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**INNATE IMMUNITY AND ITS CONTROL
IN THE PATHOGENESIS OF
INFLAMMATORY RHEUMATIC DISEASES**

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Academic Dissertation

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ORIGINAL PUBLICATIONS

- I **Anttonen K**, Orpana A, Leirisalo-Repo M, Repo H. Aberrant TNF secretion by whole blood in healthy subjects with a history of reactive arthritis: time course in adherent and non-adherent cultures. *Ann Rheum Dis* 2005;65:372-8.

- II **Kuuliala K**, Orpana A, Leirisalo-Repo M, Repo H. Neutrophils of healthy subjects with a history of reactive arthritis show enhanced responsiveness, as defined by CD11b expression in adherent and non-adherent whole blood cultures. *Rheumatology* 2007;46:934-7.

- III Repo H, **Anttonen K**, Kilpinen SK, Palotie A, Salvén P, Leirisalo-Repo M. CD14 and TNF- α promoter polymorphisms in patients with acute arthritis. Special reference to development of chronic spondyloarthropathy. *Scand J Rheumatol* 2002;31:355-61.

- IV **Kuuliala K**, Orpana A, Leirisalo-Repo M, Kautiainen H, Hurme M, Hannonen P, Korpela M, Möttönen T, Paimela L, Puolakka K, Karjalainen A, Repo H. Polymorphism at position +896 of the Toll-like receptor 4 gene interferes with rapid response to therapy in rheumatoid arthritis. *Ann Rheum Dis* 2006;65:1241-3.

- V Salvén P, **Anttonen K**, Repo H, Joensuu H, Orpana A. Endotoxins induce and interferon- α suppresses vascular endothelial growth factor (VEGF) production in human peripheral blood mononuclear cells. *FASEB J* 2001;15:1318-20.

ABBREVIATIONS

ABC	antibody-binding capacity
ACPA	anti-citrullinated protein antibody
AS	ankylosing spondylitis
BRAK	breast and kidney-expressed chemokine
CD	cluster of differentiation
cDNA	complementary DNA
Combi	combination DMARD treatment group
CR	complement receptor
CRP	C-reactive protein
CXCL	CXC chemokine ligand
DAS	Disease Activity Score
DAS28	28-joint Disease Activity Score
DC	dendritic cell
DMARD	disease-modifying antirheumatic drug
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
EDTA	ethylenediamine tetra-acetic acid
ESR	erythrocyte sedimentation rate
ESSG	European Spondyloarthropathy Study Group
FACS	fluorescence-activated cell sorter
FIN-RACo	FINnish Rheumatoid Arthritis Combination treatment study
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony stimulating factor
HLA	human leukocyte antigen
HSP	heat shock protein
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-1RA	interleukin 1 receptor antagonist
LBP	lipopolysaccharide-binding protein
LFA	leukocyte function associated antigen
LPS	lipopolysaccharide
mAb	monoclonal antibody
Mac	Macrophage antigen
mCD14	membrane-bound CD14
MCP	monocyte chemoattractant protein
M-CSF	macrophage colony-stimulating factor
MD-2	myeloid differentiation protein-2
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MPO	myeloperoxidase

mRNA	messenger RNA
MTX	methotrexate
MyD88	myeloid differentiation factor 88
NF-kB	nuclear factor-kappa B
NK	natural killer
NS	not significant
NSAID	non-steroidal anti-inflammatory drug
PAD	peptidylarginine deiminase
PAF	platelet-activating factor
PAMP	pathogen-associated molecular pattern
PBMNC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PC5	phycoerythrin-cyanin 5
PCR	polymerase chain reaction
PE	phycoerythrin
PECAM	platelet/endothelial cell adhesion molecule
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear
PRR	pattern recognition receptor
PsA	psoriatic arthritis
PSGL	P-selectin glycoprotein ligand
RA	rheumatoid arthritis
ReA	reactive arthritis
RF	rheumatoid factor
RFU	relative fluorescence unit
RNA	ribonucleic acid
RPE	<i>Rhodophyta</i> phycoerythrin
RPMI	Roswell Park Memorial Institute
RT-PCR	real time PCR
SAP	serum amyloid protein <i>or</i> shrimp alkaline phosphatase
sCD14	soluble CD14
SE	shared epitope <i>or</i> standard error
Single	single DMARD treatment group
SLE	systemic lupus erythematosus
SLex	Sialyl Lewis-X
SNP	single nucleotide polymorphism
SpA	spondyloarthropathy
ss	single-stranded
STAT	Signal Transducers and Activators of Transcription
TGF	transforming growth factor
Th	helper T cell
TLR	Toll-like receptor
TNF	tumour necrosis factor
TNF-R	tumour necrosis factor receptor
UV	ultraviolet
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor

ABSTRACT

The pathogenesis of inflammatory rheumatic diseases, including rheumatoid arthritis (RA) and spondyloarthropathies (SpAs) such as reactive arthritis (ReA), is incompletely understood. The inflammatory functions of the innate immune system in these diseases were studied in the present thesis.

Reactive arthritis (ReA) is a sterile joint inflammation, which may follow a distal infection caused by Gram-negative bacteria that have lipopolysaccharide (LPS) in their outer membrane. The LPS receptor, CD14/Toll-like receptor 4 (TLR4)/MD-2, is mainly expressed on innate immune cells and mediates, for example, the production of the essential pro-inflammatory cytokine tumour necrosis factor (TNF).

We observed that peripheral blood monocytes of subjects with previous ReA showed high secretion of TNF, which was evident in response to adherent *in vitro* conditions (mimicking the vascular endothelium made adherent by inflammatory signals) and non-specific stimulation (referring to TNF production capacity). Also, rapid induction of TNF production, which depended on adherence, was high in these subjects. Thus, enhanced TNF production capacity of adhering monocytes may be involved in the development of ReA. One possible mechanism is aberrant signalling through CD11b/CD18, the major molecule mediating monocyte adhesion to the endothelium and cell culture well surfaces.

Peripheral blood neutrophils of patients with previous ReA showed significantly high CD11b levels in response to physiological and concentrated LPS levels or adherence. Thus, circulating neutrophils that encounter bacterial material or adhere seem highly responsive, which may be associated with the pathogenesis of ReA. The increased CD11b levels may result from abnormal CD14/TLR4/MD-2 - or CD11b/CD18 -mediated signal transduction.

HLA-B27 predisposes to SpAs by an unknown mechanism. Our findings suggest that previous ReA and HLA-B27 positivity are additive

factors to bring about the elevated TNF production and CD11b expression. HLA-B27 may be involved by its functions as an antigen-presenting MHC class I molecule, as an antigen or by its tendency to misfold in endoplasmic reticulum, which could affect the transport or synthesis of other proteins.

The promoter polymorphisms *TNF* -308G/A and *CD14* -159C/T have been associated with transcriptional activity of the corresponding genes and risk of inflammatory conditions. Among our patients, all females that developed chronic SpA had the -159T allele and none had the -308A allele. This may reflect some interrelated effects of hormonal and inflammatory signals.

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterised by synovitis, progressive joint destruction and disability. Its activity should respond to the treatment rapidly in order to maintain the patient's work ability. Our results suggest that *TLR4* +896A→G (causing the Asp299Gly change in the TLR4 molecule) could be an advantageous marker when choosing the treatment strategy: patients with the G allele required a combination of disease-modifying antirheumatic drugs. Perhaps Asp299Gly alters the response to LPS or endogenous TLR4 ligands (e.g. those present in the inflamed joints).

VEGF (vascular endothelial growth factor) promotes both the increased vascular permeability characteristic of the inflamed sites and angiogenesis that takes place early in the rheumatoid synovium. We found that LPS induced and IFN- α inhibited VEGF production by peripheral blood mononuclear cells, suggesting a role for LPS-induced VEGF in the pathogenesis of systemic inflammatory disorders, such as RA, and the ability of IFN- α to prevent undue angiogenesis in the inflammatory response.

INTRODUCTION

Inflammation is the protective reaction of vascularised tissues against invading microbes and physical and chemical dangers. It is triggered by the activation of innate immunity, which discriminates rapidly and nonspecifically between self and non-self, and prevents the spreading of the damaging factor by mechanical, chemical and microbiological barriers. The effector cells of innate immunity are phagocytes: macrophages that patrol in tissues, and circulating monocytes and neutrophils, the former continuously migrating to become macrophages and the latter being quickly recruited to the inflamed sites.

A classical innate receptor that mediates phagocyte activation is CD14/Toll-like receptor (TLR) 4/MD-2, which binds lipopolysaccharide (LPS) of Gram-negative bacteria and possibly some endogenous ligands. Binding triggers intracellular signalling leading e.g. to the production of the crucial pro-inflammatory cytokine tumour necrosis factor (TNF). This and other inflammatory mediators make the local vascular endothelium express molecules that bind to CD11b/CD18 and other adhesion molecules on circulating immune cells. The subsequent binding of phagocytes and their recruitment and activation in the tissue compartment are among the most important functions of innate immunity.

The other part of the immune system, i.e. the T and B lymphocyte-based adaptive immunity, recognises specific foreign epitopes and raises a response against the invader expressing those epitopes. This effective system, however, becomes activated slower than innate immunity because, first, there are originally only a few T or B cells specific for a given antigen. Second, the adaptive effector cells, which are initially activated in secondary lymphoid tissues, must find their targets in the body thereafter. Furthermore, in order to become activated, B cells generally need help from already activated T cells. Hence, only if the mechanisms of innate immunity are evaded or overwhelmed by the invader, is there time for adaptive immunity to be launched.

To provide the host with optimal protection, innate and adaptive immunity have evolved co-operating and complementary ways of action. They interact by cell-cell communication (e.g. antigenic peptides are presented with MHC class I and II molecules and recognised by T cells), humoral communication (mostly cytokines and chemokines) and by triggering the same effective amplifying systems (e.g. the complement system). Because innate immunity initiates adaptive immunity, failures of the innate system can have profound effects on the nature of the whole response.

The pathogenesis of inflammatory rheumatic diseases, including rheumatoid arthritis (RA) and spondyloarthropathies (SpAs) such as reactive arthritis (ReA) is incompletely understood, but increasing evidence suggests that innate immune factors play a role. ReA is a sterile complication of an extra-articular infection, which is most commonly caused by Gram-negative bacteria in the gastrointestinal or urogenital tract. The strong clinical overlap of ReA and other SpAs suggests that bacterial infections may be involved in SpAs in general. HLA-B27, an MHC class I molecule, strongly predisposes to SpAs by an unknown mechanism.

Rheumatoid arthritis is a chronic systemic inflammatory disease, in which many immune cells are involved, but the synovitis is predominated by macrophages and pro-inflammatory cytokines. In addition to *HLA-DR1* alleles (MHC class II), which are known to predispose to RA, there is evidence of both microbes and gene-environment interactions in its pathogenesis.

In this study, we examined the crucial features of innate immunity; the production of TNF and the expression of the activation marker CD11b in patients with previous ReA. We also studied the diagnostic and prognostic value of *TNF* and *CD14* polymorphisms in arthritis. In early RA, markers are needed that reliably predict the outcome of alternative treatments for each individual, and here we tested the usefulness of *TLR4* +896A/G that is associated with responsiveness to LPS. We also evaluated the effect of LPS and the endogenous immunoregulatory factor IFN- α on the angiogenic vascular endothelial growth factor (VEGF) production that occurs e.g. in the rheumatic joint.

REVIEW OF THE LITERATURE

INNATE IMMUNITY

PRINCIPLES

The body distinguishes between self and harmful non-self by a wide array of approaches that can be divided into two fundamentally different strategies, innate and adaptive immunity (Chaplin 2003). Innate immunity appeared before the evolutionary split of the plant and animal kingdom, whereas adaptive immunity is only present in vertebrates and cartilaginous fish (Dempsey *et al.* 2003). The key differences between innate and adaptive immunity are listed in Table 1.

The innate immune system comprises all the ready-to-use defence mechanisms, the components of which are encoded in the germ line: mechanical barriers (epithelial cell layers, tight cell-cell contacts), secreted mucus layer (lining the respiratory, gastrointestinal and genitourinary tracts) and cilia sweeping away the mucus, and soluble proteins and bioactive small

Table 1. The diverse properties of innate and adaptive immunity.

Property	Innate immunity	Adaptive immunity
Main cell types	Neutrophils, monocytes, macrophages	T and B lymphocytes
Receptors	Fixed in the genome	Assembled from gene segments
Distribution	Non-clonal expression All cells of a class identical	Clonal expansion All cells of a class distinct
Recognition	Conserved molecular patterns (lipopolysaccharide, lipoteichoic acids, mannans, glycans)	Detailed molecular structures
Self-nonsel f discrimination	Selected over evolutionary time	Selected in individual somatic cells
MHC restriction *	No	Yes
Action time	Immediate	Delayed
Memory **	No	Yes

* The antigen fragments must be presented combined with MHC (major histocompatibility complex) molecules on antigen-presenting cells. ** The response is started more quickly and powerfully on the subsequent encounters than on the first encounter with a given antigen. Modified from Janeway and Medzhitov 2002.

molecules (such as defensins) constantly present in biological fluids. All these mechanisms prevent the invasion of pathogens (Chaplin 2003) (Table 2). More usually, however, the concept of innate immunity is confined to the innate elements that sense the presence of a pathogen and respond to it - cells, receptors, the complement system and acute phase proteins. The characteristic response triggered by pathogen recognition and/or tissue damage is called inflammation, within which innate immunity uses various cytokines and adhesion molecules to communicate with the environment and to regulate the process (Parkin and Cohen 2001).

During their life cycles, pathogens inevitably produce structural motifs, termed pathogen-associated molecular patterns (PAMPs), which are conserved in related microbial species but not present in the host. As the host has straightforward instructions for encountering PAMPs in the germ line (Dempsey *et al.* 2003) and expresses the innate recognition molecules broadly, the innate immune system is induced rapidly presenting the first line of defence (Chaplin 2003). In fact, adaptive immunity is only activated if the

Table 2. Innate mechanisms in which recognition of pathogen structures is not involved.

