucidated yet.

1.7.3 The cytokine network and haematopoietic stem cell harvesting

1.7.3.1 Pre-harvesting effects

As described earlier (p. 22), G-CSF treatment, either alone or in combination with chemotherapy or plerixafor, is used for stem cell mobilization to peripheral blood. Such treatment affects both the systemic cytokine network as well as the local bone marrow network (104, 130), thus exerting a wide range of effects. Firstly, there is an expansion of neutrophils while adhesion molecules and chemokines that maintain stem cells in the bone marrow are cleaved and thus inactivated by proteolysis. Secondly, reduced chemokine levels indirectly modulate the effects of neural innervation on various bone marrow cells, e.g. macrophages, osteoblasts, and osteoclasts (131). Use of chemotherapy (e.g. cyclophosphamide), in combination with G-CSF, as part of the mobilization regimen results in an increase in the circulating stem cell number during early haematopoietic reconstitution. This is a commonly used regimen for stem cell mobilization whereby G-CSF then

further increases the stem cell yield (104, 132, 133). However, as a chemotherapeutic and cytotoxic drug, cyclophosphamide will also exert additional effects on various immunocompetent cells, in addition to its stem cell-mobilizing effects.

1.7.3.2 Stem cell harvesting and the cytokine network

Both multiple myeloma with induction of a pro-inflammatory response as well as the pre-harvesting anti-myeloma treatment likely induce changes in the systemic, as well as the local bone marrow, cytokine network, but little is known about modulation of the cytokine network in response to stem cell mobilization and harvesting (19). One problem when treating multiple myeloma patients with autologous peripheral blood stem cell transplantation is contamination of the stem cell graft with circulating myeloma cells. This risk of contamination can be mitigated by, plasma cell reduction and depletion (negative selection) or $CD34^+$ cell enrichment (positive selection). However, neither positive nor negative selection seems to have any additional effects on patient survival, possibly because residual malignant cells in the patients are more important for time elapse until symptomatic disease progression or because the depletion and enrichment procedures do not help to improve time span until disease relapse (134, 135). A more recent strategy is purging of myeloma cells from stem cell grafts using oncolytic myxoma virus (136). Moreover, myeloma cells also respond to exogenous cytokines, as described earlier (p. 22). Chemotherapy-induced, as well as mobilization and apheresis-induced cytokine modulation may then, at least theoretically, influence the survival or proliferation of dormant myeloma cells, thereby having a prognostic impact. In this context, characterization of treatment-induced cytokine modulation in autotransplanted myeloma patients is therefore relevant.

1.8 VENOUS THROMBOSIS AND INFLAMMATORY MEDIATORS

Venous thromboembolism (VTE) is one of the most common haematological conditions. It is an important cause of death, accounting for over 500 000 deaths per year in the European Union (137). Deep vein thrombosis (DVT) and pulmonary embolism (PE) are difficult to diagnose, with undiagnosed VTE therefore representing an increased risk of death (137, 138). Improved risk stratification and diagnostic tools are important measures for VTE treatment and prevention (13).

Known risk factors for VTE include familial thrombophilia and acquired factors such as malignancies, previous VTE, reduced mobility, trauma or surgery, old age, pregnancy, heart failure, myocardial infarction, ischaemic stroke, obesity, and use of oral contraceptives (139-141). Several of these risk factors represent inflammatory conditions (5, 12). Emerging evidence suggests infection as a more important risk factor for VTE than previously recognized, and coagulation may play a major role in immune defence (3, 142). To better understand the pathophysiology of VTE and to identify improved diagnostic biomarkers for venous thrombosis, further studies on the relationship between inflammation and coagulation are needed (143).

In the following sections, findings from previous studies on inflammatory biomarkers in VTE, in particular cytokines, adhesion molecules, and MMPs, in relation to the predisposition to, and diagnosis and prognosis of, VTE are summarized.

1.8.1 Cytokines in venous thrombosis

It has been established that genetic factors affecting the coagulation system (e.g. factor V Leiden and prothrombin) are predisposing risk factors for VTE (141, 144). Case-control studies also showed that a number of single nucleotide polymorphisms (SNPs) affecting cytokine genes are associated with an increased risk, and others with a reduced risk, of VTE (141, 145-148). A number of small case-control studies demonstrated increased levels of pro-inflammatory, or decreased levels of anti-inflammatory, cytokines in patients at risk for DVT (149-151), although in a larger, prospective population-based case-control study, no such associations were found (152). Increased levels of pro-inflammatory cytokines were detected both in animal models of acute venous thrombosis (153) and clinical trials, although with no evident diagnostic value (5, 146, 154-157). Inflammation is considered as an essential response during venous thrombosis formation and resolution (158-160), and it is possible that IL-6 could represent a therapeutic target in the prevention of post-thrombotic syndrome, as suggested by a previous study using an animal model of DVT (161). Findings from selected clinical and experimental studies are summarized in Table 6 to illustrate the importance of cytokines in venous thrombosis.

Table 6. Cytokines and venous thrombosis

	Predisposing factor	Acute reaction and diagnostic use	Effect on thrombus resolution
IL-1α	**Beckers et al. (2010):** −899C/T ↓ SNP: 108 DVT vs 325 controls (145)		
IL-1β	**Zee et al. (2009):** rs1143634 ↓ SNP in DVT in larger cohort (141) **Christiansen et al. (2006):** ↔ 506 DVT vs 1464 controls (152) **van Minkelen et al. (2007):** IL1RN-H5H5 ↑ Leiden thrombophilia study (162)		
IL-4	**Beckers et al. (2010):** −589 T allele ↑ SNP: 108 DVT vs 325 controls (145)		
IL-6	**Christiansen et al. (2006):** ↔ 506 DVT vs 1464 controls (152) **Beckers et al. (2010):** −174 CC ↑ SNP: 108 VTE vs 325 controls (145) **Malaponte *et al.* (2013):** −174 G > C ↑ SNP**:** 130 DVT^+ and 190 DVT^- (cancer patients) vs 215 controls (148) **Matos et al. (2011):** −174 GC ↑ SNP: 119 VTE vs 126 controls (146) **Vormittag et al. (2006):** −174 G > C ↔ SNP: 128 DVT, 105 PE vs 122 controls ↔ IL6: 128 DVT, 105 PE vs 122 controls (144) **Mahemuti et al. (2012)/Yadav et al. (2015):** CC –572 G/C ↑ 140/246 VTE vs 160/292 controls, respectively (163, 164) **Matsuo et al (2015):** ↑ IL6, 200 ovarian cancer predictor for VTE (165)	**Vormittag et al. (2006):** −174 G > C ↔ 128 DVT, 105 PE vs 122 controls (144) **Matos *et al.* (2011):** ↑ 84 VTE vs 100 healthy (156) **Jezovnik et al. (2010):** ↑ 49 VTE vs 48 healthy controls (154) **Roumen-Klappe et al. (2002):** ↑ 40 DVT^+ vs 33 DVT^- (5) **de Franciscis et al (2015)** ↑ 201 DVT vs 60 controls (166) **Du T (2014):** ↑ abdominal cancer, post-operative (40 DVT vs 40 non-DVT vs 40 controls) (157) **Wik et al. (2016):** ↔ 181 cases vs 313 controls (167)	**van Aken et al. (2000):** ↑ 182 recurrent VTE vs 350 controls (151) **Jezovnik et al. (2012):** ↑ in post-thrombotic syndrome, 49 DVT (160) **Wojcik et al. (2011):** ↑ in post-thrombotic syndrome, 136 DVT (mice) (161) **Shbaklo et al. (2009):** ↑ in post-thrombotic syndrome, 387 DVT(159) **Roumen-Klappe et al. (2009):** ↑ in post-thrombotic syndrome, 110 DVT patients (158) **de Franciscis et al (2015):** ↑ 201 DVT vs 60 controls (166) **Wik et al. (2016):** ↔ 181 cases vs 313 controls (167) **Jezovnik et al (2017):** ↑ 43 DVT vs 43 controls(168)
IL-8/ CXCL8	**Christiansen et al. (2006):** ↔ 506 VTE vs 1464 controls (152) **Matos et al. (2011):** −251AT ↑ SNP: 119 VTE vs 126 controls (146) **van Aken et al. (2002):** ↑ 474 DVT vs 474 controls (155)	**Jezovnik et al. (2010):** ↑ 49 VTE vs 48 healthy controls (154) **Roumen-Klappe et al. (2002):** ↑ 40 DVT^+ vs 33 DVT^- (5) **Wik et al. (2016):** ↔ 181 cases vs 313 controls (167)	**van Aken 2000, 2002:** ↑ 182 recurrent VTE vs 350 controls (151, 155) **Wik et al. (2016):** ↔ 181 cases vs 313 controls (167) **Jezovnik et al (2017):** ↑ 43 DVT vs 43 controls(168)
IL-10	**Proctor et al. (2006):** ↓ in VTE group in trauma cohort (149) **Christiansen et al. (2006):** ↔ 506 VTE vs 1464 controls (152) **Zee et al. (2009):** rs1800872 ↑ SNP IL-10 in DVT cohort (22 413 women) (141) **Tang et al. (2014):** −1082GG genotype ↓ in 660 DVT vs 660 controls (169)	**Du T et al. (2014):** ↓ abdominal cancer, post-operative (40 DVT vs 40 non-DVT vs 40 controls) (157) **Wik et al. (2016):** ↔ 181 cases vs 313 controls (167)	**Wik et al. (2016):** ↔ 181 cases vs 313 controls (167) **Jezovnik et al (2017):** ↓ 43 DVT vs 43 controls(168)
IL-12p70	**Christiansen et al. (2006):** ↔ 506 VTE vs 1464 controls (152)		
IL-13	**Beckers et al. (2010):** IL-6 −174CC ↑ SNP: 108 VTE vs 325 controls (female) (145)		
CCL2/ MCP-1	**Matos *et al.* (2011):** −2518AG ↑ SNP: 119 VTE vs 126 controls (146)	**Wik et al. (2016):** ↔ 181 cases vs 313 controls (167)	**van Aken et al. 2000:** ↑ 182 recurrent VTE vs 350 controls (151) **Wojcik et al. (2011):** ↑ in post-thrombotic syndrome, 136 DVT (mice) (161) **Wik et al. (2016):** ↔ 181 cases vs 313 controls (167)
TNF-α	**Ferroni et al. (2012):** ↑ TNF-α in VTE in cancer cohort (150)	**Jezovnik et al. (2010):** ↔ 49 VTE vs 48 controls (154) **de Franciscis (2015)** ↑ 201 DVT vs 60 controls (166)	**Jezovnik et al (2017):** ↑ 43 DVT vs 43 controls(168)
IFN-γ			**Nosaka et al. (2011):** ↑ IFN-γ enhances thrombus resolution in mice through enhanced MMP9 and VEGF expression in mice (170)
TNFSF4	**Malarstig et al. (2008):** SNP ↑ (921C > T), ↓ (rs3850641) 344 DVT vs 2269 controls (147)		
NF-κB		**Du T et al. (2014):** ↑ abdominal cancer, post-operative (40 DVT vs 40 non-DVT vs 40 controls) (157)	
TGF-β1		**Wik et al. (2016):** ↔ 181 cases vs 313 controls (167)	**Wik et al. (2016):** ↔ 181 cases vs 313 controls (167)
PDGF		**Wik et al. (2016):** ↔ 181 cases vs 313 controls (167)	**Wik et al. (2016):** ↔ 181 cases vs 313 controls (167)

This table summarizes selected key human and animal studies of cytokine response in venous thrombosis. Arrows indicate the following: the cytokine/genetic polymorphism coding for the cytokine is elevated/more frequent (↑) or decreased/less frequent (↓) or unchanged (↔) in DVT cohorts as a predisposing factor (left column), as part of the acute reaction (middle column), or as a risk factor for post-thrombotic syndrome or recurrent DVTs (right column). IFN, interferon; IL, interleukin; NF-κB, nuclear factor kappa B; PDGF, platelet-derived growth factor; SNP, single nucleotide polymorphism; TGF, transforming growth factor; TNF, tumour necrosis factor; TNFSF4, tumour necrosis factor superfamily 4 .Control = healthy control.