Mechanism	Example
Mechanical barrier	Tight epithelium and keratin layer of the skin
Mechanical removal	Airway cilia Coughing Mucus on the mucosal membranes Washing effect of tears and urine Peristalsis in the gut
Chemical attack	Acidity of the stomach Fatty acids of the skin Lysozyme in saliva, tears and breast milk (degrades the peptidoglycan of bacterial cell walls) Antimicrobial peptides in the intestine Pancreatic degrading enzymes Detergent effect of bilic acids
Prevention of colonisation	Normal flora in the mouth, colon and urogenital tract Lack of specific receptors for bacterial adhesion Relatively low humidity of the skin
Deprivation of nutrients	Proteins sequestering iron (transferrin in the blood and tissues, lactoferrin in breast milk)

Modified from Tramont and Hoover 1995, Janeway *et al.* 2005a.

innate mechanisms are bypassed, evaded or overwhelmed by pathogens (Janeway *et al.* 2005a).

COMPONENTS

Cells

The main cells of the innate immune system are phagocytes, i.e. neutrophils, monocytes and macrophages. Other innate immune cells comprise basophils, mast cells and eosinophils that contribute to the response by releasing mediators, and natural killer (NK) cells, and dendritic cells that are very potent in presenting antigens to adaptive immune cells (Delves and Roitt 2000).

Monocytes and macrophages

Tissue macrophages are the first effector cells encountered by microbes that have managed to cross epithelial barriers (Janeway *et al.* 2005a, b). Macrophages are abundant in many tissues and their numbers further increase in inflammation, wounding and malignancy (Hume 2006). The source of macrophages are monocytes that continuously leave the circulation e.g. by interacting with the adhesion molecule ICAM-2 (Janeway *et al.* 2005b) and breast and kidney -expressed chemokine (BRAK; Kurth *et al.* 2001) on the resting vascular endothelium.

In general, human monocytes express CD11b, CD11c and CD14, lack B, T, NK and dendritic cell markers, and have a bean-shaped nucleus. The monocyte pool is however heterogeneous, giving rise to different macrophages, such as peritoneal macrophages, Kupffer cells in the liver, alveolar macrophages in the lungs, microglia in the brain and osteoclasts in the bones (Geissmann *et al.* 2003). The use of different adhesion molecules and chemokine receptors by different monocyte subpopulations (Ancuta *et al.* 2003), and monocyte differentiation capacity seem complex and are not entirely understood yet (Hume 2006). For instance, a monocyte subpopulation that is only minor in healthy individuals exhibits strong expression of CD16 (receptor for the Fc portion of IgG), has enhanced capacity to produce pro-

inflammatory cytokines like tumour necrosis factor (TNF) and interleukin 1 (IL-1) and consequently, is recruited to inflammatory sites (Ancuta *et al.* 2003) and expanded in inflammatory conditions. However, the expression of CD16 is increased on monocytes by the anti-inflammatory cytokines transforming growth factor- β (TGF- β) and IL-10 (Kawanaka *et al.* 2002).

The differentiation into macrophages requires macrophage-colony stimulating factor (M-CSF), the receptor of which is a marker of the monocyte-macrophage lineage (Hume 2006). Upon reaching the macrophage stage, a cell achieves a more prominent phagocytic capacity along with receptors that sense activating signals (Duffield 2003). The nature of these signals directs the type of activation. First, IFN- γ and microbial material (e.g. lipopolysaccharide) induce the development of “classically” activated macrophages, which are very effective in the inflammatory response and killing of intracellular pathogens. Second, when exposed to IL-4 or glucocorticoids, “alternatively” activated macrophages arise that produce anti-inflammatory compounds (such as IL-10) and components for extracellular matrix synthesis and tissue repair. Finally, ligation of macrophage Fc γ receptors by IgG immune complexes leads to “type II activation” of macrophages, which promotes adaptive T_H2 responses (Mosser 2003).

Tissue macrophages have an important role not only in the antimicrobial response but also in the clearance of apoptotic senescent cells. Macrophages respond by phagocytosis to various “eat me” signals, including increased oxidation and altered distribution of plasma membrane lipids (e.g. phosphatidylserine exposed on the outer leaflet) (Savill *et al.* 2002).

Dendritic cells (DCs) form a critical link between innate and adaptive immunity. They reside in tissues and continuously sample their environment by macropinocytosis. Pathogen-derived peptides and mediators induce DC maturation, whereby the stability of MHC II - peptide -complex is increased, for example, and DCs migrate to local lymph nodes to present antigenic peptides to naïve T cells (Dempsey *et al.* 2003). The markers of DCs and macrophages (such as the M-CSF receptor) are almost convergent, raising a

question if DCs represent a separate lineage or belong to the continuum of the mononuclear phagocyte system (Hume 2006).

Neutrophils

Neutrophils, i.e. polymorphonuclear leukocytes (PMNs), are not present in healthy tissues, but they are the first cells to accumulate in inflammatory foci. This rapid accumulation is partly due to the abundance of neutrophils in the circulation (representing approximately 70% of peripheral blood leukocytes in humans; Dempsey *et al.* 2003), and their stiffness that retains them in narrow capillaries readily adjacent to any inflammatory signals appearing (Witko-Sarsat *et al.* 2000). Also, macrophages of the damaged tissues produce G-CSF and GM-CSF that promote the supply of myeloid precursors from the bone marrow (Parkin and Cohen 2001). Furthermore, IL-8 and the bacterial formylated Met-Leu-Phe peptide are chemotactic especially for neutrophils (Janeway *et al.* 2005b).

The morphology of neutrophils is characterised by cytoplasmic granules containing toxic components that are mobilised intra- and extracellularly upon phagocytosis. This degranulation makes neutrophils the most rapid and prominent cells to phagocytose, and only a few microbes are able to evade killing inside them (Allen 2003). The potential of the granule contents to damage healthy host tissues is limited as neutrophils have the shortest life span among leukocytes (Dempsey *et al.* 2003, Kobayashi *et al.* 2003). As a whole, neutrophils are crucial in the first-line defence against bacteria, fungi and protozoa (Faurischou and Borregaard 2003).

Pattern recognition receptors (PRRs)

The innate immune system recognises pathogens by PRRs that are expressed on the cell surface or in intracellular compartments or secreted into the blood and tissue fluids (Table 3). The principal PRR functions include opsonisation, activation of complement, blood clotting cascades, phagocytosis and pro-inflammatory signalling pathways, and induction of apoptosis (Janeway and Medzhitov 2002). The targets of PRRs are primarily molecules

that are essential for the survival or pathogenicity of the microbes and as such, not subject to antigenic variability. The innate strategy also includes that PRRs are typically expressed by cells that are the first to encounter pathogens invading the body (e.g. surface epithelia and macrophages) (Medzhitov and Janeway 1997).

Table 3. Examples of pattern recognition receptors (PRRs).

	Non-self ligand	Self ligand	Ref.
Receptor on macrophage surface			
CD14	LPS, PGN	LBP, PGRP, apoptotic cells, LDL, HSP, fibronectin	A
TLR (selected)	LPS, PGN, LTA, unmethylated CpG, flagellin, lipoprotein, dsRNA, lipoarabinomannan	HSP-60, PGRP, fibrinogen	A
Mannose receptor	Terminal mannose, fucose, or GlcNAc	Mannose on L-selectin and lysosomal hydrolases, sulphated saccharides on lutropin and TSH	A
Scavenger receptors A I/II	Polyanionic ligands: dsRNA, lipid A of LPS, LTA	Polyanionic ligands: modified LDL, apoptotic thymocytes	A, B
MARCO	Bacterial cell walls, LPS, environmental particles	Modified LDL	A
CD11b/CD18 (CR3)	β glucan in zymosan	Promiscuous, e.g. ICAM-1 and -2, clotting factors, senescent platelets	A
β glucan receptor	β glucan in zymosan and other yeast-derived particles	Some T cells (independent of sugar groups)	A
CD1	Mycobacterial glycolipids	α Gal ceramide	A
Acute phase proteins secreted from the liver			
CRP and SAP	Phosphorylcholine on bacteria	C1q	B
MBL	Terminal mannose	C2 and C4 via MASPs	B
Cytoplasmic receptors			
NOD1 and NOD2	Peptides derived from PGN	Unknown	C

Ref., reference; LPS, lipopolysaccharide; LBP, LPS binding protein; PGN, peptidoglycan; PGRP, PGN recognition protein; LDL, low density lipoprotein; HSP, heat shock protein; LTA, lipoteichoic acid; GlcNAc, N-acetylglucosamin; TSH, thyroid stimulatory hormone; MARCO, macrophage receptor with collagenous structure; CR, complement receptor; ICAM, intercellular adhesion molecule; Gal, galactose; CRP, C reactive protein; SAP, serum amyloid protein; MBL, mannan binding lectin; MASP, MBL-associated serine protease; NOD, nucleotide binding oligomerisation domain. References: A, Gordon 2002; B, Janeway and Medzhitov 2002; C, Strober *et al.* 2006.

Pattern recognition is diverse: a single PRR usually recognises various target structures (Medzhitov and Janeway 1997), combinations of different PRRs engage complex ligands, and different cell types of innate immunity respond by distinct gene expression profiles. As the ligands also include self molecules on stressed, transformed, senescent or infected cells, or even normal self molecules, pattern recognition could be seen as a part of a wider homeostatic clearance system (Gordon 2002).

LPS receptor complex (CD14/TLR4/MD-2)

LPS is a complex glycolipid constituting the outer layer of the outer membrane of Gram-negative bacteria. Its structure was elucidated in the early 1970s (Luderitz *et al.* 1973). The three covalently linked parts of LPS are 1) the innermost region, lipid A, 2) the core oligosaccharide, and 3) the outermost region, O side chain consisting of branched tetrasaccharides (Miyake 2004).

The immune system is stimulated by LPS that is released from dying bacteria or processed by the host. LPS-binding protein (LBP) is a lipid transferase occurring in the serum. It binds to lipid A and catalyses the transfer of LPS from the bacterial membrane to the LPS receptor complex, which is mainly expressed on monocytes and macrophages (Miyake 2004). The three essential components of the LPS receptor complex (Figure 1) as known so far are CD14 (Wright *et al.* 1990), Toll-like receptor 4 (TLR4; Poltorak *et al.* 1998) and MD-2 (Shimazu *et al.* 1999).

CD14 is a glycoprotein that is either anchored to the plasma membrane via a glycosylphosphatidylinositol tail (mCD14) or circulates as a soluble molecule (sCD14) (Miyake 2004). The role of mCD14 is to bind LPS and present it to TLR4/MD-2 (Pålsson-McDermott and O'Neill 2004). sCD14 facilitates LPS clearance by delivering it to high-density lipoprotein (HDL) particles, within which LPS is neutralised and excreted from the liver (Miyake 2004).

Of the known components of the LPS receptor complex, only TLR4 has a membrane-spanning region and hence, signal transduction capacities (Pålsson-McDermott and O'Neill 2004). TLR4 is the human homologue of *Drosophila* Toll, which in turn was the first member of the Toll family of cell surface receptors discovered. *Drosophila* Toll is required in the embryonic formation of the body of the fly but also, depending on other proteins expressed, in an adult fly in antimicrobial responses (Janeway and Medzhitov 2002). Human Toll-like receptors are directly linked to pro-inflammatory signalling (Miyake 2004) and may also be activated by self molecules that indicate tissue injury or stress (e.g. heat shock proteins and hyaluronate

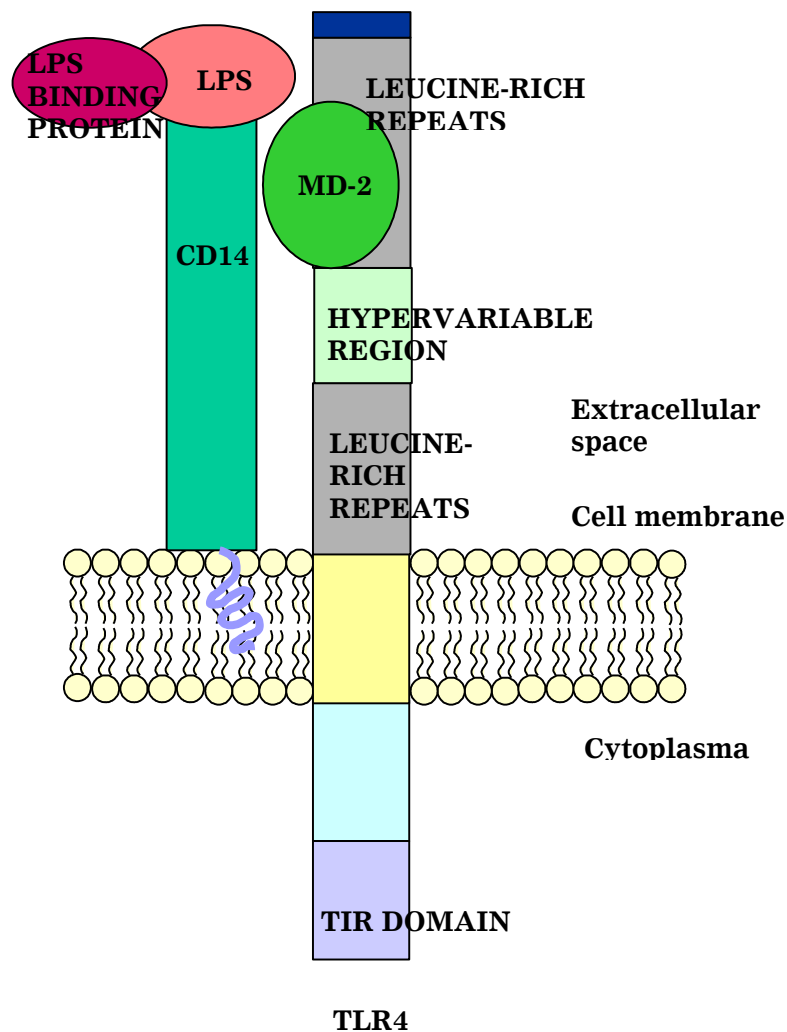


Figure 1. The LPS receptor complex. LPS, lipopolysaccharide; TIR, Toll/interleukin-1 receptor; TLR4, Toll-like receptor 4. Modified from Miller *et al.* 2005, Pålsson-McDermott and O'Neill 2004.

fragments). However, as bacterial products, foremost LPS, activate TLRs even in trace amounts, bacterial contamination as the reason for “endogenous” TLR activation results cannot be excluded (Tsan and Gao 2004).

MD-2 is a secreted adapter protein. It mediates TLR4 aggregation, which is essential for the initiation of LPS signalling (Pålsson-McDermott and O’Neill 2004). Two independent signalling pathways are triggered at TLR4: an earlier one involving myeloid differentiation factor 88 (MyD88) which acts downstream of all TLRs and the IL-1 receptor, and a delayed MyD88-independent one (Figure 2). The former pathway leads to the activation of MAP kinases and the transcription factor NF-κB, and the latter to the

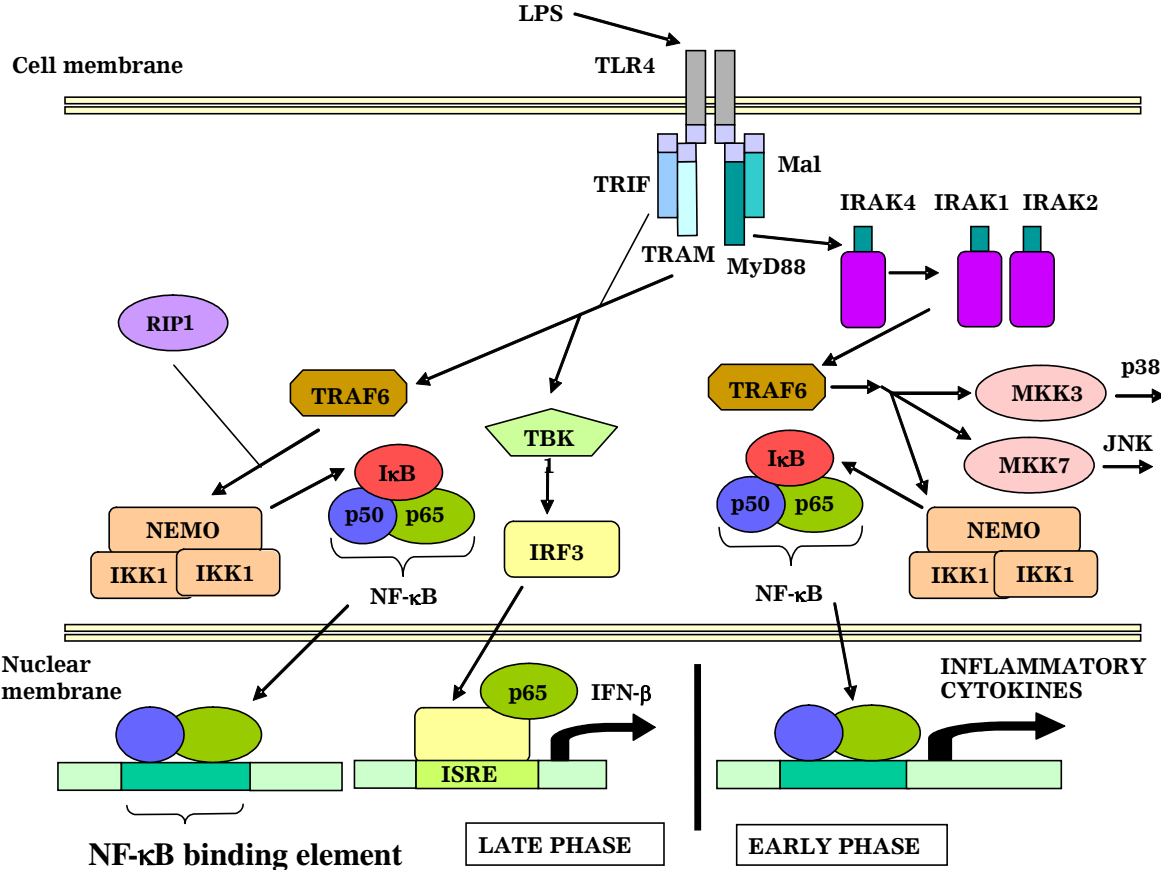


Figure 2. Simplified model of signalling from TLR-4. Negative regulators are not shown. IFN, interferon; IKK, IκB kinase; IRAK, interleukin-1 receptor -associated kinase; IRF, interferon response factor; ISRE, interferon-sensitive response element; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; Mal, MyD88 adapter-like; MKK, mitogen-activated protein kinase kinase; MyD88, myeloid differentiation marker; NF-κB, nuclear factor-κB; RIP, receptor-interacting protein; TBK, TANK-binding kinase; TLR, Toll-like receptor; TRAF6, tumour necrosis factor receptor-associated protein; TRIF, TIR-containing adapter molecule; TRAM, TRIF-related adapter molecule. Modified from Pålsson-McDermott and O’Neill 2004.

activation of interferon regulatory factor 3 (IRF3) and NF- κ B (Miyake 2004, Pålsson-McDermott and O'Neill 2004).

Intestinal epithelial cells also recognise LPS (from pathogens in food). In these cells, LPS is first internalised and then transported to the Golgi apparatus where TLR4 is located (Miyake 2004).

Inducible components

Unlike the resident cells and receptors, other innate elements are only synthesised, released or made functional when triggering signals appear. These elements usually act as mediators in the inflammatory response, and are rapidly degraded or otherwise inactivated to target the powerful response appropriately and to minimise the damage they might cause to the host (Parkin and Cohen 2001, Mitchell and Cotran 2003).

Cytokines and chemokines

Cytokines are the most prominent inducible elements of the immune system. They are primarily leukocyte-derived small molecular weight (8-80 kD) messengers that bind to specific receptors on target cells and alter cell behaviour, typically activation, division, apoptosis and movement. They act either in an autocrine (on the producer cells), paracrine (on cells near-by) or endocrine fashion (via bloodstream). An immune insult induces the production of cytokines, which in turn directs the extent and type of the developing response. Cytokines can be divided into subgroups according to their major targets or functions, e.g. interleukins (ILs) mainly influence leukocytes and interferons (IFNs) interfere with viral replication (Parkin and Cohen 2001).

Chemokines are small (8-12 kD) cytokines having a characteristic cysteine sequence, and were originally described as chemoattractants recruiting immune cells to the inflammatory site. More recently, some of them have also been shown to perform homeostatic or housekeeping functions (Borish and Steinke 2003) like BRAK mentioned above has (Kurth *et al.* 2001).

In most cases, contribution of several cytokines is required for optimal function. A given cytokine may even have opposite effects depending on the type of the target cells and the phase of the response. Most cytokines predominantly exhibit either pro- or anti-inflammatory effects (Borish and Steinke 2003).

INFLAMMATORY RESPONSE

Inflammation is the main host response to tissue injury, aimed at eliminating the triggering factor, e.g. an invading microbe, allergen, poison or mechanical trauma. The acute response to each of these different triggers is very similar, reflecting the innate non-specific nature of the early stages of inflammation and the same repertoire of the mediators used (Mitchell and Cotran 2003). Infection, i.e. inflammation caused by a microbe, can only occur if the epithelial barriers protecting the skin, respiratory, gastrointestinal and urogenital tract are colonised by the pathogen, or crossed by it via a wound, abrasion, sting, burn etc (Janeway *et al.* 2005a).

Mediators

More than a hundred compounds are known that target, regulate, modulate or complement each other in the inflammatory response (Table 4). Some of them have a broad spectrum of effects, others have only one point of action.

The effects of the mediators are observed as the four classical clinical symptoms at the inflamed site: they cause heat and redness because they induce vasodilation leading to increased blood flow, they cause swelling because they promote the infiltration of blood leukocytes (by activating the vascular endothelium), fluid and proteins (by increasing vascular permeability), and they cause pain because some mediators stimulate pain nerve endings. The fifth symptom, dysfunction, depends on the tissue/organ and the overall process (Mitchell and Cotran 2003).

Table 4. Important inflammatory mediators classified by their major effects.

Vasodilation
Prostaglandins
Nitric oxide (NO)
Increased vascular permeability
Vasoactive amines (histamine, serotonin)
Complement components C5a and C3a
Cysteinyl leukotrienes (LTC ₄ , LTD ₄ , LTE ₄)
Tumour necrosis factor (TNF), interleukin 1 (IL-1)
Bradykinin
Platelet activating factor (PAF)
Chemotaxis
Complement component C5a
Leukotriene B ₄ (LTB ₄)
Bacterial products
Chemokines (e.g. IL-8)
Fever
IL-1, IL-6, TNF
Prostaglandins
Pain
Prostaglandins
Bradykinin
Tissue damage
Degradative enzymes and oxygen metabolites of neutrophils and macrophages
NO
Regulation of leukocyte functions
Cytokines, e.g. TNF, IL-1, IL-6, IL-12
Interferons
LTB ₄
PAF
Complement component C5a

Modified from Mitchell and Cotran 2003.

Upon microbial invasion, three plasma protein systems are quickly activated. The complement system recognises antigen-antibody complexes, cell wall polysaccharides of Gram-negative bacteria and yeasts, or non-self mannose patterns in proteins and carbohydrates, causing osmotic lysis of the microbe and generating additional mediators (Delves and Roitt 2000, Parkin and Cohen 2001). The kinin system (most importantly bradykinin) is activated by negatively charged surfaces such as on collagen, the basal membrane, or LPS (Mitchell and Cotran 2003). The local blood clot created by the activated coagulation system prevents the spreading of the microbe (Janeway *et al.* 2005a).

Tissue macrophages respond rapidly to invading pathogens by producing large amounts of mediators. These guide the entry of circulating phagocytes, and activate them and tissue cells (Dempsey *et al.* 2003). Cell-derived early mediators include lipid derivatives, chemokines and cytokines. Arachidonic acid from membrane phospholipids is used in the synthesis of eicosanoids, i.e. prostaglandins and leukotrienes, and phosphatidylcholine is used in the synthesis of platelet-activating factor (PAF) (Mitchell and Cotran 2003). Important cytokines secreted by macrophages include TNF (tumour necrosis factor), IL-1, IL-6, IL-8 and IL-12 (Janeway *et al.* 2005b). TNF and IL-1 have many effects in common and bring about the majority of the inflammatory features. Typically, the pro-inflammatory cytokines regulate target cell functions by inducing the activation of certain transcription factors, which include NF- κ B, AP-1 and the STAT family (Mitchell and Cotran 2003).

TNF

In the mid-1970s, a protein with a selective antitumour effect was described and named tumour necrosis factor (Carswell *et al.* 1975) (although, in some cell types, TNF-induced killing is apoptotic rather than necrotic). TNF also revealed to contribute to severe wasting, cachexia, which often accompanies chronic infections and cancer. In particular, TNF is the main and early-induced mediator of the response against bacterial LPS (Fiers 1991). It can also be induced by other toxins, immune complexes, mechanical tissue damage and numerous other inflammatory signals (Mitchell and Cotran 2003). It is produced by monocytes and macrophages but also, after appropriate induction, by neutrophils, lymphocytes, NK cells, endothelial and mast cells (Borish and Steinke 2003).

TNF in its native conformation is a trimer with a molecular mass of 52 kDa. Of the two TNF receptors, TNF-RI is ubiquitous, while TNF-RII is more restricted to cells of hematopoietic origin and strongly expressed on T cells upon activation. The clefts between the three subunits of TNF can interact with the receptors cross-linking them, which initiates intracellular signal

transduction events (Fiers 1991). TNF enhances local inflammatory response by inducing the expression of adhesion molecules, cytokines, growth factors, eicosanoids and nitric oxide in endothelial cells, the metabolism of fibroblasts and functions of leukocytes, e.g. neutrophils (Mitchell and Cotran 2003).

TNF is also able to act from a distance to coordinate the host response (Figure 3). For instance, its effects on the hypothalamus (mediated by prostaglandin E₂) and fat and muscle cells elevate body temperature. Fever is generally beneficial to the host because it slows down the growth of most

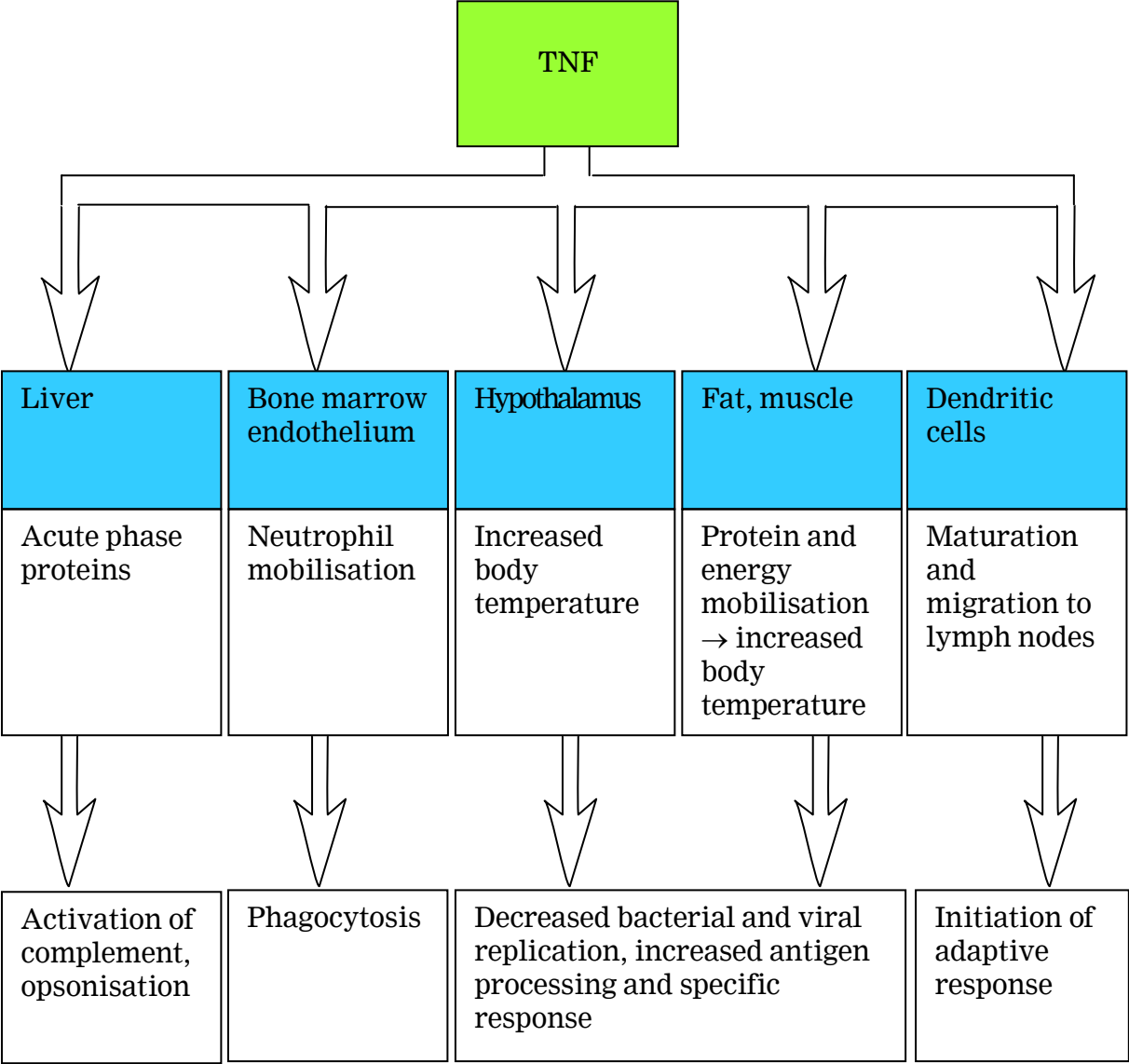


Figure 3. The ways by which the cytokine TNF coordinates the body’s response to infection. IL-1 and IL-6 share these effects, except for the effect on dendritic cells. Adapted from Janeway *et al.* 2005b.

pathogens and intensifies adaptive responses. On the other hand, TNF-promoted catabolism, fatigue and loss of appetite enhance the wasting effect. Furthermore, once a pathogen has managed to spread to the circulation, TNF production occurs systemically causing an explosive, deleterious response (Janeway *et al.* 2005b).