1.8.2 Adhesion molecules in venous thrombosis

Adhesion molecules are crucial in the development of VTE. This is true especially for P-selectin which is important both for initiating leukocyte accumulation and adhesion to the venous endothelium and for subsequent platelet accumulation in VTE development (12). Other adhesion molecules (e.g. ICAM-1, VCAM-1, and E-selectin) have not been extensively studied (154, 157, 159, 167, 171-173).

An increased P-selectin/IL-10 ratio was found to be a risk factor for venous thrombosis in trauma patients (149), whereas selected haplotypes of selectins were not found to influence DVT risk in a large population-based study (173). Several studies have demonstrated increased P-selectin levels in VTE patients, both when compared to normal controls and as a diagnostic marker in symptomatic patients (154, 171, 172, 174-179). One study suggested that P-selectin may be more specific than D-dimer as a diagnostic marker at least in specific subset(s) of patients (175), although this could not be confirmed in a subsequent study which showed that D-dimer in combination with Well's score performed as well as P-selectin combined with Well's score (174). Moreover, increased P-selectin levels, together with large thrombotic volumes, are associated with less likelihood of venous recanalization (160). Resolved thrombosis has also been found to be associated with lower P-selectin levels, compared to patients with chronic thrombosis and hence elevated P-selectin levels (180). In addition, inhibition of leukocyte adhesion and platelet recruitment by P-selectin inhibition has been studied in several animal studies (175, 181). P-selectin inhibition performed as well as treatment with low-molecular-weight enoxaparin in terms of decreased thrombus burden and inflammation, but with the advantage of no increased risk of bleeding complications. Findings from selected human and animal studies are summarized in Table 7 to illustrate the importance of adhesion molecules in VTE.

Table 7. Adhesion molecules in DVT

	Predisposing factor	Acute reaction and diagnostic use	Effect on thrombus resolution
P-selectin	**Proctor et al. (2006):** ↓ in DVT group in trauma cohort (149) **Uitte de Willige et al. (2008):** ↔ selectin haplotypes in Leiden Thrombophilia Study (173)	**Antonopoulos et al. (2013):** ↑, meta-analysis 586 DVT, 1843 controls (179) **Vandy et al. (2013):** ↑, lower extremity: 112 DVT vs 122 non-DVT ↔, upper extremity: 32 DVT vs 13 non-DVT (174) **von Bruhl et al. (2012):** ↑ mouse model (12) **Ramacotti et al. (2011):** ↑ 62 DVT vs 116 non-DVT (175) **Deatrick et al. (2011):** ↑ in DVT patients vs controls (180) **Jezovnik et al. (2010):** ↑ 49 VTE vs 48 controls (154) **Rectenwald et al. (2005):** ↑ 22 DVT vs 21 non-DVT vs 30 healthy (176) **Bucek et al. (2003):** ↔ 37 DVT vs 32 non-DVT (171) **Bozic et al (2002):** ↑ 52 DVT vs 83 non-DVT (172) **Yang et al. (2002):** ↑ platelet expressing P-selectin in post-operative DVT (177) **Blann et al. (2000):** ↑ 89 DVT vs 126 healthy (178)	**Jezovnik et al. (2012):** ↑ in acute DVT predicts post-thrombotic syndrome, 49 DVT (160) **Gremmel et al. (2012):** ↑ after anticoagulation therapy, possible therapeutic target? (182) **Deatrick et al. (2011):** ↓ P-selectin 1 month after DVT in patients with resolved vs patients with chronic thrombosis (180) **Thanaporn et al. (2003):** P-selectin inhibition decreases post-thrombotic vein wall fibrosis in a rat model (183) **Myers (2002):** P-selectin inhibition enhances thrombus resolution and decreases vein wall fibrosis in a rat model (184) **Ramacotti et al. (2010):** P-selectin/PSGL inhibitors equal enoxaparin in VTE treatment (181)
ICAM-1		**Bucek et al. (2003):** ↔ ICAM-1 37 DVT vs 32 non-DVT (171) **Wik et al. (2016):** ↑ 181 cases vs 313 controls (167)	**Shbaklo et al. (2009):** ↑ in post-thrombotic syndrome, 387 DVT (159)
VCAM-1		**Jezovnik et al. (2010):** ↔ 49 VTE vs 48 healthy controls (154) **Bucek et al. (2003):** ↔ 37 DVT vs 32 non-DVT (171) **Bozic et al. (2002):** ↑ 52 DVT vs 83 non-DVT (172) **Wik et al.(2016):** ↑ 181 cases vs 313 controls (167)	
E-selectin	**Uitte de Willige et al. (2008):** ↔ selectin haplotypes in Leiden Thrombophilia Study (173)	**Bucek et al. (2003):** ↔ 37 VTE vs 32 non-VTE (171) **Du T et al. (2014):** ↑ abdominal cancer, post-operative (40 DVT vs 40 non-DVT vs 40 healthy) (157)	

This table summarizes selected key human and animal studies of cytokine response in venous thrombosis. Arrows indicate the following: the cytokine/genetic polymorphism coding for the cytokine is elevated/more frequent (↑) or decreased/less frequent (↓) or unchanged (↔) in DVT cohorts as a predisposing factor (left column), as part of the acute reaction (middle column), or as a risk factor for post-thrombotic syndrome or recurrent DVTs (right column). The name of the genetic polymorphism is given for each study.
PSGL, P-selectin glycoprotein ligand; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.
Control = healthy control.

1.8.3 Matrix metalloproteases in venous thrombosis

Findings from studies using animal models have suggested that MMPs are important effectors during VTE resolution and reduce vessel wall fibrosis (170, 185), thus indicating that MMPs represent potential therapeutic targets (161). Genetic variants of MMP genes have been shown to be risk factors for VTE in cancer (148), and raised levels of MMPs or their inhibitors in acute and chronic thrombosis, as well as in patients with post-thrombotic syndrome, have been described (166, 180). Findings from selected human and animal studies showing the importance of MMPs in DVT are presented in Table 8.

Table 8. Matrix metalloproteinases in DVT

	Predisposing factor	Acute reaction and diagnostic use	Effect on thrombus resolution
MMP-9	**Malaponte et al. (2013):** 1562 C > T ↑ SNP: 130 DVT^+ and 190 DVT^- (cancer patients) vs 215 healthy controls (148)	**Deatrick et al. (2011):** ↑ in VTE (180)	**Nosaka et al. (2011):** ↑ IFN-γ enhances thrombus resolution in mice through enhanced MMP-9 and VEGF expression in mice (170) **Henke (2007):** Review: the role of MMPs in DVT (mouse models) (185)
MMP-1, 2, 3, 7, 8, 9 TIMP-1/2		**de Franciscis et al. (2015):** ↑ MMPs: 201 DVT vs 60 controls (166)	**de Franciscis et al. (2015):** ↑ MMP-1/8: 47 of 201 DVT developing PTS (166)

This table summarizes selected key human and animal studies of MMP response in venous thrombosis. Arrows indicate the following: the cytokine/genetic polymorphism coding for the cytokine is elevated/more frequent (↑), decreased/less frequent (↓), or unchanged (↔) in DVT cohorts as a predisposing factor (left column), as part of the acute reaction (middle column), or as a risk factor for post-thrombotic syndrome or recurrent DVT (right column).
PTS, post thrombotic syndrome; SNP, single nucleotide polymorphism; TIMP, tissue inhibitor of metalloproteases. Control = healthy control.

1.8.4 Rationale for studying the broader inflammatory response in DVT

The inflammatory response is well established as part of, and a prerequisite for, venous thrombosis, but the broader inflammatory response during DVT has not been studied in detail in patients. The close interconnection between cytokines, adhesion molecules, and the MMP network in inflammation has led to the design of the research work presented in this thesis (see Section 4.3, p. 49), with a view to examining all these components of the inflammatory response in patients with suspected DVT.

Cytokine-associated genetic factors and increased levels of the pro-inflammatory cytokines as IL-6 and CXCL8 have not been found to be useful as diagnostic tools in acute VTE (5, 146, 154-157). As for P-selectin, although initially considered as a candidate diagnostic marker, recent evidence showed it produced similar results to D-dimer (174). There have been only a few studies investigating cytokine contributions to the pathogenesis of VTE, compared to arterial thrombosis, so further studies are needed to increase our understanding of the biology of VTE and evaluate the potential of candidate diagnostic tests. Interestingly, studies using animal models of VTE have shown anti-inflammatory treatment to be effective in terms of thrombus resolution and reduction of vein wall damage, with no increase in bleeding risk during the course of treatment, so it would be important to determine whether such treatment (e.g. IL-6, IFN-γ, and P-selectin inhibition) in VTE and its outcomes are also applicable and reproducible in humans (161, 170, 174).

1.9 SEPSIS AND INFLAMMATORY MEDIATORS

1.9.1 Definition of sepsis

The term sepsis was originally used to describe the systemic response to infection. The first consensus definition published in 1992 (186) was criticized for being too sensitive and non-specific and over-focusing on inflammatory reactions. In 2001, the international sepsis definitions were revised, in which an extensive list of biomarkers and definitions of organ dysfunctions were presented (187). Both the 1992 and 2001 consensus definitions are given in Table 9. One key issue with these definitions is that they do not allow for precise characterization and staging of sepsis patients.

Table 9. **Previous definitions of sepsis**

ACCP/SCCM sepsis definition, 1992 (Sepsis 1.0)	SCCM/ESICM/ACCP/ATS/SIS clinical sepsis definition, 2001 (Sepsis 2.0)
Infection Microbial phenomenon characterized by an inflammatory response to the presence of microorganisms or the invasion of normally sterile host tissue by these organisms	**Sepsis** Infection Either documented or suspected and some of the following: *General parameters:* - fever (core temperature >38.3°C) - hypothermia (core temperature <36°C) - heart rate >90 bpm or >2 SD above the normal value for age - tachypnoea (>30 breaths per minute) - altered mental status - significant oedema or positive fluid balance (>20 ml/kg over 24 h) - hyperglycaemia (plasma glucose >110 mg/dl or 7.7 mmol/l) in the absence of diabetes *Inflammatory parameters:* - leukocytosis (white cell count >12 000/μl) - leukopenia (white cell count <4 000/μl) - normal white cell count with >10% of immature forms - plasma C-reactive protein >2 SD above the normal value - plasma procalcitonin >2 SD above the normal value *Haemodynamic parameters:* - arterial hypotension (systolic blood pressure <90 mmHg, mean arterial pressure <70, or a systolic blood pressure decrease of >40 mmHg in adults or <2 SD below normal for age) - mixed venous oxygen saturation >70% - cardiac index >3.5 l/min/m^2 - organ dysfunction parameters - arterial hypoxaemia (PaO_2/FiO_2 <300) - acute oliguria (urine output <0.5 ml/kg/h or 45 ml for at least 2 h) - creatinine increase of ≥0.5 mg/dl - coagulation abnormalities (international normalized ratio >1.5 or activated partial thromboplastin time >60 s) - ileus (absent bowel sounds) - thrombocytopenia (platelet count <100 000/μl) - hyperbilirubinaemia (plasma total bilirubin >4 mg/dl or 70 μmol/l) *Tissue perfusion parameters:* - hyperlactataemia (>3 mmol/l) - decreased capillary refill or mottling
Bacteraemia Presence of viable bacteria in blood	
Systemic inflammatory response syndrome (SIRS) Systemic inflammatory response to a variety of clinical insults. The response is manifested by two of the following: (1) temperature >38ºC or <36ºC (2) heart rate >90 bpm (3) respiratory rate >20 breaths per minute or pCO_2 <4.3 kPa (4) white cell count >12 × 10^9 or <4 × 10^9, or >10% of immature band forms	
Sepsis Systemic response to infection, manifested by two or more SIRS criteria (see listed above)	
Severe sepsis Sepsis associated with organ dysfunction, hypoperfusion, or hypotension. Hypoperfusion and perfusion abnormalities that include, but are not limited to, lactic acidosis, oliguria, or an acute alteration in mental status	
Septic shock Sepsis induced by hypotension despite adequate fluid resuscitation, along with the presence of perfusion abnormalities that include, but are not limited to, lactic acidosis, oliguria, or an alteration in mental status. Of note, patients who are given inotropic or vasopressor agents may not be hypotensive at the time when perfusion abnormalities are measured	
Sepsis-induced hypotension Systolic blood pressure of <90 mmHg or a reduction of ≥40 mmHg from baseline, in the absence of other causes of hypotension	
Multiple organ dysfunction syndrome (MODS) Presence of altered organ function in an acutely ill patient, such that homeostasis cannot be maintained without intervention	

ACCP, American College of Chest Physicians; ATS, American Thoracic Society; ESICM, European Society of Intensive Care Medicine; SCCM, Society of Critical Care Medicine; SD, standard deviation; SIS, Surgical Infection Society. Adapted from references (186, 187). Footnotes to the 2001 definition are not included.