Acute phase response

The so-called acute phase response is launched within 48 hours of pathogen exposure. Acute phase proteins are defined as hepatocyte-derived plasma proteins whose concentration is increased or decreased by at least 25% by the effect of the cytokines TNF, IL-1 and especially IL-6 (Gabay and Kushner 1999). CRP was the first acute phase protein discovered (Tillett and Francis 1930) and currently, elevated CRP level is the most widely used indicator of an ongoing inflammatory reaction. CRP recognises phosphocholine of bacterial and fungal cell wall lipopolysaccharides and when bound to these it is able to opsonise and activate the complement cascade. MBL is also increased in inflammation, and it activates complement by binding to microbial mannose residues and acts as an opsonin for monocytes (Janeway *et al.* 2005b).

Recruitment of phagocytes

Under normal conditions, circulating leukocytes travel in the fastest blood flow in the center of the vessel lumen. In the early stages of inflammation, physical changes and the appearance of adhesion molecules and chemokines on the vascular endothelium take place, guiding the phagocytes to attach to the vessel walls and be recruited to the inflamed sites. This is one of the most important functions of innate immunity (Janeway *et al.* 2005b).

The migration of phagocytes (and other leukocytes) out of blood vessels, i.e. extravasation, occurs in four steps (Figure 4). The first step, tethering, is mainly guided by glycoconjugate-binding molecules, selectins. Most circulating leukocytes express L-selectin and glycoproteins such as P-

selectin glycoprotein ligand-1 (PSGL-1) on the tips of their microvilli. L-selectin recognises sulphated sialyl-Lewis^x (sLe^x) -like sugars in glycoproteins or -lipids on the inflamed endothelium and in PSGL-1 on adherent leukocytes (Luster *et al.* 2005).

On the endothelial side, P-selectin is unloaded from cytoplasmic granules to the cell surface within minutes of exposure to TNF, LTB₄, C5a or histamine. TNF and LPS also trigger the transcription of the E-selectin gene. The interactions between selectins and their ligands are not strong enough to anchor phagocytes but instead, make phagocytes “roll” on the endothelium,

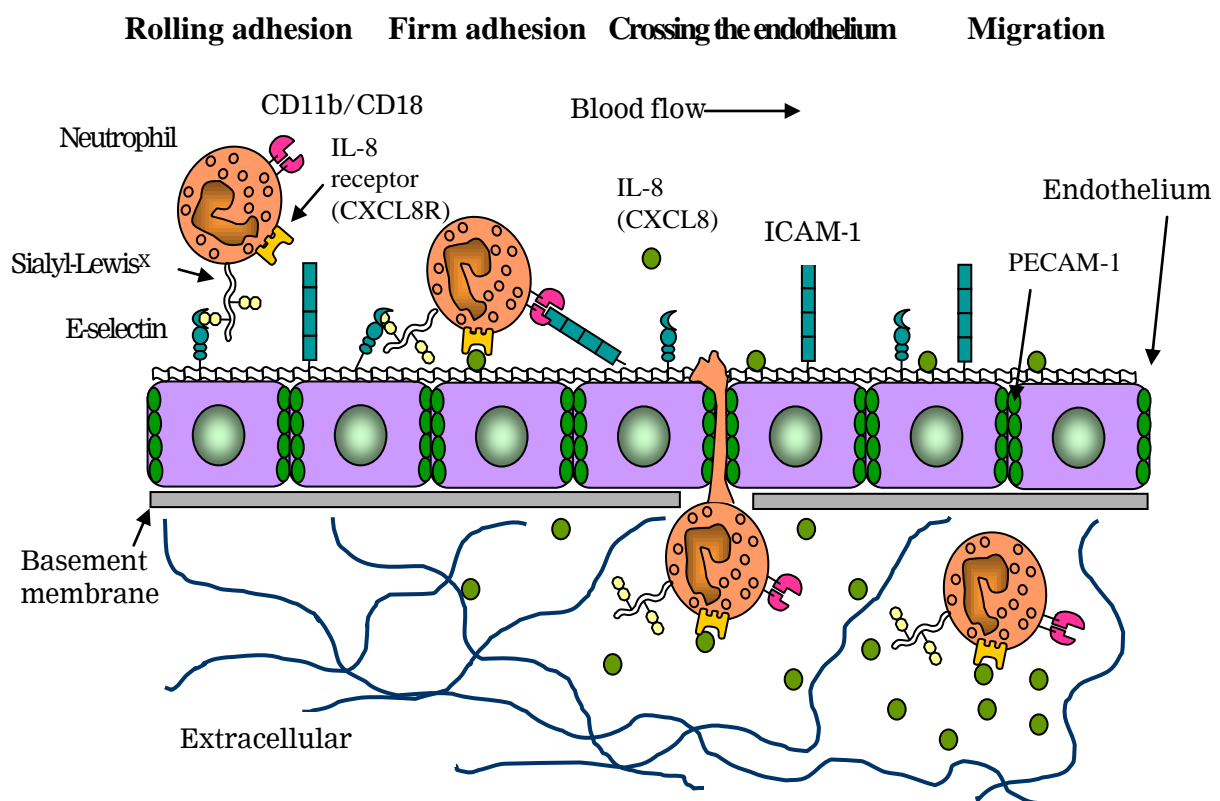


Figure 4. Extravasation of a neutrophil across the vascular endothelium in four steps. 1) Rolling adhesion. Reversible binding of carbohydrate ligands on the leukocyte and selectins on the endothelium makes neutrophils roll on the endothelium. 2) Firm adhesion. Chemokines (such as IL-8) and rolling enable the neutrophil to form stronger interactions between integrins (CD11b/CD118, CD11a/CD18) and immunoglobulin-like ligands (ICAM-1) on the endothelium, and to arrest rolling. 3) Crossing the endothelium. Homophilic PECAM-1 interactions and integrins are needed for crossing the endothelial barrier. Penetrating the basement membrane (diapedesis) requires degradative enzymes on the leukocyte surface. 4) Migration. The concentration gradient of chemokines guides the migration of neutrophils towards the pathogen in the tissue. Leukocyte enzymes degrade and modify extracellular matrix for the migrating neutrophil to move onwards. Modified from Janeway *et al.* 2005b.

continually making and breaking contacts. Inflammatory chemokines (such as IL-8) are trapped by endothelial proteoglycans to become bound by chemokine receptors on rolling phagocytes. Receptor-mediated intracellular signals are rapidly transmitted to cell surface integrins, resulting in a conformational change that enhances the ability of integrins to bind to their ligands (members of the immunoglobulin superfamily) on the endothelium. The involvement of integrins arrests rolling and launches the second step of extravasation, firm adhesion. The most relevant phagocyte integrins in the inflammatory migration are β_2 integrins CD11b/CD18 (Mac-1, CR3) and CD11a/CD18 (LFA-1). They bind to ICAM-2 and ICAM-1, which is induced on the endothelium by TNF (Janeway *et al.* 2005b).

In the third step phagocytes cross the endothelium paracellularly, i.e. by squeezing between endothelial cells, which is facilitated by the increased expression of ICAM-1 and VCAM-1 on the endothelium and the regulated phosphorylation of various proteins at the intercellular junctions (Luster *et al.* 2005). Also, molecules on the phagocyte surface interact with junctional molecules thus replacing the homophilic molecular interactions between adjacent endothelial cells. For instance, PECAM-1 (platelet/endothelial cell adhesion molecule 1) and CD11b/CD18 on a phagocyte interact with PECAM-1 and JAM-C (junctional adhesion molecule C) on the endothelial junctions, respectively. Leukocyte integrins and the modification of the cytoskeleton drive the formation of lamellipodia, which the leukocytes use for crawling onwards. Following the passage of leukocytes, endothelial junctions are resealed (Imhof and Aurrand-Lions 2004).

In the fourth step leukocytes migrate in the tissue towards the inflammatory focus. There the concentration gradient of extracellular matrix (ECM)-bound chemokines. The early-released IL-8 attracts mostly neutrophils, which usually peak within the first six hours of the response. Monocytes are predominantly recruited later e.g. through the release of MCP-1 (Janeway *et al.* 2005b). The use of PECAM-1 on leukocytes increases the avidity of specific β_1 and β_2 integrins for ECM constituents. Also, in order to remodel and

penetrate ECM barriers, particular proteases and glycosaminoglycan-degrading enzymes are upregulated in leukocytes. The localised proteolysis can also expose cryptic ECM ligands (Luster *et al.* 2005). Receptor recycling and clustering of anti-adhesive cell surface molecules (e.g. leukosialin) favour the detachment from adhesion substrates, which is also needed for moving (Witko-Sarsat *et al.* 2000).

CD11b/CD18

Integrins are adhesion molecules required for interactions of leukocytes with endothelial cells, other cell types and ECM. Upon binding their ligands, integrins cluster and become activated, and signals generated by certain other cell surface receptors are also mediated to integrins to increase their ligand avidity. The intracellular targets of integrins include tyrosine kinases, MAP kinases, cytoskeletal components and adaptor molecules. The signalling events are coordinated to bring about the appropriate responses, foremost cell motility, growth, differentiation, survival and the specialised functions of phagocytes (Williams and Solomkin 1999).

Each leukocyte subtype expresses one or more members of the β_2 integrin family. β_2 integrins are heterodimeric glycoproteins consisting of either an α_L (CD11a), α_M (CD11b), α_X (CD11c) or α_D (CD11d) chain, and a common β_2 chain (CD18). Within minutes after stimulation, CD11b/CD18 ($\alpha_M\beta_2$ or Mac-1) is increased on the plasma membrane of circulating phagocytes from cytoplasmic storages, and CD11b/CD18 can be used as an early and sensitive phagocyte activation marker (Repo and Harlan 1999). It is an extremely promiscuous receptor, being able to interact with over 30 protein and non-protein ligands (Table 5). Accordingly, it is utilised in all steps of phagocyte extravasation after tethering (Yakubenko *et al.* 2002).

The major ligand-binding region of CD11b/CD18 is the so-called I (inserted) domain in the α_M chain, in which the consensus sequence forms a loop and a contiguous α helix (Figure 5). However, the molecular basis of the degenerate binding is poorly understood (Yakubenko *et al.* 2002). Some ligands of CD11b/CD18 can be competitive, and their spatial and temporal distribu-

Table 5. Examples of extracellular CD11b/CD18 ligands.

Endothelial molecules	Phagocyte enzymes
ICAMs 1-3	Elastase
E-selectin	Myeloperoxidase
Junctional adhesion molecule C (JAM-C)	Catalase
Plasma proteins	Extracellular matrix proteins
Fibrinogen	Fibronectin
Kininogen	Laminin
Factor X	Collagens
Complement fragment iC3b	Vitronectin
Complement factor H	Matrix metalloproteinase-9 (MMP-9)
Transferrin	Pathogen structures of
Bovine serum albumin	<i>Candida albicans</i>
Ovalbumin	<i>Bordetella pertussis</i>
Leukocyte surface receptors	<i>Leishmania</i>
CD14	Non-proteins
Fc receptors (e.g. CD16)	Heparin
Urokinase-type plasminogen activator receptor	β -glucan
	Plastic

Yakubenko *et al.* 2002, Imhof and Aurrand-Lions 2004 (JAM-C) Fagerholm *et al.* 2006 (MMP-9).

tion may determine which of them are primarily bound (Pluskota *et al.* 2003). A unifying feature of the ligands might be the presence of acidic residues positioned in a certain conformation (Harris *et al.* 2000, Stefanidakis *et al.* 2003). The phosphorylation state of specific cytoplasmic α chain residues is likely to influence the conformation of the extracellular part and the binding preference for the endothelium or iC3b, for example (Fagerholm *et al.* 2006).

Phagocyte functions

Phagocytosis

Upon phagocytosis, a phagocyte engulfs the microbial target into an intracellular vacuole, phagosome, which “matures” to form the “killing compartment”, where oxygen-dependent and -independent mechanisms are activated. In macrophages, the phagosome matures by fusing with endosomes and lysosomes acquiring lysosomal hydrolases and vacuolar ATPase, which creates optimal acidic conditions for the activity of the hydrolases (Lee *et al.* 2003). In neutrophils, cytoplasmic granules are rapidly unloaded to the phagosome and plasma membrane (Allen 2003).