1.9.2 The PIRO system—Predisposition, Insult, Response, and Organ dysfunction

The additional staging system PIRO was therefore proposed as an analogue to the TNM classification of human malignancies (Table 10). The proposed PIRO system has the intention to build a framework to stratify sepsis patients based on four main parameters as described in detail in Table 10: Predisposing conditions, Insult, host Response and Organ dysfunction (187). Although this should be regarded as a framework for a better definition and description that can be used both in clinical research and routine clinical practice, a validated and clearly described model is still not available. This model was not included in the third definition; however the PIRO system could still be a useful hypothesis-generating model when planning studies to reveal the biology of sepsis (187).

Table 10. The PIRO system

Domain	Present	Future	Rationale
Predisposition	Premorbid illness with reduced probability of short-term survival. Cultural or religious beliefs, age, gender	Genetic polymorphisms in components of inflammatory response (e.g. TLR, TNF, IL-1, CD14). Enhanced understanding of specific interactions between pathogens and host diseases	Premorbid factors impact on the potential attributable morbidity and mortality of an acute insult (present). Deleterious consequences of insult heavily dependent on genetic predisposition (future)
Insult (infection)	Culture and sensitivity of infecting pathogens, detection of disease amenable to source control	Assay of microbial products (LPS, mannan, bacterial DNA), gene transcript profiles	Specific therapies directed against inciting insult require demonstration and characterization of that insult
Response	SIRS, other signs of sepsis, shock, CRP	Non-specific markers of activated inflammation (e.g. PCT or IL-6) or impaired host responsiveness (e.g. HLA-DR). Specific detection of target of therapy (e.g. protein C, TNF, PAF)	Both mortality risk and potential to respond to therapy vary with non-specific measures of disease severity (e.g. shock). Specific mediator-targeted therapy is predicated on presence and activity of mediator
Organ dysfunction	Organ dysfunction as number of failing organs or composite score (e.g. MODS, SOFA, LODS, PEMOD, PELOD)	Dynamic measures of cellular response to insult—apoptosis, cytopathic hypoxia, cell stress	Response to pre-emptive therapy (e.g. targeting microorganism or early mediator) not possible if damage already present. Therapies targeting the injurious cellular process require the presence of causative insult

CRP, C-reactive protein; IL, interleukin; LODS, logistic organ dysfunction system; MODS, multiple organ dysfunction syndrome; LPS, lipopolysaccharide; PAF, platelet-activating factor; PCT, procalcitonin; PELOD, paediatric logistic organ dysfunction; PEMOD, paediatric multiple organ dysfunction; SIRS, systemic inflammatory response syndrome; SOFA, sequential (sepsis-related) organ failure assessment; TLR, Toll-like receptor; TNF, tumour necrosis factor. Adapted from reference (187).

A new set of definitions of sepsis (Table 11) has recently been published, called Sepsis 3.0 (16, 188), which states that sepsis is a syndrome, and not a specific illness, and emphasizes the heterogeneity and many unknown pathophysiological processes in severe infection (16). In contrast to previous definitions, Sepsis 3.0 includes only patients in the previous category of severe sepsis, i.e. only those patients with established organ dysfunction. Also, in Sepsis 3.0, organ failures are strictly

defined according to the Sequential (sepsis-related) Organ Failure Assessment (SOFA) score (189). However, the new definitions of Sepsis 3.0 are still under debate and are not substantially more precise than the former definitions. Definitions given in Sepsis 3.0 are presented in Table 11.

Table 11. Sepsis 3.0: the third international consensus definitions for sepsis and septic shock

Sepsis	
Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection.	
Organ dysfunction	**Septic shock**
Organ dysfunction is identified as an acute change in the total SOFA score of ≥2 points as a result of the infection. - The baseline SOFA score is assumed to be zero in patients not known to have pre-existing organ dysfunction - A SOFA score of ≥2 reflects an overall mortality risk of approximately 10% in a general hospital population with suspected infection. Even patients presenting with modest organ dysfunction can deteriorate further, emphasizing the seriousness of this condition and the need for prompt and appropriate intervention, if not already instituted. In lay terms, sepsis is a life-threatening condition that arises when the body's response to an infection injures its own tissues and organs.	Septic shock is a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase the risk of mortality. - Patients with septic shock are identified with a clinical construct of sepsis that includes persisting hypotension requiring vasopressors to maintain an MAP of 65 mmHg and the presence of a serum lactate level of >2 mmol/l (18 mg/dl) despite adequate volume resuscitation. With these criteria, hospital mortality rate is in excess of 40%.
qSOFA	
Patients with suspected infection who are likely to have a prolonged ICU stay or die in hospital can be promptly identified at the bedside with qSOFA, i.e. an alteration in mental status, a systolic blood pressure of 100 mmHg, or a respiratory rate of 22 breaths per minute.	

ICU, intensive care unit; MAP, mean arterial pressure; qSOFA, quick SOFA; SOFA, sequential (Sepsis-related) organ failure assessment. Adapted from reference (16).

1.9.3 Detection of microbes

The innate immune system is the first-line defence against invading pathogens, as described in Section 1.3, p. 6 (Figure 1) where the pathogens and damage inflicted by them are recognized by PRRs via PAMPs and DAMPs, respectively. The adaptive immune response develops following antigen recognition, followed by B- and T-cell activation and maturation.

1.9.3.1 Initiation of innate immune responses by different PRR systems

TLRs play a crucial role in pathogen detection and are widely distributed in the innate immune system (neutrophils, monocytes, macrophages, B-cells, DCs, various epithelial cell types). So far, 11 receptor types involved in detecting different pathogens have been identified (97). The various TLRs share the main downstream signalling pathways and, once activated, initiate pro-inflammatory responses (190).

The best described TLR activator is lipopolysaccharide (LPS), a major constituent of the outer membrane of Gram-negative bacteria, which activates TLR4 (191). Other important sensor systems are NOD-like receptors (NLRs) that recognize bacteria, RIG-I-like receptors (RLRs) and DNA-sensing molecules that detect viruses, and C-type lectin receptors (CLRs) that sense fungi and mycobacteria (192, 193).

Superantigen toxins are the most potent toxins produced by bacteria which bypass the conventional immune response. They bind directly to T-cell receptor molecules, which are important in T-cell activation. The molecules are able to induce T-cell activation by binding to, and crosslinking, the co-stimulatory molecule CD28 and T-cell receptor chains. This is in contrast to a normal response where presentation of the antigen to a MHC class II molecule in an antigen presenting cell is a prerequisite for activation (191). Among the superantigen-producing bacteria are *Staphylococcus aureus* that produces the staphylococcal enterotoxins (SEs) A–E as well as toxic shock syndrome toxin-1 (TSST-1), and *Streptococcus pyogenes* that produces streptococcal pyrogenic exotoxin A and C (SpeA and SpeC) and streptococcal mitogenic exotoxin Z (SmeZ) (191, 194). Superantigen binding results in activation of >20% of the T-cell population, compared to normal antigenic recognition that activates <0.01% of the T-cell population (191). Animal models of necrotizing soft tissue infection and polymicrobial and Gram-negative infections have demonstrated beneficial effects from treatment with a CD28 antagonist (195, 196), and results from a recent randomized clinical trial also suggest that this therapeutic strategy is effective in patients with necrotizing fasciitis (197).

1.9.3.2 Clinical studies describing the immune response to different microbes

Different microbes can be detected by different receptors. It may therefore be assumed that different bacteria will elicit different immune responses, e.g. Gram-positive compared with Gram-negative bacteria. Gram-negative bacteria express LPS which binds to TLR4, thereby inducing cytokine release by monocytes. In contrast, Gram-positive bacteria express superantigens that crosslink T-cell receptor chains with CD28, thus initiating a T-cell cytokine response. It has also been shown that the gene expression pattern in leukocytes may differ, depending on the type of infection (198-200), and some minor differences in cytokine expression have also been observed (201).

Pathogen recognition, together with the ensuing damage caused by the pathogen, during infection initiates a pro-inflammatory, as well as an anti-inflammatory, response, with the latter helping to balance against the pro-inflammatory reaction (2). The pro-inflammatory reaction includes the widespread activation of leukocytes, complement, and coagulation, thus increasing the risk for ‘collateral’ damage of normal tissue. Immune suppression is therefore an important strategy to keep immune reactions in balance, consisting of inhibition of immunocompetent cells, altered neuroendocrine regulation, and a more general inhibition of pro-inflammatory gene transcription

(Figure 1). In addition, during the course of sepsis, a secondary immune response is often seen in the late phase, during which the risk for secondary infections and late mortality is increased (202-205).

The immune response during sepsis represents a complex interaction between several biological mediators. Studies of single cytokines and adhesion molecules have been reviewed elsewhere (85, 188, 206, 207). Clinical studies that have examined the interactions or crosstalk between different pro-inflammatory soluble mediators will be discussed in the following sections.

1.9.4 Combining several cytokines

Several studies of cytokine profiles have been conducted, both in the emergency department (ED) and intensive care unit (ICU) settings, with a view to establishing a prognostic role for these profiles (44, 208-214). While most studies have described a discriminative ability between different cytokine profiles, the use of differing methods and panels of inflammatory mediators, as shown in Table 12, has rendered any meaningful comparisons difficult. In addition, the number of mediators investigated can range anywhere from six to 150. For statistical evaluation of data, most studies used multiple logistic regression for combinations of mediators, while others used hierarchical clustering or principal component analysis (PCA).

None of the previous studies have included soluble adhesion molecules or protease inhibitors. A study of a patient cohort from the emergency department that focuses on differences in type of infection (Gram-positive vs. Gram-negative) and the severity of intravascular bacterial load (bacteraemia vs. non-bacteraemia) has not been examined previously by using multiplex and hierarchical clustering analyses.

Table 12. Clinical studies of multiple cytokine combinations in sepsis cohorts

Study	Cytokines examined Patient cohort	Statistical method	Main findings
Bozza et al. (2007) (208)	Multiplex 17 cytokines Prospective cohort study 60 patients—ICU	Mann–Whitney *U*-test and Kruskal–Wallis test ROC Univariate and multivariate logistic regression	Distinct cytokine profiles associated with sepsis severity, evolution of organ failure, and death
Shapiro et al. (2009) (209)	Exploratory 150 biomarkers in 250 patients Nine biomarkers selected and analysed in 971 patients—ED	Multivariate logistic regression was used to identify an optimal combination of biomarkers to create a panel	Predictive of severe sepsis, septic shock, and death in ED patients with suspected sepsis
Mera et al. (2011) (44)	Multiplex 17 cytokines 30 patients—ICU Daily analyses, days 1–7	Multivariate logistical regression ROC	Simultaneous evaluation of multiple cytokines in sepsis can identify complex cytokine patterns that reflect the systemic response associated with shock and mortality
Lvovschi et al. (2011) (210)	Multiplex 25 cytokines 126 patients—ED	Univariate and multivariate logistic regressions, PCA, and agglomerative hierarchical clustering	Univariate analysis revealed weak associations between cytokine levels and sepsis. Multivariate analysis revealed independent association between sIL-2R ($p = 0.01$) and severe sepsis, as well as between sIL-2R ($p = 0.04$), IL-1b ($p = 0.046$), and IL-8 ($p = 0.02$) and septic shock. However, neither PCA nor AHC distinguished profiles characteristic of sepsis
Fjell et al. (2013) (211)	39 cytokines measured 363 patients—ICU Vasopressin in Septic Shock Trial (VASST)	Hierarchical clustering was performed on plasma values to create patient subgroups Logistic regression was performed to assess the importance of cytokines for predicting patient subgroups	A distinct pattern of cytokine levels measured early in the course of sepsis predicts disease outcome. Subpopulations of patients have differing clinical outcomes that can be predicted accurately from small numbers of cytokines
Xu et al. (2013) (212)	6 cytokines 111 haematology/oncology paediatric patients with septic shock	Logistic regression or Cox proportional hazards regression model ROC	Cytokine scoring system which performs well in disease severity and fatality prediction in paediatric/haematology/oncology patients with septic shock
Jekarl et al. (2015) (213)	13 cytokines 127 patients with SIRS syndrome, 97 with sepsis—ED	Hierarchical clustering analysis	No relationship between cytokine profiles and sepsis
Mickiewicz et al. (2015) (214)	Multiplex 45 cytokines/chemokines and 60 metabolites 57 patients: 37 septic shock and 20 ICU controls	PCA, supervised orthogonal partial least squares discriminant analysis (OPLS-DA) and regression component, AUROC	Integration of quantitative metabolic and inflammatory mediator data can be utilized for the diagnosis and prognosis of septic shock in the ICU

AHC, agglomerative hierarchical clustering; AUROC, area under receiver operating characteristic; PCA, principal component analysis; ROC, receiver operating characteristic; SIRS, systemic inflammatory response syndrome.

2 METHODS

2.1 PATIENT SELECTION AND STUDY DESIGN

As mentioned in Section 4 below, a total of four studies are described in this thesis, three of which have been published in peer-reviewed articles (papers I to III) and one is being considered for publication (paper IV). All four studies included defined patient cohorts that were admitted to the Haukeland University Hospital, as well as, where appropriate, control groups composed of healthy subjects, and the patients followed prospectively after inclusion. All patient cohorts were relatively small in size, compared to the larger number of analytes, resulting in a lower statistical power. Therefore, all four studies in this thesis are hypothesis-generating, i.e. they are initial studies exploring the relevance of inflammatory responses using newer multiplex technology (215).

The study presented in paper I examined a small population of patients with multiple myeloma undergoing stem cell harvesting, as well as a small population of healthy controls undergoing platelet apheresis. Ideally, this study should have included a control group of healthy individuals undergoing stem cell harvesting. However, such subjects were not available at the time of inclusion. The studies presented in papers II and III examined patient cohorts admitted with suspected DVT, with the study in paper III including healthy controls. As shown in Tables 6, 7, and 8, most previous studies only compared DVT patients with healthy controls; however, a comparison of DVT patients with those admitted with suspected DVT would give a more realistic approach to evaluating the potential of using soluble mediators for diagnostic evaluation. Finally, the study presented in paper IV examined a cohort of 80 patients admitted with sepsis and proven bacterial infection. This approach emphasizes the bacteriological differences and their resulting impact on the inflammatory reaction, as opposed to other studies which examined sepsis by generally comparing broader cohorts (Table 12).

2.2 SELECTION OF INFLAMMATORY MEDIATORS TO BE ANALYSED

The inflammatory response consists of a highly complex and broad network of interacting and interconnected inflammatory mediators, including cytokines, coagulation factors, and proteins, as described previously in Sections 1.1 to 1.6 in the Introduction. The development of multiplex analysis has enabled simultaneous testing of various mediators in one same sample. However, the challenge remains in the selection of mediators to be analysed among the vast array of potential mediators available. Firstly, the study designs and selection of mediators for analysis were based on previous published studies, in order to fill the gaps in existing knowledge. As shown in Tables 6, 7, and 8, detailed analysis of cytokines, adhesion molecules, and MMPs has not been performed in patients with

DVT, and therefore, the design for two of the four studies presented in this thesis included patients with DVT. In addition, for sepsis, most previous studies investigated either single or a small range of inflammatory mediators for their importance in the inflammatory network. As shown in Table 12, there are no studies investigating detailed systemic inflammatory profiles, including adhesion molecules and MMPs, in patients with sepsis.

Inflammatory mediators included in the four studies were selected based on: (i) the availability of highly standardized and well-characterized assays suitable for high-throughput analyses of limited sample volumes and (ii) the biological functions of these mediators and how they relate to the function of different cells or different mechanisms that are important in the inflammatory process.

The choice of the right methodology will always depend on what is feasible, practically achievable, and economically available. Studies presented in this thesis mainly used enzyme-linked immunosorbent assay (ELISA) kits and multiplex sets from known manufacturers (mainly from R&D Systems, but also from Millipore and Bio-Rad) with established experience in product development and a proven track record in maintaining high product quality. The availability of different multiplex analysis kits using Luminex® technology was initially restricted to fixed sets where a defined group of specific mediators were analysed, although recently it has also been possible to order custom combinations of mediators from manufacturers. Moreover, although it is possible to construct local in-house combinations of mediators, despite being limited by the risk of cross-reactivity and the presence of auto-antibodies that might affect the results (216), development of such custom bead sets requires extensive and rigorous internal quality control which is beyond our capability to perform at the present time. Therefore, mainly fixed or custom combinations of mediators, as recommended by the manufacturers, were used in the work presented here.

2.3 BIOLOGICAL VARIABILITY

Inflammatory mediators show considerable biological variability. Many cytokines show substantial intra-individual and inter-individual variability in healthy controls (e.g. IL-6, TNF-α, and IL-17A) (217), and there is even greater variability seen in haemodialysis patients (e.g. CCL5 and IL-10) (218). Serum concentrations of mediators in healthy subjects can sometimes be below the detection limit (217), thus making it difficult to determine the normal variability of these mediator levels, as encountered for a range of cytokines in the studies presented here. Another important aspect of biological variability is circadian variation of inflammatory mediators such as adhesion molecules and cytokines (IL-1β, IL-6, TNF, IL-10, and chemokines) (219, 220).

2.4 SAMPLE HANDLING AND STORAGE STABILITY

Several endothelial cell biomarkers have been investigated in clinical studies of inflammatory conditions, most of which are based on their detection in plasma or serum samples. The concentrations of these biomarkers depend on the sampling method, i.e. use of serum vs. plasma samples (221).

It is important to standardize the handling of biological samples with regard to the time duration until cryopreservation, preparation procedure, storage time, and platform(s) used for analysis (221-230). Time taken until separation of serum and plasma samples can have a significant impact on the results, and it is recommended that blood samples are centrifuged within 2 h. There are several reports describing cytokine decay after 2 years of storage, even when stored at −80°C (222, 229). Variations in the type and concentrations of soluble mediators can also vary when collecting blood samples in different media (serum, heparin, or EDTA (ethylenediaminetetraacetic acid) plasma), thus making it difficult to compare results obtained from these different media (221).

In the four studies here, all plasma samples were standardized using citric acid as anticoagulant and thereafter transferred into plastic tubes without additives and centrifuged twice at 2500 g for 15 min at room temperature within 120 min of sampling. Plasma supernatants were transferred into cryotubes and frozen immediately before storage at −80°C until analysis. More than 90% of all samples were frozen within 60–75 min after their collection, and therefore this initial sampling would not be expected to have any major influence on the study results (222, 229).

For the reasons outlined above, it is important to emphasize that for most inflammatory mediators, direct comparison of results obtained from the different studies should be interpreted with caution, as samples from each study might have been handled differently (221, 222).

2.5 LUMINEX® AND MULTIPLEX ANALYSIS

Various methods are available for analyses of inflammatory mediators such as cytokines. In the studies here, Luminex®, a bead-based multiplex immunoassay, was used to analyse most mediators, and ELISA was used for a selection of mediators. Other possible multiplex methods include microtitre plate-based arrays, slide-based arrays, and reverse-phase protein arrays (216). Luminex® is a multistep procedure, similar to ELISA, as illustrated in Figure 3.

2.5.1 Cross-reactivity and auto-antibodies

Cross-reactivity between antibodies and serum or plasma constituents will always be a potential complication when dealing with antibody-dependent capture (216, 231). Rigorous testing for cross-reactivity both within multiplex sets and against other serum constituents is required. The possible presence of auto-antibodies is also a key issue of concern, which can be circumvented by using different assay diluents, and this is therefore a vital part of all antibody-dependent diagnostics. This phenomenon of auto-antibodies is seen particularly in patients with autoimmune diseases such as rheumatoid arthritis (231).

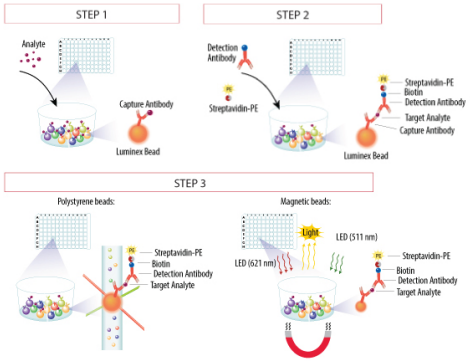


Figure 3. Luminex® analysis.
Step 1. The sample is added to each well where a mixture of pre-coated, colour-coded beads, available as pre-mixed from the manufacturer, and antibodies bind to specific analytes.
Step 2. Detection antibodies (with biotin) bind to analytes and form an antibody–antigen sandwich. Streptavidin conjugated with phycoerythrin (PE) binds to biotin on the detection antibody.
Step 3. Left: A dual laser flow-based detection instrument reads the polystyrene beads where one laser classifies the bead and the other determines the magnitude of the PE-derived signal (directly proportional to the amount of analytes bound). Right: A procedure using magnetic beads and two spectrally different LEDs (imaging of each well using a CCD camera is an alternative, but not used in the experiments).
(Picture obtained from R&D Systems, with permission.)

2.5.2 Analytic variability

Strong correlations between ELISA and multiplex bead arrays from different manufacturers have been reported, but with poor concurrence of quantitative values (232). However, strong correlations and similar quantitative values are obtained when comparing ELISA and multiplex kits that use identical capture and reporter antibodies, as well as similar diluents and serum blockers, although multiplex kits show a larger dynamic range (232).

2.5.3 Inter-assay and intra-assay variability

In general, multiplex kits have acceptable well-to-well and day-to-day reproducibility, but differences between product lots and changing storage times may influence the results (228). The multiplex assays and ELISA kits used in the studies here have an intra- and inter-assay variability of between 5% and 10%, according to the manufacturer's information, which is similar to the variability percentages obtained when analysing duplicate samples in our experiments. From previous experience in running duplicate samples (both ELISA and multiplex kits), the CV (coefficient of variation) values for most samples are below 5%, some between 5% and 10%, and a few above 10%. CV values of over 10% occur predominantly when readings are near the upper or lower detection range of the standard curve.

Both methods using ELISA and Luminex® are highly operator-dependent, which is therefore always a possible source of error. However, the risk of error will be reduced by rigorously following protocols and always running samples in duplicates. Previous personal experience using these assays have shown that inter-assay plate variability is small, compared with larger intra-assay plate variability.

Therefore, when running more than one assay plate, it is preferable to run all plates on the same day to reduce this possible problem. Also, when setting up the experiments, the possible impact of intra-assay plate is also decreased if samples are analysed in a random order. Moreover, for each of the four studies here, samples with similar storage times were consistently used for comparisons, and used the same lot number of the Luminex kits throughout the study to avoid lot-to-lot differences.

2.5.4 Reading below lower or above upper limits

Each kit is calibrated by the producer, and a recommended dilution of the samples is also given. We always followed the instructions provided by the producer, although a considerable number of cytokine levels were below the detection range, and these cytokines had to be excluded from the statistical assessment. Values above the detection range were a minor problem. Procedures for interpretation of missing values are described in the following section. Especially in paper I (stem cell harvesting) and paper III (DVT) we experienced problems with multiple mediators that were below the detection limit, and in paper IV (sepsis) we have therefore reduced the number of cytokines examined.

2.6 STATISTICAL CONSIDERATIONS

2.6.1 Interpretation of missing values

Obtaining missing values > 20% of the samples, these data sets were excluded from further analysis. In order to be able to perform statistical procedures including patient samples that had missing values the threshold for all missing values was set at 25% lower than the detection limit (or higher in a few cases). When conducting non-parametric analyses, this will have no effect on the results, as the rank-sum test was used and therefore these values will be ranked below the others. Those values defined to represent the missing values would have a greater impact on the results when executing logistic regression and hierarchical clustering, and hence it will be a matter of debate which values were the most appropriate to use under such circumstances.

2.6.2 Statistical methods

Since most data obtained did not follow a normal distribution, non-parametrical methods of analysis were used. Wilcoxon–Mann–Whitney signed-rank test was used for group-to-group comparisons and Kruskal–Wallis test was used when comparing multiple groups. Pearson correlation and univariate and multivariate logistic regression were also used.

In all studies, a large number of mediators from relatively small patient cohorts were analysed and corrected for multiple comparisons using Bonferroni correction to avoid type I errors (false positive), although this method increases the risk for type II errors (false negative) (233, 234). Bonferroni correction is a conservative method for power correction, and alternative methods for power calculation are likely to be better to reduce the risk for false negatives (215).