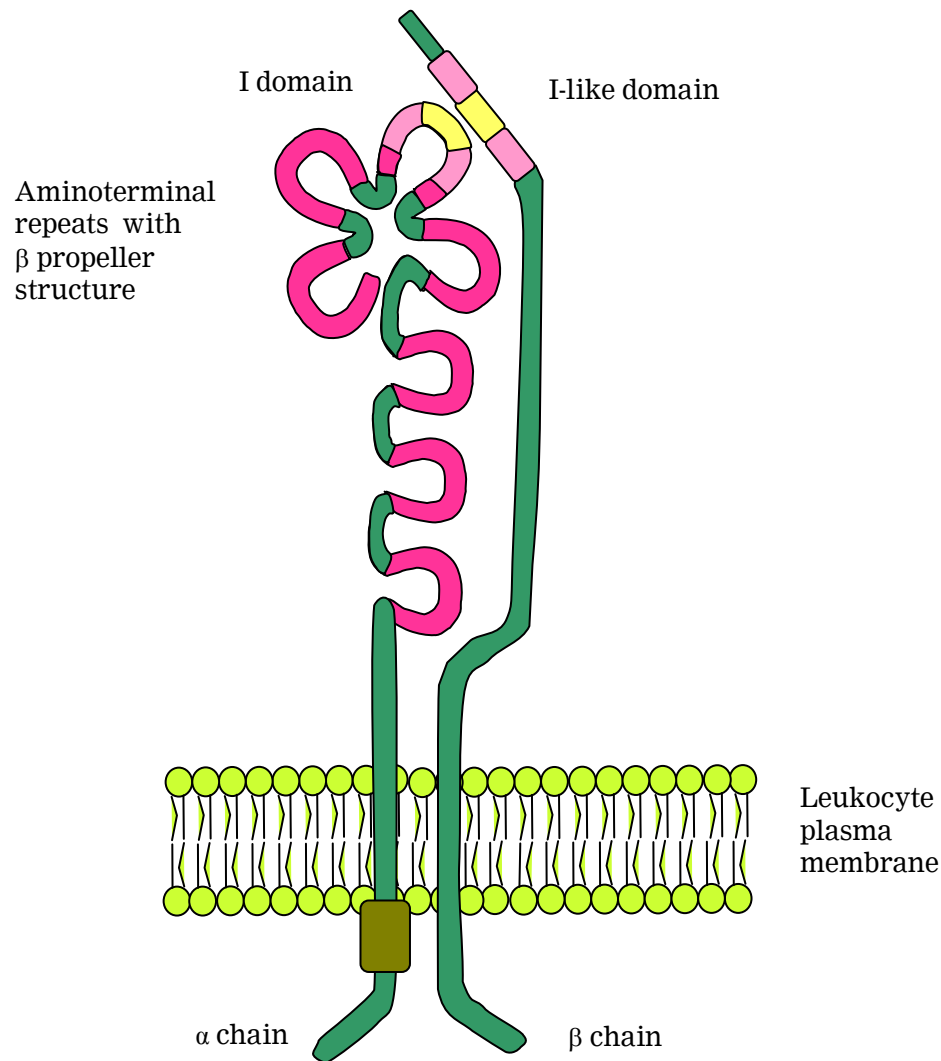


Figure 5. Structure of β_2 integrins. The α chain includes seven extracellular N-terminal homologous repeats with a β propeller structure. The lighter coloured regions in the heterodimer represent the α chain I domain and the β chain I-like domain, each with the embedded metal ion-dependent adhesion site (MIDAS). The heterodimer is illustrated in the “closed”, i.e. low-affinity state. Quaternary changes triggered by activating signals shift the I-like domain, allowing the I domain to accept a more open, high-affinity state via tertiary changes. Adapted from Harris *et al.* 2000.

Phagocytosis is mediated by an array of different PRRs on a phagocyte that concomitantly interact with the target structure. Opsonisation, most importantly mediated by Fc receptors and, in innate immunity, complement receptors, is essential for recognising particles that are not immediate ligands for phagocyte receptors (Stuart and Ezekowitz 2005).

Oxygen-dependent antimicrobial response

The NADPH oxidase complex is assembled and activated on the plasma membrane and within phagosomes as phagocytosis begins (Weiss 1989). It catalyses the production of superoxide ($\cdot\text{O}_2^-$), which particularly attacks bacterial enzymes involved in amino acid biosynthesis, and reacts further to produce stronger oxidants, hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot\text{OH}$). These attack microbial proteins, DNA and lipids (Miller and Britigan 1997).

Myeloperoxidase (MPO) uses H_2O_2 and halides to produce hypohalous acids that peroxidate, oxidise and decarboxylate cell membranes and oxidise the respiratory chain (Miller and Britigan 1997). Hypochlorous acid reacts with amines to generate chloramines, some of which are especially long-lived oxidants (Weiss 1989). The MPO- H_2O_2 system also generates tyrosyl radicals and reactive aldehydes from substrates other than halide ions (Witko-Sarsat *et al.* 2000).

Macrophages are considered to exhibit a less marked oxidative killing capacity than neutrophils primarily because of the significantly lower expression of MPO (Allen 2003). However, macrophages are able to produce nitric oxide ($\text{NO}\cdot$), which interferes with the proteins of the respiratory chain and DNA synthesis machinery. It can also react further with $\cdot\text{O}_2^-$ to generate peroxynitrite ($\text{ONOO}\cdot$) that oxidates DNA bases and peroxidates lipids (Miller and Britigan 1997).

Oxygen-independent antimicrobial response

The oxygen-independent response of phagocytes includes proteases, antimicrobial peptides and membrane-bound receptors for endothelial, extracellular matrix and bacterial ligands (Lee *et al.* 2003). In neutrophils, these are located in four types of cytoplasmic granules: azurophil (primary), specific (secondary) and gelatinase (tertiary) granules and secretory vesicles (Faucschou and Borregaard 2003).

Following even a low-level inflammatory stimulation, secretory vesicles are exocytosed exposing, for example, CD11b/CD18 to its endothelial

ligands. Gelatinase granules have the second highest propensity for extracellular release, and their typical contents, matrix metalloproteases, are needed in extravasation. Specific granules and last, azurophil granules undergo partial exocytosis, unloading matrix-binding and -degrading enzymes and antimicrobial substances such as lysozyme. The contents of azurophil and specific granules are also released and activated in phagosomes containing bacterial structures (Faucus and Borregaard 2003).

The proteins of the host are protected from the action of neutrophil proteases by ubiquitous endogenous inhibitors. Sufficient phagocyte migration is apparently retained as the large inhibitors are excluded from the space between the phagocyte and ECM, and as serine proteases are bound on the phagocyte surface. The term “neutrophil proteases” is often used although low levels of elastase and cathepsin G are also produced in macrophages (Pham 2006).

Resolution of inflammation

In order to reach complete healing, the inflammatory response must be repressed. Data on neutrophils suggest that a coordinated resolution program initiates already during the first hours of the response as the pathways leading to PGE₂ and PGD₂ production also give rise to other classes of lipid mediators. These include lipoxins generated from arachidonic acid, and resolvins and protectins from omega-3 polyunsaturated fatty acids. Lipoxins retard the entry of neutrophils, reduce vascular permeability and stimulate macrophages to ingest apoptotic neutrophils. PGD₂ and its metabolites override neutrophil survival signals, triggering caspase-dependent apoptosis (Serhan and Savill 2005).

Macrophage activation is considered to comprise early pro-inflammatory and later anti-inflammatory phases. Alternatively activated macrophages play a major role in all resolution: down-regulation of inflammation, angiogenesis, and elimination of tissue debris, apoptotic cells and bodies (Porcheray *et al.* 2005). The phagocytic removal of apoptotic cells not encouraging inflammation is essential for the re-establishment of tissue

homeostasis. Phagocytes that have engulfed apoptotic cells begin the production of cytokines such as VEGF, which is critical for the repair of endothelial and epithelial injury, and TGF- β 1, which suppresses TLR signalling (Serhan and Savill 2005). Reparatory macrophages produce intracellular repressors of signal transduction, enzymes modifying chromatin structure, and inhibitors of signalling between cell surface receptors and proteoglycans (Wells *et al.* 2005), and the resolution program ends as the macrophages withdraw and depart through the lymphatics (Serhan and Savill 2005).

VEGF

Vascular endothelial growth factor (VEGF) was first described as vascular permeability factor (VPF), the most potent promoter of vascular permeability and tumour-associated ascites fluid accumulation (Senger *et al.* 1983), and thereafter, a factor essential for growth and differentiation of endothelial cells (Leung *et al.* 1989). VEGF stimulates angiogenesis (sprouting of new vessels from pre-existing vasculature) by promoting the migration of endothelial cells and vascular smooth muscle cells, and vasculogenesis by recruiting endothelial progenitor cells from the bone marrow. Of the several splicing variants of VEGF, a protein of 165 amino acids is the predominant and also the strongest isoform in stimulating angiogenesis (Byrne *et al.* 2005).

The action of VEGF is needed in normal physiology and physiological responses. It is required in establishing the vascular network of an embryo. In addition to its angiogenic role in the healing of injured tissues or wounds, VEGF also promotes the migration of mononuclear phagocytes and neutrophils thus contributing to the acute inflammatory response. VEGF is also expressed in the female reproductive tract, being highest in the early development of the corpus luteum (Byrne *et al.* 2005).

Elevated local and serum VEGF levels are associated with the pathogenic processes in certain diseases, e.g. excessive endothelial proliferation and joint destruction in the joints of patients with rheumatoid arthritis (Paleolog 2002), ocular neovascularisation and hemorrhages in patients with

diabetic retinopathy (Hoeben *et al.* 2004), and abnormal dermal blood vessels and reddening of the skin in patients with psoriasis (Xia *et al.* 2003).

Tumour cells require nutrients and oxygen via blood vessels in order to grow beyond 2-3 mm³ in size. VEGF is also secreted by tumour cells and harnessed by them in building an intratumour vascular supply. VEGF also stimulates the migration and invasion of some tumour cells, which, together with the new vasculature of the tumour, facilitate the forming of distant metastases (Byrne *et al.* 2005).

IFN- α

Interferon- α is a type I interferon and mainly produced by DCs, monocytes, macrophages, T cells, NK cells and fibroblasts. It is essential for innate antiviral defense and efficient in inhibiting neoplastic growth, and found to be a useful clinical immunotherapeutic. Its well-known effects on target cells include enhancement of antigen presentation by upregulating the processing of viral antigens and expression of MHC class I molecules, stimulation of NK cell-mediated cytotoxic activity, and induction of apoptosis by activating caspases and (in the presence of dsRNA) inhibiting translation. Furthermore, IFN- α -induced IFN- γ expression in CD4⁺ and CD8⁺ T cells and NK cells may be a nexus for the innate-to-adaptive transition. Identifying the specific or direct effects of IFN- α is complicated because it regulates several other cytokines and their receptors (Brassard *et al.* 2002), and because *IFN- α* can be induced by signalling routes that also lead to other immune genes, e.g. in the late phase of LPS-induced signalling, including IRF-3 and *IFN- β* induction (Hata *et al.* 2002) (Figure 2).

In acute inflammation, high levels of IFN- α may promote the responses that protect the host (e.g. activation of regulatory T cells, reduction of the TNF response to LPS, and increase of redox reactions that prevent oxidative stress). Interestingly, when the inflammatory stimuli are becoming overcome, the lowering of IFN- α levels may provide a signal for major shift to non-inflammatory state. Low IFN- α levels decrease the cell surface expression of CD14 and other PRRs, for example (Amadori 2007). IFN- α also inhibits

angiogenesis (Brassard *et al.* 2002) and thus, the decline in IFN- α levels in the healing process may contribute to the induction of angiogenesis. On the other hand, higher IFN- α levels could counteract angiogenic pathophysiology.

Systemic inflammation

Systemic inflammation is a consequence of the activation of innate immunity throughout the circulation, i.e. systemically. If the local response fails to limit the pathogen, it can spread into the bloodstream and the condition is called sepsis. Hereby, TNF is released by macrophages in the liver, spleen and other organs and tissues. This leads to systemic increase in vascular permeability with subsequent tissue edema, loss of plasma volume and eventually, shock. As disseminated blood clotting is also triggered by TNF, the clotting factors are consumed massively, and the ability to clot appropriately when needed is lost. This often leads to bleeding and failure of vital organs such as the kidneys, liver, heart and lungs (Janeway *et al.* 2005b). The mortality in septic shock is high, 50-90%. In such a critical state as sepsis, even the anti-inflammatory mechanisms aimed at resolution can be fatal, because they may lead to immune suppression, which increases the risk of secondary infections and organ failure (Takala *et al.* 2002).

GENETIC VARIATION

Single nucleotide polymorphisms (SNPs)

SNPs are defined as base pair positions in DNA at which the rarest alternatives (alleles) exist at least in 1% of normal individuals. Thus they are the most common type of genetic variation in humans. An SNP occurs in approximately 1 of 1000 base pairs, depending on the genome region. Mechanisms that modify demographic history, such as selective pressure from infectious microbes (Aguillón *et al.* 2006), bottlenecks, admixture, inbreeding, migration, immigration and assortative mating have produced distinctive inheritable SNP patterns in different populations (Brookes 1999).

SNPs are said to be functional if they affect gene products and biological processes. First, when an SNP in the coding region causes an amino

acid change, the structure and function/activity of the corresponding protein can be altered. Second, promoter SNPs that modulate the binding affinity of transcription factors potentially influence gene expression levels. Third, variation in the 5' untranslated region (UTR) may disrupt mRNA translation, and variation in the 3' UTR may affect mRNA stability and export. Fourth, changes in the reading frame or preterm transcription termination signals usually lead to a defective or truncated protein. Finally, splice site changes can interfere with mRNA processing or protein function (Arcaroli *et al.* 2005).

SNPs in innate immune genes may influence the risk, activity, and adaptive downstream responses of diseases with an inflammatory component (Lazarus *et al.* 2002). Usually, the combination of SNPs and environmental factors has the potential to determine disease appearance (Brookes 1999).

TLR4 +896A→G

The human TLR4 gene contains 44 SNPs (Lazarus *et al.* 2002). Of these, the +896A→G substitution leads to the Asp299Gly change in the extracellular domain of the TLR4 protein. This may reduce LPS-mediated cellular responses, possibly by impairing ligand binding or by disturbing the transport of TLR4 to the cell surface (Arbour *et al.* 2000, Kiechl *et al.* 2002). *TLR4 +896G* is associated with inflammatory conditions (Table 6), e.g. increased risk of Gram-negative infections (Agnese *et al.* 2002) and septic shock (Lorenz *et al.* 2002) and in some cases, recognition of ligands other than LPS (e.g. Chlamydial or endogenous heat-shock proteins, oxidised lipids) (Edfeldt *et al.* 2004, Schröder and Schumann 2005).