As previously mentioned, the studies presented here using multiplex sample analyses are only hypothesis-generating, and power calculations were not done a priori. Power calculations were performed post hoc for results presented in paper II, although this retrospective technique has been debated (215). All studies here aimed to assess for any large differences in mediators that may be clinically relevant and thus should show statistical significance despite low power; therefore, it is possible smaller differences might have been overlooked due to false-negative results.

2.6.3 Hierarchical clustering

Hierarchical clustering is based on predefined criteria whereby samples and variables are grouped and presented. The clustering algorithms result in a two-dimensional figure that expresses co-variation of a larger data set where similarity between different variables are expressed as dendograms and individual samples are expressed as a heat-map with high and low values visualised by different colours (20, 235).

In data mining, this is a relatively free methodology that allows experimentation of different mathematical approaches for calculation of co-variation of samples and variables (235). Scaling differences between different variables will have a significant impact on the results, which can be minimized using log-conversion and normalization. Data can be z-transformed or median- or mean-normalized (20, 21, 236), and this transformation allows comparison of results obtained for different inflammatory mediators in the same clustering analysis. Median or mean normalizing means that all samples are divided by the median or mean, respectively, and all results are then log-transformed before the final clustering analysis. As most of the data are non-parametrical, median normalization was initially used (papers I to III). In paper IV, we used z-transformation that also corrects for the standard deviation. However, the general impression obtained from using different normalization procedures is that results from these different approaches are relatively similar despite their difference in methodology. Median normalizing and $\log_2$ conversion give values that are more intuitively understandable, as the values are related to the median, and it is therefore possible to calculate directly from the heat-map how the value of each inflammatory mediator for each patient compares with the median. In addition, z-transformation includes $\log_{10}$ transformation and gives more coherent clusters, as outliers are also taken into consideration.

Both distance calculation and clustering can be performed using different methodologies. For distance calculation between each sample and variable, Euclidean and Pearson correlations are the most frequently used methods, whereas for clustering methodology (tree formation), complete linkage, single linkage, and wards methodology are more commonly used (235). Although the data here were examined using different methodologies, Euclidean correlation was consistently used for distance calculations and complete linkage was used as the standard clustering method for our final analyses, as

these methods produced results with the most homology between the inflammatory mediators in the clusters and also were more clinically relevant.

The heat-map displays a small square for each inflammatory mediator for each patient, and the colour of the squares represents the concentration of the mediator, compared with its median or mean levels (or corrected for standard deviation when the values are z-transformed) (Figure 4). To the left, a tree is formed where patients with similar mediator co-variations cluster together, and another tree is formed at the top of the figure that displays the co-variation of different mediators. The methodology used to calculate the distance between each square and that used for tree formation are given in the bottom left corner. To the right of the figure, clinically relevant information is shown for each patient.

Clinical interpretation of hierarchical clustering results can be challenging, as it is a relatively free methodology, and conclusions should therefore be drawn with caution. However, hierarchical clustering can be useful as a hypothesis-generating methodology to reveal new patterns and relationships between inflammatory mediators being assessed, hence its application in the studies presented here which include large data sets of inflammatory mediators (20, 21, 235, 237).

2.6.4 Software programs used for hierarchical clustering analyses

Various software programs are available to perform statistical analysis. For the studies here, the traditional statistical methods Graph Pad Prism and SPSS were used. A range of software for hierarchical clustering analysis have been developed since the 1960s (238), of which the J-express software developed at the University of Bergen (UiB), and now managed in collaboration between four universities in Norway (UiB, NTNU, UiT and UiO), was used for the analyses here (20). The most common software used by statisticians for hierarchical clustering analysis comprise appropriate packages run in R, although it is also found in other commonly used statistical programs such as SPSS, Stata, and MATLAB (239-241). The J-express was used as a platform, as this program is user-friendly, with possible access to software support during regular workshops at the University of Bergen, although all these other programs (or similar), which use well-established principles, as stated above, are also available.

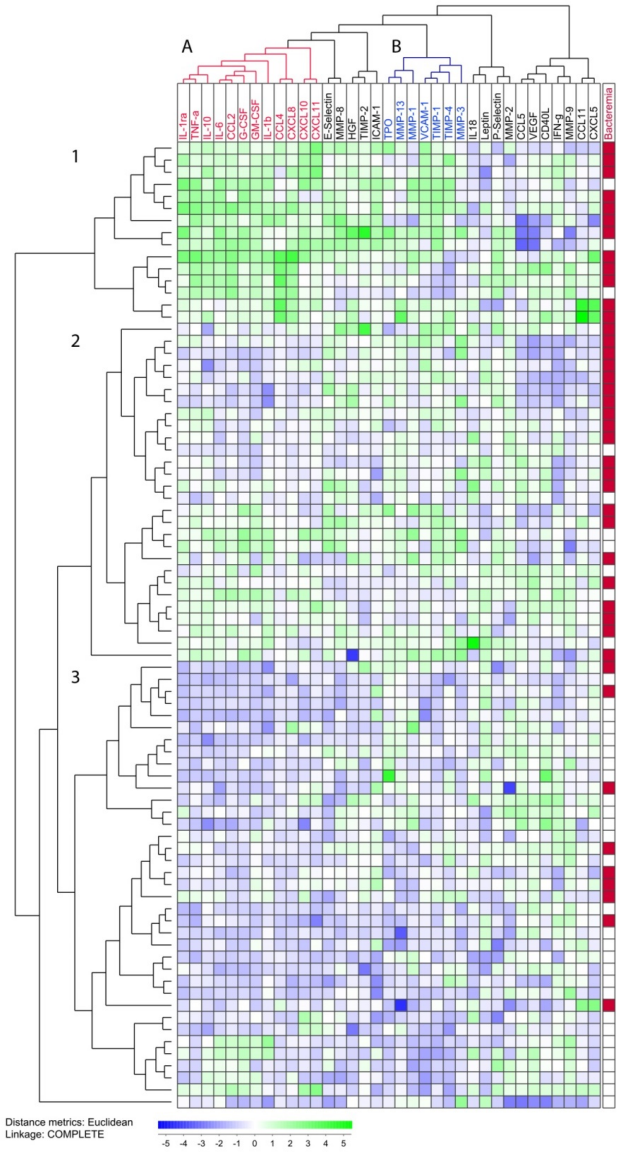


Figure 4. How to read a hierarchical clustering heat-map.
An example of a clustering heat-map, as presented in paper IV. Each mediator value is displayed as a coloured square where green represents high values and blue low values. The scale is given at the bottom of the figure. Here, the data are z-transformed, i.e. each individual value is normalized to the mean (log_{10}) and corrected for standard deviation. A value with maximum intensity (green 5) falls on a logarithmic scale, 5 higher than the mean for that given mediator, corrected for standard deviation. In papers I–III, median normalization is used where each value is normalized to the median of each mediator and log_2-converted, as described in the main text. The distance between each mediator is calculated by Euclidian correlation, as described in the main text. Individual mediators are clustered horizontally (top of figure), and patients are clustered vertically (left of the figure), forming trees that indicate how close the mediators and patients co-variate, respectively. Tree formation is based on the complete linkage equation, as given above. Patients form three main clusters, whereas mediators form several less well-defined clusters. It is also possible to show clinical characteristics for each patient in the right field. See the individual papers I–IV for further description and discussion.

3 AIMS OF THE THESIS

The objectives of the research presented in this thesis are:

(i) to give a broader description of the inflammatory responses that take place in well-defined clinical conditions characterized by sterile inflammation or bacterial infections

(ii) to investigate how inflammation is reflected through systemic mediator profiles, and

(iii) to determine whether such systemic inflammatory profiling has prognostic or diagnostic potential, using multiplex technology and advanced bioinformatical analyses.

The work presented in this thesis comprises a total of four studies, in which the following well-defined clinical conditions were used to examine the inflammatory response elicited, with a view to providing answers to key questions outlined below:

(i) The inflammatory response to harvesting of G-CSF-mobilized peripheral blood stem cells from patients with multiple myeloma. Does this procedure lead to a clinically relevant inflammatory response?

(ii) The inflammatory response in patients admitted to hospital with suspected DVT. Can systemic mediator profile analysis be used in the diagnostic evaluation of these patients?

(iii) The inflammatory response in sepsis. Can the inflammatory profile be used for the diagnostic and/or prognostic evaluation of patients admitted to hospital with sepsis, i.e. for improved patient stratification according to the severity of infection (i.e. bacteraemia vs. no bacteraemia) or type of infection (i.e. Gram-positive vs. Gram-negative bacteria)?

4 SUMMARY OF RESULTS

4.1 PAPER I: STEM CELL MOBILIZATION AND HARVESTING BY LEUKAPHERESIS ALTERS SYSTEMIC CYTOKINE LEVELS IN PATIENTS WITH MULTIPLE MYELOMA

The inflammatory response during stem cell mobilization and harvesting by peripheral blood leukapheresis in patients with myeloma is not known in detail. In the present study, the effects of these interventions were investigated on a larger group of cytokines by using Luminex® multiplex analysis. In addition, the 15 patients with multiple myeloma who underwent peripheral blood stem cell harvesting were also compared with healthy donors who underwent platelet apheresis.

Patients with myeloma show an overall increase in inflammatory response following both stem cell mobilization and peripheral stem cell leukapheresis. Following stem cell mobilization with chemotherapy plus G-CSF, increased levels of several CCL (CCL2/3/4) and CXCL (CXCL5/8/10/11) chemokines, as well as thrombopoietin, IL-1 receptor antagonist, IL-4, G-CSF, and hepatocyte growth factor (HGF), were obtained. Following peripheral stem cell leukapheresis, further altered plasma levels of several inflammatory mediators were obtained: CD40 ligand, IL-1 receptor antagonist, CCL5, and CXCL5/8/10/11. Thrombapheresis in healthy individuals had only minor effects on plasma cytokine levels.

Prognostic differences by chemokine profiling in graft supernatants is hypothesized. Stem cell graft supernatants showed high levels of several cytokines, particularly CCL and CXCL chemokines. Analyses of chemokine profiles in pre-apheresis plasma and graft supernatants suggested that such profiling can be used to detect prognostically relevant differences between patients with multiple myeloma.

Taken together, our results demonstrate a procedure-related inflammatory reaction, in which patients with multiple myeloma have an altered cytokine network during stem cell mobilization. The network is further altered during stem cell harvesting by leukapheresis. These treatment- or procedure-induced changes involve several inflammatory mediators known to affect myeloma cell proliferation, migration, and survival.

4.2 PAPER II: SYSTEMIC LEVELS OF ENDOTHELIUM-DERIVED SOLUBLE ADHESION MOLECULES ENDOCAN AND E-SELECTIN IN PATIENTS WITH SUSPECTED DEEP VEIN THROMBOSIS

Initial evaluation of patients with suspected DVT focused on the use of biomarkers reflecting the activation of the coagulation system (D-dimer), whereas inflammatory biomarkers were not a major part of the evaluation. In addition, as the thromboembolic process and neighbouring inflammatory responses also affect endothelial cells, endothelial cell markers that may be altered by DVT were also examined. Thus, in this single-centre study, we investigated the plasma levels of the endothelium-specific biomarkers soluble E-selectin and endocan in a consecutive and unselected group of 120 patients admitted to hospital for suspected DVT.

Elevated plasma levels of either endocan or E-selectin did not differentiate the presence of DVT from other inflammatory and non-inflammatory conditions

Patients with DVT showed evidence of an acute phase reaction with increased serum CRP levels, although this was similar to CRP levels obtained in other patients admitted with suspected, but not confirmed, thrombosis. There was no difference in plasma levels of endocan and E-selectin in patients with thrombosis compared with healthy controls and patients with no confirmed thrombosis (i.e. patients with other underlying causes for their symptoms, including various inflammatory and non-inflammatory conditions. In contradiction to these findings the traditional biomarker of inflammation, CRP, differentiate most of the inflammatory conditions from the non-inflammatory conditions (Table 2, paper II).

Combined use of endocan, E-selectin, D-dimer, and CRP could help identify the presence of DVT

A hierarchical clustering model showed that the endothelial biomarkers endocan and E-selectin, in combination with CRP and D-dimer, could be used to identify patient subsets with different frequencies of venous thrombosis (Figure 2, paper II). Therefore, analysis of plasma biomarker profiles that include endothelial cell markers could prove useful in the initial evaluation of patients with DVT.

4.3 PAPER III: ALTERED LEVELS OF CYTOKINES, SOLUBLE ADHESION MOLECULES, AND MATRIX METALLOPROTEASES IN VENOUS THROMBOSIS

The inflammatory response in DVT has been assessed in several previous studies of either single or a small number of inflammatory mediators, but not of the broader inflammatory response. Here, we examined the plasma levels of a total of 43 inflammatory mediators in a cohort of 89 consecutive patients with suspected DVT and 20 healthy controls using Luminex® multiplex analyses: 13 ILs, 3 immunomodulatory cytokines, 8 chemokines, 8 growth factors, 3 adhesion molecules, and 8 MMPs. Selected mediators were also analysed in a second cohort of 80 consecutive patients.

Only four mediators show significant differences between patients with and those without DVT. The levels of only P-selectin ($p < 0.0001$), VCAM-1 ($p = 0.0009$), MMP-8 ($p = 0.0151$), and HGF ($p = 0.0415$) showed statistically significant differences in patients with, compared to those without, DVT. Subgroup comparisons are summarized in Table 13 (unpublished comparisons not included in the article). This table illustrates that it was particularly difficult to differentiate those patient subsets showing the highest degree of activated inflammation, i.e. relatively few mediators showing significant differences when comparing DVT patients to patients with inflammatory or infectious diseases. However, these comparisons have to be interpreted with great care because several groups are relatively small.

A range of mediators show significant differences when comparing DVT patients with healthy controls. When patients with DVT were compared with healthy controls, significant differences for several mediators were observed, with P-selectin ($p = 0.0009$), VCAM-1 ($p < 0.0001$), all MMPs (all $p < 0.0014$), and HGF ($p < 0.0001$) showing the strongest significant differences.

Unsupervised hierarchical clustering. Unsupervised hierarchical clustering analyses based on biomarkers showing differences between patients with and those without DVT could be used to identify patient subsets that differed significantly in their DVT frequency. As shown in Table 13, clustering analysis showed that the higher the degree of inflammation in a patient, the more similar the inflammatory profile is to that in patients with DVT.

Conclusion. Patients with venous thromboses show altered systemic levels of several inflammatory mediators with different biological functions. This systemic inflammatory response shows similarities with the responses detected in patients with infections.

Table 13. Inflammatory mediators in patients with suspected DVT

Mediator, median (range)	Patient groups (*n*)					
	DVT (21)	**Non-inflammatory (24)**	**Thrombophlebitis (5)**	**Stasis (8)**	**Inflammatory (21)**	**Infection (9)**
			INTERLEUKINS			
IL-1ra[1]	737.1 (381.3–2505)	602.0 (153.4–1817)	600.4 (489.4–2307)	675.9 (432.5–1730)	762.3 (358.6–7116)	1137 (552.9–1520)
IL-6[1]	1.240 (nd to 14.30)	nd (nd to 36.50)	3.57 (nd to 8.95)	2.020 (nd to 23.59)	nd (nd to 18.88)	**9.960 (nd to 26.50)** ***p*** **= 0.0165**
			GROWTH FACTORS			
G-CSF[1]	22.30 (8.080–66.71)	21.92 (5.32–82.65)	21.92 (1.22–8.59)	28.37(13.90–38.14)	19.52 (3.860–59.05)	30.04 (16.62–46.08)
EGF[1]	3.400 (nd to 21.61)	3.4 (nd to 14.67)	13.24 (nd to 22.22)	2.955 (nd to 16.06)	3.58 (nd to 35.74)	7.42 (nd to 19.27)
HGF[1]	212.6 (78.33–1177)	**140.7 (55.33–2210)** ***p*** **= 0.0035**	156.3 (47.62–317.2)	260.4 (109.8–996.5)	169.5 (72.11–11 588)	244.4 (93.23–3 195)
TPO[1]	305.7 (57.53–2 734)	**242.6 (20.20–414.7)** ***p*** **= 0.0395**	258.6 (151.7–411.1)	232.9 (183.9–403.8)	265.9 (76.22–515.1)	262.6 (40.82–586.4)
Leptin[1]	19 902 (3 317–148 212)	23 968 (1 395–99 928)	38 758 (10 885–131 575)	15 673 (1 231–66 806)	17 039 (1 182–85 826)	20 418 (2 568–104 039)
VEGF[1]	8.380 (nd to 28.42)	**nd (nd to 13.38)** ***p*** **= 0.0122**	11.71 (nd to 21.86)	nd (nd to 13.61)	3 450 (0.022–25.96)	7 010 (nd to 55.13)
			CHEMOKINES			
CCL2[1]	154.1 (98.51–270.2)	147.2 (69.91–317.7)	197.6 (125.3–277.3)	139.7 (122.0–297.2)	150.5 (60.05–226.0)	179.2 (87.48–255.1)
CCL3[1]	nd (nd to 162.5)	nd (nd to 258.5)	nd (nd to 120.2)	41.50 (nd to 110.7)	59.00 (24–234.4)	110.7 (24–201.9)
CCL4[1]	25.21 (nd to 120.9)	19.97 (nd to 343.6)	14.23 (nd to 54.79)	25.07 (4 222–65.15)	19.31 (1.22–618.1)	21.72 (8.190–68.30)
CCL5[1]	7 074 (1789 to ad)	6 318 (1 835 to ad)	6 393 (2 550–11 984)	5 651 (1 891 to ad)	6 782 (3 351 to ad)	7 722 (3 459 to ad)
CCL11[1]	71.08 (nd to 264.7)	58.7 (nd to 184.8)	80.77 (61.0–133.9)	112.0 (30.66–160.3)	67.77 (nd to 245.1)	84.47 (nd to 156.3)
CXCL5[1]	208.4 (60.85–458.7)	182.8 (41.34–633.6)	228.4 (49.50–454.6)	153.7 (106.1–396.3)	276 (35.87–1 168)	240.0 (nd to 744.9)
CXCL8[1]	4.010 (nd-48.40)	**0.58 (nd to 14.53)** ***p*** **= 0.0168**	0.44 (nd to 3.73)	4.005 (nd to 23.06)	4 010 (nd to 41.75)	4 010 (1 610–21.89)
CXCL10[1]	59.33 (24.06–177.6)	53.66 (16.61–248.4)	57.11 (29.56–743.7)	131.6 (44.28–733.4)	41.13 (18.31–193.1)	**147.3 (39.12–383.6)** ***p*** **= 0.0374**
CXCL11[1]	18.90 (nd to 150.3)	**nd (nd to 36.05)** ***p*** **= 0.0316**	6 780 (nd to 119.6)	9.285 (nd to 162.2)	9.920 (nd to 134.4)	**49.54 (3.380–79.18)** ***p*** **= 0.0265**
			IMMUNOMODULATORY CYTOKINES			
CD40L[1]	383.1 (121.7– 747.2)	323.2 (25.84–1 114)	292,3 (203,5-653,2)	234,3 (42,66-694,1)	365,2 (75,56-1170)	481,2 (94,27-791,3)
			ADHESION MOLECUELS			
ICAM-1[1]	162 439 (51 019–247 545)	128 165 (93 058–219 547)	122 880 (112 772–184 012)	165 145 (115 396–213 254)	135 833 (91 593–213 748)	203 164 (128 522–275 090)
E-selectin[1]	25 869 (8 253–55 843)	29 296 (13 071–51 722)	24 755 (20 745–52 701)	30 468 (14 957–36 613)	30 817 (14 366–62 494)	33 981 (21 516–72 473)
P-selectin[1]	59 798 (8 397–128 459)	**31 571 (19 086–52 555)** ***p*** **<0.0001**	**39 879 (20 393–44 753)** ***p*** **= 0.0374**	**34 633 (29 869–70 084)** ***p*** **= 0.0180**	**34 226 (15 054–54 887)** ***p*** **= 0.0003**	**33 493 (25 566–44 793)** ***p*** **= 0.0011**
VCAM-1[1]	850 161 (104 311–1 571 607)	**589 684 (290 605–999 319)** ***p*** **<0.0001**	**602 782 (508 885–843 992)** ***p*** **= 0.0192**	1 337 133.5 (751 713–2 793 861)	**555 472 (306 268–1 113 602)** ***p*** **= 0.0005**	832 150 (630 719–1 024 449)
			MATRIX METALLOPROTEASES			
MMP-1[1]	361.5 (61.15–1971)	**152.8 (nd to 1 191)** ***p*** **= 0.0185**	463.3 (110.4–1 571)	711.8 (169.5–1 925)	440.1 (nd to 2 393)	304.3 (nd to 1 967)

MMP-2[1]	276 951 (15 025–317 761)	259 712 (91 283–303 335)	254 667 (242 689–311 204)	**319 839 (259 314–354 352)** ***p*** **= 0.0299**	272 875 (58 098–317 870)	237 318 (19 643–336 247)
MMP-3[1]	17 272 (6 452–40 971)	**8 699 (3 373–25 074)** ***p*** **= 0.0112**	**7 907 (3 474–18 840)** ***p*** **= 0.0437**	13 660 (7 599–64 046)	13 462 (5 974–72 879)	12 560 (6 951–31 654)
MMP-7[1]	3 219 (994.5–22 261)	**1 761 (677.7–4 640)** ***p*** **= 0.0032**	**1 571 (1 387–2 583)** ***p*** **= 0.0270**	2 674 (1 353–5 237)	2 825 (1 005–8 208)	4 362 (166–12 246)
MMP-8[1]	2 273 (1 087–6 942)	**1 244 (356.9–5 649)** ***p*** **= 0.0005**	1 986 (791.8–4 310)	2 763 (637.8–4 140)	1 791 (534.2–5 737)	1 051 (687.8–40 873)
MMP-9[1]	31 640 (13 282–110 149)	27 010 (12 253–100 882)	29 214 (22 302–75 027)	33 828 (17 483–51 961)	32 498 (12 076–157 415)	28 367 (12 019–161 669)
			TRADITIONAL MEDIATORS			
D-dimer[2]	8.59 (0.6500 to >20.00)	**0.425 (<0.22 to 2.110)** ***p*** **<0.0001**	**1.380 (0.27–2.11)** ***p*** **= 0.0112**	**0.5400 (<0.22 to 3.050)** ***p*** **= 0.0005**	**1.030 (nd to 5.640)** ***p*** **<0.0001**	**1.77 (0.34–5.440)** ***p*** **= 0.0018**
CRP[2]	13 (<1 to 170.0)	**2 (1–19)** ***p*** **= 0.0002**	8 (1–36)	7.5 (<1 to 26.00)	7 (1–62)	**57 (5–143)** ***p*** **= 0.0372**

The table shows differences in results for each inflammatory mediator between DVT and different diagnostic groups. The analyses were performed using Mann–Whitney test and were not corrected for multiple testing. This table was not published in paper III due to small-sized patient groups and difficulties with multiple testing. It indicates expectedly that the conditions with most inflammation (infection and other inflammatory conditions) have fewer mediators that differentiate against DVT.

Values are given as the median (range) in pg/ml[1] and mg/l[2]. *p*-values are given where the test shows statistically significant results (*p*-value <0.05).

ad, above detection limit; CRP, C-reactive protein; EGF, epidermal growth factor; G-CSF, granulocyte colony-stimulating factor; HGF, hepatocyte growth factor; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; nd, not detected; TPO, thrombopoietin; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor.

4.4 PAPER IV: CYTOKINE PROFILES CAN IDENTYFY BACTERAEMIA IN PATIENTS WITH SERIOUS INFECTIONS

Only a few studies have investigated multiple cytokine combinations as part of a broader inflammatory response (Table 12). One of the studies presented in this thesis aimed to assess for differences in inflammatory profile in patients with sepsis showing different microbiology backgrounds (i.e. bacteraemia vs. non-bacteraemia and Gram-positive vs. Gram-negative). This approach differs from that in most other studies where differences in physical deterioration, organ failure, and prognosis were compared. In this prospective observational study here, 80 septic patients with proven bacterial infection and no immunosuppression during the first 24 h of hospitalization were included. Luminex® analysis of a total of 35 mediators was performed: 16 cytokines, six growth factors, four adhesion molecules, and nine MMPs or TIMPs.