CD14 -159C→T

37 SNPs have been identified within the human CD14 gene (Lazarus *et al.* 2002). The -159C→T exchange in its promoter region may be associated with increased transcriptional activity, probably by weakening the interaction between the so-called GC box and the inhibitory transcription factor Sp3 (Le Van *et al.* 2001). -159T was first reported to be a risk factor for myocardial infarction (Unkelbach *et al.* 1999). Thereafter, it has been

Table 6. Studies investigating the potential association of inflammatory conditions with the *TLR4* +896G allele.

Condition	Association	Population studied	Reference
Gram-negative infection	Risk ↑	Combined American	Agnese <i>et al.</i> 2002
Septic shock	Risk ↑	White French	Lorenz <i>et al.</i> 2002
Meningococcal disease	NS	Gambian	Allen <i>et al.</i> 2003
RSV infection	Risk ↑	Israeli Jewish	Tal <i>et al.</i> 2004
Crohn's disease (CD) and ulcerative colitis (UC)	Risk ↑	Belgian, Ashkenazi Jewish, Moroccan	Franchimont <i>et al.</i> 2004
	CD: Risk ↑, UC: NS	Dutch	Braat <i>et al.</i> 2005
Asthma	Risk ↑	Swedish	Fagerås-Böttcher <i>et al.</i> 2004
Atherosclerosis	Progression ↓	Italian	Kiechl <i>et al.</i> 2002
Cardiovascular events	Risk ↑	Dutch	Boekholdt <i>et al.</i> 2004
Myocardial infarction	NS	White American men	Zee <i>et al.</i> 2005
	Risk ↑ in men	Swedish	Edfeldt <i>et al.</i> 2005
Spondyloarthropathies	NS	Hungarian	Gergely <i>et al.</i> 2006
Rheumatoid arthritis	NS	Spanish	Sánchez <i>et al.</i> 2003
SLE	NS	Spanish	Sánchez <i>et al.</i> 2003
Diabetic neuropathy	Risk ↓	White German	Rudofsky <i>et al.</i> 2004

NS, not significant; RSV, respiratory syncytial virus; SLE, systemic lupus erythematosus.

Table 7. Studies investigating the potential association of inflammatory conditions with the *CD14* -159T allele.

Condition	Association	Population studied	Reference
Myocardial infarction	Risk ↑ in subjects w/o other risk factors	White German men	Unkelbach <i>et al.</i> 1999
Acute coronary syndromes	Risk ↑ (TT homozygotes)	White British men	Arroyo-Espliguero <i>et al.</i> 2005
Atopic asthma	Risk ↓	Indian	Sharma <i>et al.</i> 2004
Atopy	Children: Risk ↓, adults: NS	Australian	O'Donnell <i>et al.</i> 2004
	NS	Swedish	Fagerås-Böttcher <i>et al.</i> 2004
Gram-negative infections	Risk ↑	White Canadian	Sutherland <i>et al.</i> 2005
Septic shock	Risk ↑, mortality ↑	White French	Gibot <i>et al.</i> 2002
Crohn's disease	Risk ↑	German	Klein <i>et al.</i> 2002
	NS	Hungarian	Klausz <i>et al.</i> 2005
Ulcerative colitis	Risk ↑	Japanese	Obana <i>et al.</i> 2002
Rheumatoid arthritis	NS	White German	de la Fontaine <i>et al.</i> 2005
Parkinson's disease	Risk ↑ (TT homozygous women)	Taiwanese	Lin <i>et al.</i> 2006
Alcoholic liver disease	Risk ↑	Finnish	Järveläinen <i>et al.</i> 2001

w/o, without; NS, not significant.

associated with several inflammatory diseases (Table 7), e.g. Gram-negative infections (Sutherland *et al.* 2005). *CD14* can also be linked to adaptive TH2 responses in allergy. Low LPS exposure in early childhood may predispose to

high IgE production with concomitant susceptibility to allergic disorders (Sharma *et al.* 2004). -159TT homozygotes seem to have increased sCD14 levels, paralleled by decreased IgE levels (Baldini *et al.* 1999).

TNF promoter polymorphisms

At least 7 distinct SNPs are located within the proximal promoter of the TNF gene (Flori *et al.* 2005). Positions -308, -238 and -376, for example, may be functional. -308G/A was the first *TNF* SNP discovered, and several studies have confirmed that the A allele increases stimulated and unstimulated TNF production at transcriptional (Wilson *et al.* 1997, Abraham and Kroeger 1999) and translational level (Louis *et al.* 1998). -238G/A is located near a repressor-binding site (Flori *et al.* 2005) and -376G/A within a region capable of multiple

Table 8. Studies investigating the potential association of disease conditions with *TNF* promoter SNPs.

SNP	Disease	Association	Population studied	Reference	
-308A	Malaria	Risk and mortality ↑	Gambian	McGuire <i>et al.</i> 1994	
	Septic shock	Risk and mortality ↑	White French	Mira <i>et al.</i> 1999	
		Mortality ↑	Chinese	Zhang <i>et al.</i> 2003	
		NS	White British and Australian	Gordon <i>et al.</i> 2004	
		Rheumatoid arthritis	Risk ↓, severity ↑	Swedish	Cvetkovic <i>et al.</i> 2002
	Psoriasis, PsA	NS	Polish	Pawlik <i>et al.</i> 2005	
		Risk ↓	Estonian	Mössner <i>et al.</i> 2005	
		Irritable bowel sdr.	Risk ↑	Dutch	van der Veek <i>et al.</i> 2005
		Metabolic syndrome	Risk ↑	Combined	Sookoian <i>et al.</i> 2005
		Non-Hodgkin lymphoma	Mortality ↑	French	Juszczynski <i>et al.</i> 2002
Hepatocellular ca.		Risk ↑	Taiwanese	Ho <i>et al.</i> 2004	
-238A	Rheumatoid arthritis	Severe disease ↓	Italian	Fabris <i>et al.</i> 2002	
		Joint erosions ↓	Dutch	Brinkman <i>et al.</i> 1997	
	Psoriasis	Risk ↑	Estonian	Mössner <i>et al.</i> 2005	
	Psoriasis, PsA		German	Höhler <i>et al.</i> 1997	
	Ankylosing spondylitis	NS	Dutch	Kaijzel <i>et al.</i> 1999	
	Uveitis	Risk ↑	Austrian	El-Shabrawi <i>et al.</i> 2005	
	SLE	Risk ↑	Mexican Mestizo	Zúñiga <i>et al.</i> 2001	
	Malaria	Parasitemia ↑	Burkinabe	Flori <i>et al.</i> 2005	
-376A	Malaria	Severe disease ↑	Gambian, Kenyan	Knight <i>et al.</i> 1999	

SNP, single nucleotide polymorphism; PsA, psoriatic arthritis; sdr., syndrome; ca., carcinoma; SLE, systemic lupus erythematosus; NS, not significant.

DNA-protein interactions (Knight *et al.* 1999). Disease conditions with which *TNF* SNPs might be associated include malaria, septic shock and rheumatoid arthritis (Table 8).

INFLAMMATORY RHEUMATIC DISEASES

JOINTS

Normal anatomy

Joints are the structures connecting bones. Synovial joints are those that allow the movements of the limbs, support in exercise and have a synovial cavity between the adjacent bones (Figure 6). Immovable (e.g. sutures of the skull, and the sacrum) or slightly movable joints (e.g. those between the vertebrae) provide support and protection, and contain connective tissue, cartilage or bone tissue between the bones (Mankin and Radin 1989).

The synovial joint is surrounded by the synovial capsule composed of the outer collagen-rich fibrous joint capsule (e.g. forming the ligaments that guide the direction and magnitude of movements) and the inner synovial

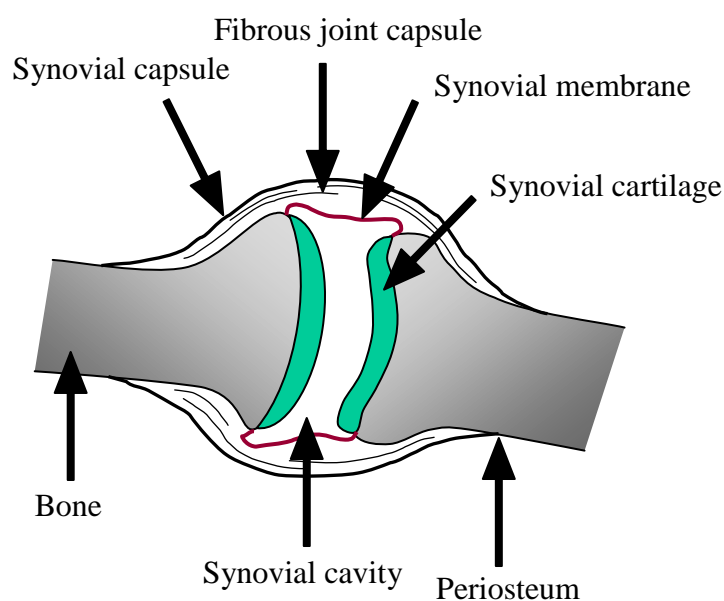


Figure 6. Parts of the synovial joint. Helminen and Tammi 2002.

membrane (Figure 8). The main cells of the synovial membrane, synoviocytes, are either macrophage- or fibroblast-like and surrounded by glycosaminoglycans and thin fibres of connective tissue. The synovial cavity normally contains only a few milliliters of synovial fluid that acts as a lubricant and nourishes the cartilage that has no blood vessels. The highly hydrophilic hyaluronate makes the fluid gel-like. In the cartilage matrix, large aggregates of hyaluronate and proteoglycans (typically aggrecan) give elasticity and diminish friction. Their anionic groups attract cations, increasing osmotic pressure and promoting elasticity. The collagen framework surrounding the aggregates gives tensile strength (Mankin and Radin 1989).

Pathologic changes

The first inflammatory changes in the joint are tissue edema and fibrin deposition. When the synovitis continues, the synovial membrane thickens, consisting of layers of synoviocytes and inflammatory cells. Early in the chronic disease, endothelial cells of synovial vessels transform into high endothelial venules, which are normally found in secondary lymphoid organs. Active angiogenesis provides the hypertrophic synovium with oxygen, nutrients and additional inflammatory cells (Lee and Weinblatt 2001). VEGF and its receptor are present in the rheumatoid synovium (Müller-Ladner *et al.* 2005).

Pannus, i.e. hypertrophic synovial membrane growing over the cartilage surface, is a characteristic rheumatic lesion. By time, the incongruence and destruction of the synovial surfaces, muscle pull and damages of the capsule, ligaments and tendons cause the typical deformities. Spreading of pannus-like tissue into periarticular tissues leads to ankylosis of the joint (Hough and Sokoloff 1989).

Cartilage degradation is started by the activity of proteases, which cleave proteoglycans from the matrix, impairing the tissue's mechanical capacity. This damage can still be reversed by proteoglycan synthesis of chondrocytes. In turn, collagen degradation, performed by metalloproteases, is thought to be irreversible (Zvaifler 1989).

The balance of bone formation/degradation is shifted towards degradation in chronic joint inflammation. The bone-resorbing cells, osteoclasts, are equipped with enzymes and a proton pump that enable them to degrade bone matrix and solubilise calcium, respectively (Schett 2007).

There are numerous pain nerve endings, nociceptors, throughout the joints (with the exception of the cartilage). Inflammatory mediators, cartilage destruction products and edema-associated intra-articular pressure sensitise the nociceptors to normal movements. Many mediators also excitate the nerves, and proteases can evoke spontaneous neural activity (McDougall 2006).

SPONDYLOARTHROPATHIES

Spondyloarthropathies (SpAs) are a heterogeneous group of inflammatory diseases characterised by peripheral oligoarthritis, spinal inflammation, enthesitis (inflammation of attachments of ligaments or tendons to bones) and, at times, mucocutaneous, ocular and/or cardiac manifestations. SpAs show striking familial aggregation and are typically associated with the HLA-B27 allele, but their pathogenesis is not fully understood. SpAs include ankylosing spondylitis (AS), reactive arthritis (ReA), psoriatic arthritis (PsA), enteroarthritis/spondylitis associated with inflammatory bowel diseases (IBDs), juvenile spondyloarthropathy, and undifferentiated spondyloarthropathy encompassing patients who express features of other SpAs still not fulfilling their criteria. Isolated acute anterior uveitis and spondylitic heart disease associated with HLA-B27 can also be classified as SpAs (Reveille and Arnett 2005).

SpAs are quite common, occurring in approximately 2% of the population, at least, as especially the undifferentiated form often remains underrecognised (Stafford and Youssef 2002). In Finland, the incidence of SpAs in adults is 218/100 000 annually. The most typical age of onset lies between 20 and 40 years (Isomäki *et al.* 1978).

Diagnosis and clinical manifestations

The lower extremities of the body are typically affected in SpAs, and the pattern of inflamed joints is asymmetrical. Enthesitis, such as Achilles tendonitis or plantar fasciitis, is an important hallmark. Widely accepted diagnostic criteria are shown in Table 9. Radiological sacroiliitis develops slowly and therefore, is usually not helpful in early diagnosis (Stafford and Youssef 2002).

Table 9. The criteria for diagnosing spondyloarthropathies according to the European Spondyloarthropathy Study Group (ESSG).

Inflammatory spinal pain or synovitis (asymmetrical, predominantly in the lower limbs), and one or more of the following
Positive family history
Psoriasis
Inflammatory bowel disease
Urethritis, cervicitis or acute diarrhoea within 1 month before arthritis
Buttock pain alternating between right and left gluteal areas
Radiological sacroiliitis
Enthesopathy

Dougados *et al.* 1991.

There are no specific laboratory tests for diagnosing SpAs. Findings supporting the SpA diagnosis include HLA-B27 positivity, absence of rheumatoid factor, elevation of the erythrocyte sedimentation rate (ESR) or CRP level, and anemia (Kataria and Brent 2004).

Reactive arthritis (ReA) is usually a mild form of SpA with a self-limiting course of 3-12 months. It is an aseptic arthritis developing 1-4 weeks after an infection outside the joints, usually in the gastrointestinal or genitourinary tract. Up to 50% of the patients have extra-articular manifestations, such as conjunctivitis. The triad of urethritis, conjunctivitis and arthritis is called Reiter's syndrome. Dactylitis ("sausage digit", inflammation of an entire digit) is also common (Kataria and Brent 2004). When ReA following an intestinal infection is suspected, bacterial culture finding is of high diagnostic value but only received when the pathogen is still present in the intestine (Leirisalo-Repo 2005).