Six mediators show significant differences in levels between patients with and those without bacteraemia. Of a total of 80 patients with sepsis, 42 patients (53%) had positive blood cultures, with the remaining 38 showing negative blood cultures. The levels of six mediators showed statistically significant differences between the group of patients with and those without bacteraemia, using Mann–Whitney test (p <0.0014): TNF-α, CCL4, E-selectin, VCAM-1, ICAM-1, and TIMP-1. In addition, ten mediators also showed statistically significant differences in levels, with p-values ranging between 0.05 and 0.0014: IL-1ra, IL-10, CCL2, CCL5, CXCL8, CXCL11, HGF, MMP-8, TIMP-2, and TIMP-4. Of note, VCAM-1 showed the most robust results using univariate and multivariate logistic regression.

Hierarchical clustering shows that patients with bacteraemia have higher levels of inflammatory mediators and are clustered together. Unsupervised hierarchical clustering revealed that the six inflammatory mediators TNF-α, CCL4, E-selectin, VCAM-1, ICAM-1, and TIMP-1 could be used to discriminate between the two patient groups, i.e. those with and those without bacteraemia. Patients with bacteraemia were mainly clustered in two separate groups (two upper clusters, 41/42 patients, 98%) with higher inflammatory mediator levels. In contrast, most patients without bacteraemia (23/38, 61%) were clustered in the lower cluster, compared with only one patient with bacteraemia (2%) clustered in this group (chi-squared test, p <0.0001).

Gram-positive and Gram-negative bacterial infections do not show significant differences in inflammatory profiles in a consecutive sepsis population with proven bacterial infection. Only minor differences in inflammatory mediator levels were obtained in patients with Gram-positive bacterial infections compared with those with Gram-negative infections. Of the inflammatory mediators analysed, differences in levels were found only with CCL4, CXCL10, and leptin, with CCL4 levels being the highest in the Gram-negative group, and CXCL10 and leptin levels being the

highest in the Gram-positive group. However, these differences were not significant after Bonferroni correction.

Comments and conclusion. In this study we compared the inflammatory responses for patient with Gram-negative and Gram-positive infections, and these two patient subsets did not differ significantly. This is probably due to the expression of molecules with strong proinflammatory effects by both types of bacteria (see Section 1.9.3.2 p.34 LPS and various superantigens, respectively), although the molecular mechanisms behind the proinflammatory effects differ between them. Furthermore, as will be discussed later (Section 5.2.3.3) bacteraemia is more common for patients with severe sepsis and especially for patients with septic shock. Even though differences in systemic inflammatory profiles did not have an independent prognostic impact in our study, our observations suggest that the systemic inflammatory profile may be used as a clinical tool for early identification of a patient subset with an increased frequency of bacteraemia and thereby increased risk of later complications. However, this question has to be further addressed in future clinical studies before a final answer can be achieved.

5 DISCUSSION

The work presented in this thesis has explored and compared the broader inflammatory profiles, as well as their possible prognostic and diagnostic implications and relevance, in four different patient cohorts.

5.1 METHODOLOGICAL CONSIDERATIONS

5.1.1 Pre-analytical variability in patient cohorts

As described in the Methods section, several factors can contribute to pre-analytic variability. Inflammatory mediators show considerable biologic intra- and inter-individual variability in healthy controls as well as in various patient cohorts with stable non-inflammatory diseases. This indicates that the inflammatory process is highly dynamic, varying from one individual to another, which represents a major challenge when examining the inflammatory response in different patient cohorts (as described in Section 2.1 on p. 37). Moreover, in all patient cohorts studied, their inflammatory conditions (i.e. DVT and sepsis) improved following treatment administration or after the completed procedure (i.e. apheresis) (224). Thus, this raises the question of whether biologic variability of inflammatory mediators is a major confounding factor in our patient cohorts. Most studies of inflammatory mediators have examined inflammatory profiles at a single time point (85, 188, 206, 207), as was also the case for studies described in papers II, III, and IV. One basic assumption when planning the studies in this thesis has was that biologic inter- and intra-individual variability will have lesser impact on the inflammatory profiles than on the clinical condition itself. Still a cautious approach to the issue of variability is needed, as it is difficult to discount or disregard any potential impact from biologic variability, whether intra-individual (differences in disease development or expression of inflammatory mediators) or inter-individual (diurnal, disease fluctuation). Inflammatory profiles in cases of sepsis, for example, might be more prone to high biologic variability, as the timing of sample collections can vary, e.g. whether taken during daytime or at various stages in the course of the condition (242). On the other hand, studies of inflammatory profiles in healthy subjects and conditions with lower inflammation as shown in Paper III might be more likely to be exposed to both biologic and analytical variability since relatively low levels of inflammatory mediators are involved. If there is less inflammation and lower levels of inflammatory mediators, other intermittent inflammatory conditions could give larger relative both intra-individual and inter-individual variation because the base value is lower. There will also be larger risk for larger variability when analysing lower levels of mediators because analyses with ELISA and Luminex will give less accurate values in the fringes of the assay range.

For the apheresis cohort described in paper I, low pre-analytical variability was expected, as these patients underwent a standardized procedure at the same time point in the day for sampling. In fact, of the four studies presented, this study had the most rigorously standardized conditions regarding timing of collections and sample handling methods. However, as reported in a previous study (130), there were considerable difficulties here in comparing results for the stem cell harvesting group which received pre-treatment (i.e. G-CSF and cyclophosphamide) that could have affected the inflammatory profile and results for the thrombapheresis group which received no treatment.

For the studies presented in papers II and III, most of the patients with DVT were referred to the hospital in the afternoon, although this was not an absolute rule. Since treatment was expected to directly affect the results by reducing the thrombotic burden (5), all samples were standardized and taken before the start of treatment.

For the study described in paper IV, samples were collected from sepsis patients as soon as possible within the first 24 h of hospitalization. However, as there is greater fluctuation in the inflammatory process in sepsis, this makes the interpretation of results more challenging when only measuring samples taken at one particular time point. Ideally, sample collections would have been taken at more time points; however, to avoid increased patient distress, sampling was restricted to a single time point only.

The sample handling method (time to centrifugation, storage medium) and storage time will directly affect the inflammatory mediator levels (224). Here, samples collected in citrate plasma and with similar storage times were compared. When investigating patient cohorts for inflammatory profiles, one major difficulty lies in obtaining fresh samples for immediate processing and subsequent testing within a short time period. However, most of the study samples here were analysed within 2 years of collection, so cytokine degradation was not expected, although this cannot be fully excluded (222, 229). Of note, cytokines such as IL-1α, IL-1β, IL-10, IL-15, and CXCL8 can degrade by up to 75% after 4 years (224).

5.1.2 Analytical variability

An important question is whether the results obtained by antibody-dependent analytical methods reflect the actual concentration of the different inflammatory mediators. According to comparative analyses between different ELISA and multiplex bead array assays from different manufacturers, quantitative levels of inflammatory mediators cannot be compared directly, implying that the actual concentrations obtained depend on the specific assay kit used (228).

In addition, proteolytic cleavage of inflammatory mediators by MMPs may lead to either activation or inactivation of the mediators. Thus, if antibody binding sites are altered during proteolysis, theoretically, previously inactivated forms could be measured as now active forms by

antibody-dependent methods. For the studies presented here, according to R&D Systems, the precise effects of possible truncation of inflammatory mediators measured using their ELISA and/or Luminex® kits are not known.

5.1.3 Different statistical approaches

Biological systems are highly complex, and it is often difficult to adjust for all determinants that result in the final outcomes. Increasing possibilities for exploring larger parts of biological systems (e.g. genomics, proteomics, metabolomics, etc.) have led to the development of more holistic approach to studying biological systems, often referred to as systems biology (9, 237). One of the statistical approaches used to explore larger data sets includes hierarchical clustering (20, 235, 237), while several other approaches have also been applied to multiplex cytokine data such as PCA and self-organizing maps (235). Examples of these other approaches have already been described in Section 1.9.4, p.35 where different statistical approaches used to analyse multiple inflammatory mediator profiles in sepsis patients are displayed. This table show that traditional statistical methods such as Mann-Whitney and logistic regression have been widely used in clinical studies of sepsis cohorts (also in our studies), while hierarchical clustering is not so commonly used. PCA was used in studies with even larger datasets than ours, and as we managed to describe the datasets with easier tools, we did not use yet another methodology. ROC-analysis was performed in our datasets, but was excluded from the final presentation of the data as the datasets were small and as well as fear of overestimating the diagnostic impact from this analysis.

5.1.4 Does hierarchical clustering provide more than just redundant information?

The hierarchical clustering methodology does not give any further information on statistical differences between different groups, compared to what can be obtained using Mann–Whitney test and logistic regression (i.e. here bacteraemia, Gram-positive/-negative, organ failure). The reason for performing hierarchical clustering is to obtain a more complementary picture useful in heterogeneous data sets. In our studies, this methodology was used to explore inflammatory mediators as well as patient covariates, as it gives an overview of both mediator and patient covariation in one single picture that is difficult to obtain using traditional statistics. In turn, this allows the generation of hypotheses using combinations of multiple mediators that would need confirmation using more traditional methodology.

5.2 CLINICAL AND BIOLOGICAL IMPLLICATIONS OF THE RESULTS

5.2.1 Paper I: Stem cells

Paper I describes changes in several inflammatory mediators in multiple myeloma patients undergoing stem cell mobilization and harvesting, although there were methodological difficulties with the control

group, as discussed earlier and also in the paper, since both G-CSF and cyclophosphamide also induce an inflammatory response (130, 131). Multiple myeloma is not considered a curable disease, and stem cell treatment represents an important component in myeloma treatment, as described earlier in Section 1.7 on p. 22 (104). Several aspects of the effects of stem cell treatment in myeloma are unclear, and an understanding of the broader inflammatory response during the procedure adds new knowledge of the biological mechanisms underlying the treatment and the disease itself.

5.2.1.1 Possible implications of altered inflammatory profiles

When interpreting the study results, it should be noted that inflammation seen in cancer is caused by the disease itself, and therefore these patients are primed for additional modulation of the cytokine network by inflammatory stimuli.

Clinical context of multiple myeloma and cytokine reaction in patients with multiple myeloma. Myeloma cells are highly dependent on a rich support milieu of cells, and high levels of inflammatory mediators have been associated with a worse prognosis of myeloma (4, 104, 130, 170). As myeloma cells are dependent on, and sensitive to, cytokines and since stem cell treatment is a vital part of multiple myeloma treatment, it is not unthinkable that inflammatory changes during treatment could impact the malignant cells, other immune cells, and any subsequent treatment. Findings here showed that there are differences before stem cell harvesting, compared to healthy controls and changes during stem cell harvesting. Therefore, this could directly affect dormant cancer cells residing in the bone marrow and in the stem cell graft itself (134, 135).

5.2.1.2 Prognosis estimation from chemokine patterns in stem cell grafts

Despite the fact that higher levels of inflammatory mediators are correlated with poor prognosis, study findings here showed that lower levels of inflammatory chemokines in the apheresis products were associated with poorer prognosis (Figure 3, paper I). However, patient numbers were small, and since the clustering methodology should be regarded as hypothesis-generating, conclusions should be drawn with caution, as emphasized in the paper. The reduced chemokine levels may be due to the immunocompromised status (e.g. myeloma-induced hypogammaglobinaemia, chemotherapy-induced $CD4^+$ T-cell defects) in many of these patients that might be related to disease progression, which could further explain the poorer prognosis (121-123). Of note, patients were treated before the currently used staging systems, i.e. initially the International Staging System for multiple myeloma (ISS) and subsequently the revised International Staging System for multiple myeloma R-ISS, were implemented in the clinical practice here (104, 243).

Paper I illustrates the importance of considering the overall clinical context, including diagnostic and therapeutic methods, when interpreting a systemic inflammatory mediator profile.

5.2.2 Papers II and III: DVT

5.2.2.1 Deciphering the inflammatory process in a heterogenic patient cohort with suspected DVT

The heterogeneity of diagnoses in the patient cohort is a strength as well as a weakness. This unselected and consecutive cohort mimics more closely the actual clinical situation, compared to most studies comparing DVT patients with normal controls. In line with previous research as shown in Section 1.8 p. 26, a larger part of the mediators differentiates between healthy controls and DVT patients. Although there are few patients in the different diagnosis groups, the main trend emerging shows that the more inflammation from causes other than DVT, the more difficult it is to differentiate DVT from the other diagnoses. This illustrates that thromboses and inflammation are tightly interconnected, a point emphasized in Section 1.2 in the Introduction (p.3).

Detailed assessment of the inflammatory mediators showed that only few of these mediators can differentiate DVT from other diagnoses in the clinical situation, and they all showed less discriminatory potential than the currently used D-dimer. The most promising inflammatory mediator of all those tested is possibly P-selectin, although direct studies of P-selectin have not shown convincingly better results, compared with D-dimer (179).

5.2.2.2 Problems with diagnosing DVT

The patients were diagnosed with DVT through ultrasound examination, only a few patients underwent conventional or CT venography which have greater sensitivity for leg vein thrombosis (244). It is therefore difficult to exclude smaller leg vein thromboses, however none of the patients were readmitted with DVT after initial diagnostic assessment, although some underwent repeated ultrasound examination. Performing venography in all patients would have caused more patient discomfort and risk for complications and is not recommended as a routine examination method (244).

5.2.2.3 Inflammation—cause or consequence?

Inflammatory conditions are considered as a risk factor for venous thromboses (138-141). However, some larger studies of altered inflammatory mediators indicated that both changes in inflammatory mediator levels (e.g. ↓ IL-10, ↑ TNF-α) (149, 150) and several SNPs of inflammatory mediators are associated with an increased risk for venous thromboses (e.g. ↓ IL-1α, ↓ IL-1β, ↑ IL-6, ↑ IL-10) (141, 145).

Mechanistic animal studies support that venous thromboses are dependent on inflammatory activation through the endothelium, neutrophils, and monocytes (12). Of note, more studies have been carried out showing elevated inflammatory mediator levels in acute DVT than investigating

inflammatory mediators as risk factors. This might be because it is methodologically more difficult to study inflammatory mediators as risk factors. However, taken together, all these studies consistently demonstrate that inflammation increases during DVT and decreases as the thrombotic burden is reduced. Thrombosis per se will also accentuate the inflammatory response (5), and several studies have described higher levels pro-inflammatory mediators in acute DVT (5, 146, 154, 157) and also in patients with an increased risk for post-thrombotic syndrome (151, 158, 160). Interestingly, it has also been shown in a recent study that patients 5 years after their diagnosis of DVT still have elevated levels of inflammatory mediators (168), and the authors postulate that inflammation is a risk factor for DVT.

The patient cohorts studied in our studies did not undergo assessment before clinical presentation and had no follow-up after presentation. Therefore, it was not possible to determine whether there were any pre-existing inflammatory conditions already present that could have had an impact on the results. However, no major differences were obtained in the inflammatory response related to the extent of the thromboses, as shown in Figure 2 in paper III.

5.2.2.4 Does clustering analysis give any additional information in these patient cohorts?

The unsupervised hierarchical clustering models used here revealed patterns of inflammatory mediators, identifying groups with increased frequency of thromboses (Figure 5a, paper III). The combination of inflammatory mediators with D-dimer gave better discriminatory results (Figure 2, paper II; Figure 5b, paper III). While future approaches could include the possible use of clustering models for diagnosis, findings using the models described here indicated that such models have major limitations, as DVT was identified even in the group of patients with very low levels of inflammatory mediators. Taken together with results obtained using the Mann–Whitney test, no methods tested so far seem to give better discriminatory values than the currently used D-dimer, in agreement with previous studies. Moreover, addition of D-dimer also gives more coherent clusters of other diagnoses, as described at the previous page.

Furthermore, the clustering models also provided information on inflammatory mediators that covariate in the patient cohort (Figure 3, paper III), suggesting an association between different mediators. Accordingly, results showed both adhesion molecules (marked red) and MMPs (marked blue) clustering closely, in agreement with the background knowledge shown in Table 4 and 5 and Figure 2. In addition, most chemokines were shown to cluster in another separate cluster (marked blue). As discussed earlier, conclusions from hierarchical clustering should always be made with caution.

Findings discussed so far have demonstrated a broader inflammatory response during venous thrombosis. However, as already mentioned, coagulation activation is part of several inflammatory processes, and therefore it is difficult to envisage using inflammatory mediators as sole diagnostic

biomarkers. It is still important to use a systematic combination of clinical signs (Wells score), biomarkers, and radiological evidence (ultrasound or venography) for diagnosing venous thromboses (245).

5.2.3 Paper IV: Sepsis

5.2.3.1 Main problem with sepsis studies: a heterogeneous patient cohort is associated with multiple confounding factors

The precise pathophysiological mechanisms underlying sepsis are poorly understood (16). In addition, the group of patients with sepsis is highly heterogeneous. Due to this heterogeneity, the sepsis definition has been extensively debated and changed over the years, till the present Sepsis 3.0 definition. Direct comparisons between different studies of sepsis have been hampered by the frequent changes in the definition of sepsis as well as by the non-specificity of the definitions. To overcome these difficulties, our study was designed to investigate a more well-defined group of patients (i.e. those with bacterial infections) within a larger sepsis cohort by assessing the effects of different types of infections (i.e. Gram-positive vs. Gram-negative and bacteraemia vs. non-bacteraemia) on the inflammatory response. Despite this effort, the patient cohorts were still found to remain heterogeneous (Table 1, paper IV).

5.2.3.2 Altered sepsis definition during the publication process

The latest sepsis definition Sepsis 3.0, which includes patients with infection and organ failure with a total SOFA score increase of ≥2 (16, 188), was used our study. The cytokine profile divided the patients into subsets, in which most of those with organ failure were found in the two clusters with the highest inflammatory response. As it was difficult to define organ failure using the previous Sepsis 2.0 definition, the SOFA score was therefore used, which was prior to the publication of the new Sepsis 3.0 definition. The Sepsis 3.0 definition gave a scoring of organ failure that is closer to our clinical impression of the patients and also a better coherence with the inflammatory mediators.

5.2.3.3 Bacteraemia—does it help to know?

Bacteraemia has been shown to be associated with disease severity in sepsis patients; 17% of sepsis patients, 25% of severe sepsis patients, and 67% of septic shock patients were shown to have bacteraemia in a larger sepsis cohort (246). Bacteraemia is associated with mortality from univariate analyses, although not after correction for organ failure and early appropriate antimicrobials (247-249). In light of this, it can be argued that irrespective of whether the patient has bacteraemia or not, this will not affect patient management as long as appropriate antibiotic treatment is administered. However, the rationale behind the study here was to determine the impact of the infection itself on the inflammatory response, for a better understanding of the pathophysiological mechanism underlying sepsis.

The Sepsis 2.0 definition proposed PIRO as a framework for the study of factors influencing the development of sepsis: Predisposition (host) – Insult (microbe and extension) – Response (inflammatory response) – Organ failure. The model data obtained here suggested that the inflammatory response was also dependent on the insult, in this case the insult being bacteraemia, whereby the inflammatory response does not necessarily only correlating with the degree of physical derangement and organ failure. In a scenario where impaired organ function is the main predictor of mortality, a factor such as bacteraemia that results in an increased inflammatory response, but has no effect on survival, will be a major confounder if inflammatory mediators were used as predictors of survival. This is reflected in the Sepsis 3.0 definition that focuses mainly on organ failure, and less so on the inflammatory response, as a better predictor of survival (16).An open question with the Sepsis 3.0 definition is how this will impact treatment of sepsis. In the absence of organ failure, a high inflammatory response, as described in paper IV, will support a high probability of bacteraemia, indicating the necessity for broad antibiotic cover for bloodborne infections, and conversely patients with low inflammatory parameters and no organ failure would likely need less broad antibiotic cover.

5.2.3.4 Differences in mediators between patients with and without bacteraemia

Of the 80 consecutively included patients with confirmed bacterial infections, differences in mediators were found mainly between the patients with or without bacteraemia. Of note, patients with bacteraemia were older and had more sepsis-related organ failures and higher total SOFA scores, compared with patients without bacteraemia (Table 1, paper IV). In itself, this is not surprising, as age is an important risk factor for the development of sepsis and the risk for bacteraemia increases with increased severity of sepsis (16, 246, 249). Therefore, caution to adjust for these differences is needed when evaluating the isolated effect of bacteraemia on inflammatory mediators. For the studies presented here, a multiple logistic regression model was developed to assess the impact of age and severity of sepsis on inflammatory mediators. Due to the low patient number, the number of confounding factors that could be corrected for in the logistic regression model was limited; therefore, only these two factors were corrected for in the model including the six most significant mediators (hence a total of eight factors). In this model, results showed that changes in the SOFA score and age did not affect the inflammatory mediators (Table 2, paper IV). The hierarchical clustering model used here (Figure 3, paper IV) suggested that both bacteraemia and organ failure were found in those patients with higher levels of inflammatory mediators (upper and middle clusters), although there were a higher number of patients with organ failure than with bacteraemia in the group with the lowest levels of inflammatory mediators (lower cluster).

5.2.3.5 Magnitude of the inflammatory response—what are the main drivers

The SIRS criteria were replaced by the SOFA scoring system for diagnosing sepsis, as the new qSOFA criteria have been shown to be better predictors of an unfavourable prognosis in larger sepsis

databases, and organ failure is chosen as the central sepsis-defining criterion (16). Our findings support the assumption that not all patients with high levels of inflammatory mediators necessarily have organ failure, and vice versa. With the Sepsis 3.0 definition included in the last study, 65% of patients showed sepsis-induced organ failure. The hierarchical clustering model (Figure 3, paper IV) that displayed both organ failure and bacteraemia showed that there were fewer patients with bacteraemia in the lower cluster with the lowest levels of inflammatory mediators, compared with patients with organ failure.

5.2.3.6 Gram-positive and Gram-negative infections

Only minor differences in inflammatory mediator levels were obtained between Gram-positive and Gram-negative infections. Few studies have been performed comparing the inflammatory response in Gram-positive and Gram-negative infections (201, 250). One study showed higher levels of inflammatory mediators in Gram-positive infections (patients with various infection foci) (201), whereas another reported higher mediator levels in Gram-negative infections (patients with bacterial peritonitis only) (250). These discrepancies could be explained by the heterogeneity of infection sites in the Gram-positive cohort that could influence the results, compared to the more homogeneous Gram-negative cohort.

5.2.3.7 Diagnostic potential and comparison with previous studies

The Sepsis 3.0 definition strongly suggests multiple mediator profiles as a possible future diagnostic possibility (16). The mediator profile that distinguishes the presence of bacteraemia in sepsis patients is perhaps the most promising of all profiles described in the four papers presented in this thesis. The results of our studies also suggest that mediator profiles should include soluble mediators with different molecular functions to reflect various aspects of the inflammatory process. For example, procalcitonin, which, on its own, could have a similar predictive value for bacteraemia, as shown in some studies (85, 188, 206, 207), will not be detected as early as the cytokines and adhesion molecules tested here (188, 206, 207). Study results presented here suggest VCAM-1 as a possible predictive marker for bacteraemia (85, 207).

Still, the approach described here using multiple mediator profiles is not ready for direct application into daily clinical practice. Single biomarkers have so far not demonstrated satisfactory diagnostic value, and therefore biomarker combinations should be investigated further (188). Careful and thorough patient history taking, clinical examination, and judicious monitoring to reveal clinical signs of an infection focus, inflammatory response, and organ failures will always remain the cornerstones of diagnosis and treatment in severe infections. Correct identification of the infection focus and early administration of appropriate antibiotics are vital determinants of prognosis in patients with sepsis (248, 251).

6 FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

It has now been over three decades since the first interleukins were described (252, 253). Even though a wide range of cytokines have been identified since and despite their role in the pathogenesis of many diseases, analysis of the cytokine system has not become part of routine clinical practice, with a few exceptions, e.g. in the diagnosis of haemophagocytic lymphohistiocytosis (254, 255).

Results presented in this thesis, as well as previous studies, have demonstrated that cytokines form an interacting biological network together with many other soluble mediators. The possible clinical impact of this cytokine network should be analysed not as the effect of a single individual cytokine, but rather as the effect of the overall network function, e.g. evaluation of the systemic or cytokine profile or analysis of the cytokine profile in biologically defined compartments.

Diagnostics through pattern recognition is not an established methodology. Although there is continuing progress and development in bioinformatical capacities and even artificial intelligence, such models are heavily dependent on the quality of the initial data provided (256, 257). Clustering can reveal patterns to generate hypotheses that can be tested through more sophisticated approaches (258).

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