Ankylosing spondylitis (AS) is the most HLA-B27 -dependent (up to 95% of the patients), severe and chronic form of SpA. It is three times more common in men than in women. Typically, it begins before the age of 30 with inflammatory lower back pain, which is worst in the early morning and improves with activity. Pain progresses upwards the spine and is relieved by stooping. Consequently, the spine may ossify into a kyphotic position. A totally ossified, rigid spine is seen in radiographs as “bamboo spine” (Kataria and Brent 2004). Acute anterior uveitis is the most frequent extra-articular manifestation. AS complicated by spinal fracture, cardiovascular and intestinal diseases or amyloidosis (which is becoming less common) is associated with increased mortality. However, AS is highly variable and may also heal (Khan 1998a).

Pathogenesis

The exact pathogenic mechanisms of SpAs are unknown. It is likely that an interplay between genetic, environmental and immunological factors is responsible for the clinical manifestations (Kataria and Brent 2004).

Triggering bacteria

So far, ReA is the only SpA whose pathogenesis has been proved to involve specific microbes. These are able to invade mucosal surfaces and replicate intracellularly and have LPS in their outer membrane (Reveille and Arnett 2005) (Table 10). *Yersinia*, typically contaminating pork, can grow at refrigeration temperature and induces a remarkable proportion of enteroarthritis in countries where food is stored in the fridge. As tourism is increasing, ReA following a *Salmonella*- or *Campylobacter*-induced food poisoning is becoming more common. Over 80% of *Salmonella* enteritis cases in Finland have been acquired abroad (Vesikari 2003). *Campylobacter*, usually growing in incorrectly handled or raw poultry products (Moore *et al.* 2005), is the most frequent trigger of enteroarthritis. *Chlamydia trachomatis* causes the common sexually transmitted disease and is also the most common trigger

of uroarthritis and ReA altogether. On the whole, 1 to 15% of those having a mucosal infection develop arthritis (Leirisalo-Repo 2005).

Antigens of ReA-associated microbes have been detected in the synovium of ReA patients even years after the initial infection, suggesting a pathogenic role for prolonged bacterial persistence. Circulating IgA antibodies and synovial T cell response against the initial microbe have also been observed (Reveille and Arnett 2005). As IgA antibodies are typical of mucosal defence, the hypothesis of the association between inflammation in the joints and colonic mucosa is supported (Figure 7). Intestinal bacterial antigens may be circulated by mononuclear phagocytes between the gut and joints (Baeten *et al.* 2002b) and the breakdown of the gut-blood barrier could be an inciting or/and perpetuating event in SpAs (Reveille and Arnett 2005).

Table 10. Microbes associated with reactive arthritis.

Enteroarthritis
<i>Campylobacter</i>
<i>Salmonella</i>
<i>Yersinia</i>
<i>Shigella</i> (mostly <i>Sh. flexneri</i>)
<i>Clostridium difficile</i> (rare)
Uroarthritis
<i>Chlamydia trachomatis</i>
<i>Neisseria gonorrhoeae</i> (?)
<i>Mycoplasma genitalium</i> (?)
<i>Ureaplasma urealyticum</i> (?)
Arthritis following an upper respiratory tract infection
β -haemolytic streptococci (more usually triggering rheumatic fever)
<i>Chlamydia pneumoniae</i>

Modified from Leirisalo-Repo 2005.

Genetic predisposition

In 1973, *HLA-B27* was reported to be strongly associated with AS (Brewerton *et al.* 1973, Schlosstein *et al.* 1973) and to date, it is the only documented susceptibility gene for other SpAs, too (Breban *et al.* 2006) (Table 11). The frequency of *HLA-B27* in a given population loosely correlates with the frequency of SpAs. For instance, 40% of the Alaskan Eskimos, 24% of the

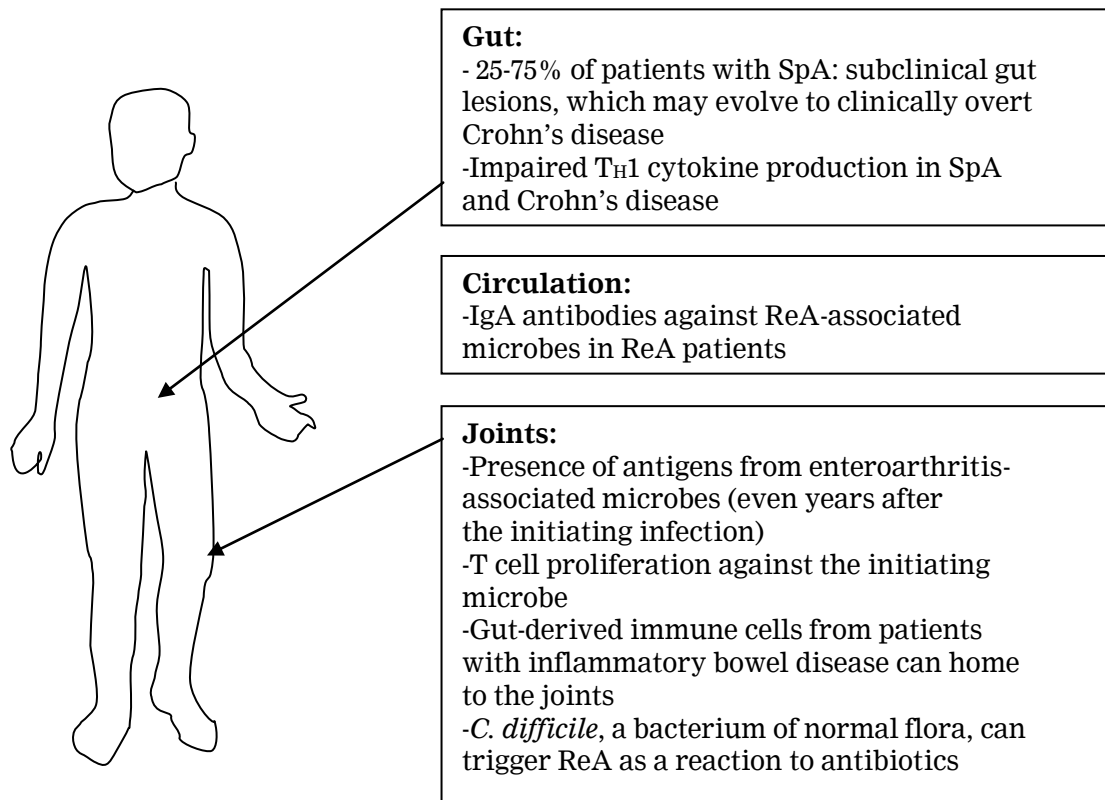


Figure 7. Evidence of the association between spondyloarthropathies (SpAs) and inflammation in colonic mucosa. ReA, reactive arthritis. References: Söderlin *et al.* 1999, the reviews by Baeten *et al.* 2002a, Reveille and Arnett 2005.

Table 11. Percentage of HLA-B27 positive individuals of patients with spondyloarthropathies in Europe.

Ankylosing spondylitis	95%
Reactive arthritis	70%
Psoriatic spondylitis	60%
Peripheral psoriatic arthritis	25%
Inflammatory bowel disease –associated spondylitis	70%
Acute anterior uveitis without other stigmata of spondyloarthropathies	50%

Reveille and Arnett 2005.

Sami and 8% of the caucasoids in Western Europe and the USA are B27 positive with the AS frequency being 2.5%, 1.8% and 0.2%, respectively (Khan 1998b).

HLA-B molecules belong to the MHC class I proteins, which are expressed on all normal nucleated cells as heavy α chain - β_2 microglobulin heterodimers. The classical function of MHC class I proteins is to present

intracellularly produced foreign (i.e. viral, bacterial or tumour) peptides to CD8⁺ T cells and to activate killing-inhibiting receptors on NK cells so that only abnormal cells become killed (Reveille and Arnett 2005). *HLA-B27* is one of around 490 polymorphic HLA-B alleles recognised. Polymorphism is concentrated in regions involved in peptide binding (Chaplin 2003). However, it is not known how HLA-B27 increases the risk of SpAs and why it is only associated to certain organ systems (Reveille and Arnett 2005). The most studied hypotheses are the arthritogenic peptide and molecular mimicry theories (Table 12).

According to the arthritogenic peptide hypothesis, HLA-B27 has a unique ability to bind a joint-specific peptide recognised by autoreactive CD8⁺ T cells. The molecular mimicry hypothesis suggests that bacterial peptides and either an HLA-B27 peptide or a self peptide presented by HLA-B27 may cross-react, which could break tolerance. More recent theories bring up the amino acid residues in the B pocket of HLA-B27, which predispose to slow or incorrect folding during the B27 assembly in the endoplasmic reticulum (ER). Thereby, two heavy chains tend to form homodimers, which could induce proinflammatory ER stress (Hacquard-Bouder *et al.* 2006). Free heavy chains that still have the peptide-binding groove may also be expressed on the cell

Table 12. Hypotheses to explain the role of HLA-B27 in spondyloarthropathies.

Hypothesis	Implication of HLA-B27	Immunological mechanism
Antigen-specific		
Arthritogenic peptide	Presentation of self peptide deriving from target tissue antigen	Activation of autoreactive CD8 ⁺ T cells against self peptide
Molecular mimicry	Mimicry between self peptide derived from/presented by HLA-B27 and bacterial antigen	Cross-reaction between bacteria responsible for infection and HLA-B27
Independent of antigen specificity		
Misfolding	Slow folding of HLA-B27 in the endoplasmic reticulum	Instability of HLA-B27, stress response, NFκB activation, inflammatory response
HLA-B27 heavy chain dimerisation	Disulfide bond formation between 2 cysteine 67	Unusual reactivity between HLA-B27 and CD4 ⁺ or natural killer cell receptors

Hacquard-Bouder *et al.* 2006.

surface. Altogether, SpAs may be associated with the recognition of different HLA-B27 forms by leukocyte receptors (Reveille and Arnett 2005).

According to family studies, *HLA-B27* contributes to about 37% of the overall genetic risk of SpA. The entire effect of the MHC region is 50%, including genes that encode e.g. HLA-DR proteins of MHC class II, TNF, complement components, heat shock protein 70, and proteins involved in antigen presentation by MHC class II molecules. Evaluation of the effect of individual loci is complicated by strong linkage disequilibrium and *HLA-B27* (Reveille and Arnett 2005, Breban *et al.* 2006) and the fact that the risk alleles are apparently associated with specific symptoms rather than SpA subtypes (Breban *et al.* 2006).

Genome-wide scans have revealed non-MHC regions that may contain SpA-associated genes. A significant association has been found between AS and the IL-1 gene cluster in chromosome 2. Currently, the most promising region is probably the 9q31-34 (Breban *et al.* 2006).

Innate immune responses

The synovium and entheses of the SpA patients are rich in T cells, particularly CD8⁺ cells (Baeten *et al.* 2002a). However, synovial infiltration of phagocytes may reflect the global disease activity of SpAs, and the innate immune cells play a prominent role in SpA synovitis while lymphocytes cause only secondary alterations (Baeten *et al.* 2005). Macrophages expressing the haptoglobin scavenger receptor CD163 are abundant in the synovium of SpA patients. Cross-linking of CD163 mediates e.g. the secretion of IL-6 and GM-CSF (Baeten *et al.* 2002b), and CD163⁺ cells exhibit high HLA-DR expression and TNF production after LPS stimulation. Chronic exposure to high levels of TNF can lead to impairment of T_H1 response and insufficient clearance of intracellular bacteria. Consistently, the use of TNF blockers has provided very beneficial effects on arthritic symptoms in AS, PsA and undifferentiated SpA, and on enthesitis and uveitis (Baeten *et al.* 2002a).

Self molecules characteristic of the joints, such as proteoglycans, have been proposed to activate TLR4 signalling, and peripheral blood mononuclear

cells from SpA patients can have a nearly 50% increase in TLR4 expression when compared with healthy individuals (De Rycke *et al.* 2005). Intact extracellular matrix may inhibit TLR4 activation, and the inhibition is abrogated when proteases are released upon infection- or tissue injury-triggered matrix degradation. LPS may also bypass the suppressive mechanism (Brunn *et al.* 2005).

Treatment and outcome

The treatment of SpAs includes patient education, physical therapy, exercise and most importantly, relief of pain and stiffness in which non-steroidal anti-inflammatory drugs (NSAIDs) are usually effective. Disease-modifying antirheumatic drugs (DMARDs, especially sulphasalazine and methotrexate) quite commonly cause adverse effects, and are therefore reserved for the patients who do not benefit from NSAIDs or who develop erosions. Intra-articular corticosteroid injections can be used to control local synovitis and relieve pain (Stafford and Youssef 2002). The use of the new and expensive biological drugs, TNF blockers, is considered when refractory active disease does not respond to traditional drugs (Reveille and Arnett 2005). There are probably no living bacteria in the joints of ReA patients and consistently, long-term antibiotics are not advantageous (Baeten *et al.* 2002a).

15-30% of ReA patients develop chronic arthritis or radiologically detectable sacroiliitis (Kataria and Brent 2004). The prognosis is slightly worse in uro- than enteroarthritis. Therefore, early diagnosis and treatment of *C. trachomatis* infection is essential. Genotyping of HLA-B has a prognostic value, since HLA-B27 positivity is often associated with a more difficult acute ReA, extra-articular symptoms, and development of chronic disease and spinal inflammation (Leirisalo-Repo 2005). Currently, early diagnosed AS only seldom results in severe disability. Patients with hip joint involvement or kyphotic spine have a worse prognosis (Khan 1998a). 55% of undifferentiated SpA patients and 17% of PsA patients have reached remission after 2-3 years (Stafford and Youssef 2002). Patients with polyarticular form of early PsA are at risk of progressive disease (Gladman *et al.* 2005) IBD-associated SpA usually

subsides in 6-8 weeks and only 10% develop chronic arthritis (Kataria and Brent 2004). There are quite few studies on the outcome of juvenile SpA, but it seems that more than 50% of the patients have active arthritis still in the adulthood (Minden *et al.* 2000).

RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterised by chronic synovial joint inflammation that leads to cartilage and bone destruction (Aguillón *et al.* 2006). Despite the highly varying activity of RA, the disease frequently causes deformities of the joints, functional disability and sometimes extra-articular manifestations such as rheumatoid nodules, vasculitis, scleritis, pleuritis, pneumonitis, pericarditis, and ocular and haematological disorders (Lee and Weinblatt 2001). The primary cause and pathogenesis of RA remain unresolved. In general, it is defined as a result of a sustained immune response to an unknown antigen in a genetically susceptible host (Aguillón *et al.* 2006).

RA affects approximately 1% of the adult population worldwide, being most common among Pima and Chippewa Indians in North America, and rarest in rural Africa (Lee and Weinblatt 2001). Its prevalence in Finland is 0.8% with the incidence of 5000/100 000 adults each year. The typical age of onset lies between 60 and 70 years, and RA is three times more common in women than in men (Isomäki *et al.* 1978).

Diagnosis and clinical manifestations

The onset of RA is usually insidious, although a rapid multisystem inflammation is also possible. The predominant symptoms are pain, swelling and stiffness of peripheral joints (e.g. feet, fingers, wrists and knees are commonly affected), palpation tenderness, and morning stiffness caused by edema that accumulates in the tissues during sleep. Non-specific symptoms (tiredness, loss of appetite and weight) may also occur (Lee and Weinblatt 2001). The diagnostic criteria used worldwide are shown in Table 13.

Table 13. Diagnostic criteria for rheumatoid arthritis established by American College of Rheumatology in 1987. Four or more of the criteria should be fulfilled, and the criteria 1-4 must have been present for at least six weeks.

-
1. Morning stiffness (for at least 1 hour)
 2. Arthritis of 3 or more joint areas
 3. Arthritis of hand joints
 4. Symmetric arthritis
 5. Rheumatoid nodules
 6. Rheumatoid factor in the serum
 7. Radiological changes (e.g. erosions) in hand and/or wrist joint
-

Arnett *et al.* 1988.

Serum autoantibodies are common in RA. The classic example, rheumatoid factor (RF), is present in 70-80% of the patients. It may appear years before the clinical symptoms take place, and permanent RF seropositivity is useful in distinguishing RA from other rheumatic conditions. RF recognises altered, aggregated or immunocomplexed mammalian IgG. However, RF is not a very specific marker for RA. Instead, novel and more efficient diagnostic tools, anti-citrullinated protein antibodies (ACPAs), are highly specific for RA, easily detected by citrullinated substrate constructs and good predictors of progressive joint damage (Weissmann 2006).

ACPAs attack epitopes in which arginine residues have been converted to citrulline by peptidyl deiminase (PAD) enzymes (Cantaert *et al.* 2006). Other post-translational modifications may also create epitopes contributing to the autoreactivity in RA. The patients often have autoantibodies against cartilage collagen, the arthritogenicity of which may be attributed to glycosylation (Fournier 2005). In practice, a seropositive polyarthritis with radiologic erosions, unclear etiology, and existence of serum autoantibodies is regarded as RA, even if the clinical symptoms were minor (O'Dell 2004).

Pathogenesis

The disease process of RA has been proposed to consist of three phases (Table 14). Phases 2 and 3 are regarded as a T_H1-type disease (Arend 2001). This is supported by the fact that in patients with RA and AIDS, RA can entirely

heal when the number of CD4+ T cells declines as AIDS progresses (Weyand and Goronzy 1992). However, in RA, a high proportion of the synovial leukocytes are innate immune cells and the prevailing cytokines are derived from monocytes and macrophages (Lee and Weinblatt 2001). Furthermore, T cell activation in the synovium appears to be B cell-dependent (Müller-Ladner *et al.* 2005). Thus, multiple cell types and mechanisms and factors influencing these are likely to contribute to the pathogenic process of RA.

Several environmental and genetic factors have been associated with increased or decreased risk of developing RA. Interactions between these factors may be highly important, though mostly unknown as yet (Oliver and Silman 2006).

Environmental factors

According to the most common view, an infection is the environmental component needed to launch the inflammatory process in RA, and the most consistent evidence points to Epstein-Barr virus (EBV). RA patients may exhibit a decreased T cell response to the EBV protein gp110, which controls EBV replication. Such an insufficient response could lead to poor control of the infection and chronic exposure to EBV antigens. However,

Table 14. Phases of the pathophysiology in rheumatoid arthritis. The initial phase may be largely subclinical and multiple mechanisms are possible. In the perpetuation phase, adaptive immunity is launched.

Initiation

Mechanisms are unknown

May include local cytokine release by non-specific activation of resident macrophages and dendritic cells in the joints

Perpetuation

Priming of autoreactive T cells in lymph nodes → migration → activation of memory T cells within the joints

Subsequent stimulation of B cells and macrophages with rheumatoid factor production and further cytokine release

Chronic inflammation

Dysregulation of multiple immune cell types

Abnormal fibroblast growth

T cell responses to altered self antigens

Arend 2001.

any specific infection has not been confirmed to precede the onset of RA. Instead, there is indirect evidence of the involvement of different microorganisms. First, as there is similarity between human and microbial molecules (e.g. heat shock proteins), the immune system may fail in maintaining tolerance, leading to autoimmune reactions (Oliver and Silman 2006). Second, pet ownership has been suggested to be a risk factor, as pets are believed to harbour environmental agents that can trigger RA. Animal antigens *per se*, however, are an unlikely risk factor, as RA is more common in urban than in rural populations. Third, the recent decline in RA incidence in high-risk populations might reflect the changing of the habits of life, e.g. the intestinal flora is encountered by different pathogens than before (Aguillón *et al.* 2006).

Smoking is one of the most studied lifestyle risk factors for RA. Males have an almost three-fold risk of developing RA if they are smokers, and heavy smoking can increase the risk of both sexes over 10-fold (Oliver and Silman 2006). Of the occupational exposures, inhaled silica particles may increase the risk of RA among rock drilling and stone crushing crew, presumably via the activation of alveolar macrophages (Stolt *et al.* 2005). Mineral oils cause polyarthritis in rats and increase the risk of RA among men exposed to motor or hydraulic oil. In rats, mineral oil is rapidly transported to the lymph nodes and may also trigger acute phase reactions (Svelander *et al.* 2001).

Diet can affect the onset and persistence of RA. The so-called Mediterranean diet containing plenty of oily fish rich in omega-3 fatty acids may protect against inflammation, and antioxidants from fruit and vegetables may strengthen the advantageous effect (Oliver and Silman 2006).

Genetic predisposition

Based on large twin studies in the UK and Finland, heredity accounts for around 50-60% of the predisposition to RA (Oliver and Silman 2006). Ethnic differences in haplotype distribution and linkage disequilibrium complicate the discovery of the ultimately responsible genetic factors (van der Helm-van Mil *et al.* 2005). Known risk genes/alleles for RA are listed in Table 15.

Table 15. Examples of genes affecting the susceptibility or severity of rheumatoid arthritis.

Genetic factor	Proposed role in the pathogenesis	Remarks	References
HLA-DRB1 alleles *0101, *0102, *0401, *0404, *0405, *0408, *1001, *1402	Shared epitope that influences antigen binding	Susceptibility ↑, risk of erosions ↑	van der Helm-van Mil <i>et al.</i> 2005
<i>TNF</i> -308G→A SNP	Increased TNF production	Susceptibility ↓, disease severity ↑	Wilson <i>et al.</i> 1997, Louis <i>et al.</i> 1998, Cvetkovic <i>et al.</i> 2002
<i>PAD4</i> (peptidylarginine deiminase) SNPs	Increased citrullination	Susceptibility ↑ (controversial results from different populations)	van der Helm-van Mil <i>et al.</i> 2005
<i>PTPN22</i> (hematopoietic protein tyrosine phosphatase) SNPs	Aberrant T cell activation	Susceptibility ↑	Oliver <i>et al.</i> 2006
<i>SLC22A4</i> (cation transporter) and <i>RUNX1</i> (hematopoietic transcription factor) SNPs	Cumulative effect on disrupting transcription	Susceptibility ↑	van der Helm-van Mil <i>et al.</i> 2005
<i>ICAM-1</i> , <i>VEGF</i> (vascular endothelial growth factor) and <i>IL-1RA</i> (receptor antagonist) SNPs	Inflammatory changes	Susceptibility ↑ (non-replicated results)	van der Helm-van Mil <i>et al.</i> 2005
<i>IL-1β</i> , <i>IL-2</i> , <i>IL-4</i> and <i>IL-6</i> SNPs	Inflammatory changes	Disease severity ↑	Oliver <i>et al.</i> 2006
<i>IL-10</i> microsatellite haplotypes	(Anti-) inflammatory changes	Disease severity ↑, association only in females	van der Helm-van Mil <i>et al.</i> 2005

MHC class II genes are the most powerful genetic contributors to RA recognised so far, covering at least 30% of the total genetic effect. Complexes of MHC class II proteins and peptides that are derived from endocytosed or phagocytosed antigens are recognised by CD4⁺ T cells on the surface of antigen-presenting cells, i.e. dendritic cells, macrophages and B cells. There is no need to express MHC class II molecules on more cell types, because activated CD4⁺ T cells produce cytokines, which amplify the response by activating a wide range of cells (Parkin and Cohen 2001, Chaplin 2003).

The HLA-DRB1 alleles *0101, *0102, *0401, *0404, *0405, *0408, *1001 and *1402 encode shared epitope (SE), i.e. a conserved amino acid sequence (QKRAA, QRRAA or RRRAA) at a position that contributes to the peptide-presenting site. Alleles encoding SE are associated with higher susceptibility and severity of RA. *HLA-DRB1* haplotypes that encode the sequence DERAA instead of the shared epitope may protect from RA (van der Helm-van Mil *et al.* 2005).

Within the HLA region, another potential candidate for RA is *TNF* -308G→A. In several populations, the -308A allele is more common in healthy individuals than in RA patients, but it is also associated with a more severe form of RA (Cvetkovic *et al.* 2002). This may reflect the different need of TNF in acute defence and the chronic phase of the disease.

Female hormonal environment is likely to play a role in the pathogenesis, evidenced by the higher RA incidence in women and the pro-inflammatory effects of estrogens, the receptors of which are expressed on synovial macrophages (Kinne *et al.* 2000). Also, the onset of RA is reduced during pregnancy, and increased again postpartum particularly among breastfeeders, which may be explained by increased secretion of the pro-inflammatory hormone prolactin. The use of oral contraceptives also seems to protect from RA, possibly by postponing pregnancy (Oliver and Silman 2006).

Interactions between genetic and environmental factors

Recently, a large Swedish case-control study revealed for the first time gene-environment interactions that operate in the etiology of RA. Smokers

carrying two copies of SE had a 21-fold risk of RA when compared to non-smokers without susceptibility alleles. Long-term exposure to cigarette smoke may induce mechanisms that accelerate deimination of arginine to citrulline in autoantigens present in the lungs, possibly by up-regulation of PAD activity in macrophages. As citrullination increases the binding of peptides to SE-containing HLA-DR, the subsequent immune response may be induced in individuals carrying SE (Klareskog *et al.* 2006).

Innate immune responses

The severity of RA correlates with the abundance and activity of synovial macrophages, which produce large amounts of inflammatory cytokines, chemoattractants and proteases at the site of tissue destruction. In active RA, an altered cytokine or growth factor milieu may be built up in the circulation, leading to rapid generation of CD14 and HLA-DR positive myelomonocytic cells in the bone marrow and systemic monocyte activation (Kinne *et al.* 2000).

Paracrine, autocrine and cellular interactions between synovial fibroblasts and macrophages induce cartilage destruction, in which the most prominent mediators are IL-1 and TNF (Kinne *et al.* 2000). They stimulate the production of matrix-degrading enzymes (such as MMPs) in fibroblasts. Cellular hyperplasia in rheumatic joints partly results from prevention of apoptosis as the expression of Bcl family members and soluble Fas ligand is increased in fibroblasts (Müller-Ladner *et al.* 2005). Also, pannus formation might be driven by penetration of synoviocytes that produce high amounts of MMPs (Lee and Weinblatt 2001).

The synovial endothelial cells are an active target for cytokines and mitogens, which crucially contributes to the expansion of synovial tissue and the subsequent increased need for blood vessels. Hypoxia induces the production of angiogenic VEGF in the synovial membrane, and pannus is highly vascularised (Paleolog 2002).

Of the other cell types, neutrophils have the greatest capacity to cause joint damage, and they are abundantly present in synovial fluid and at the site

of early erosions, pannus-cartilage junction (Witko-Sarsat *et al.* 2000). Synovial DCs may constantly receive non-specific stimulation (e.g. from minor trauma) in the rheumatoid synovium and migrate to the regional lymph nodes (Arend 2001).

Because only a few protective mechanisms operate in the joints, imbalance of any of the regulatory systems may shift the balance between matrix production and degradation in the direction of RA chronicity. For instance, the ratio of IL-1 and its physiological inhibitor IL-1RA is elevated in RA (Kinne *et al.* 2000).

The innate receptors TLR4 and TLR2 may mediate RA-associated signals. Endogenous TLR4 ligands are abundant in inflamed joints, and an association between RA susceptibility and the TLR4 Asp299Gly variant has been suggested. Due to the heterodimerisation of TLR2 with either TLR1 or TLR6, TLR2 ligands are a diverse group, including native and modified bacterial lipoproteins, peptidoglycan and endogenous HSPs. Increased numbers of CD16+ monocytes that produce high amounts of pro-inflammatory cytokines in response to TLR2 agonists have been detected in peripheral blood of RA patients (Brentano *et al.* 2005).

Treatment and prognosis

In early RA, the therapeutic strategies are aimed at obtaining remission and preventing joint destruction as soon as possible. These goals can be reached with conventional DMARDs or new biological agents. Effective early treatment is essential as the majority of the damages can occur within the first five years (Morel and Combe 2005). The adoption of standardised and validated clinical outcome measures (e.g. the disease activity score DAS in Europe) that inform about inflammation, function and quality of life, have greatly improved the assessment of treatment. These measures are combined with radiological scores (e.g. Larsen score) that assess the severity of erosions and joint-space narrowing (Lee and Weinblatt 2001). Physical or occupational therapy and patient education accompany the drug therapy (O'Dell 2004).

To date, the most common DMARDs are methotrexate (MTX) and sulphasalazine. MTX is a folic acid antagonist, which prevents purine and pyrimidine synthesis and thereby cell proliferation at high doses (in cancer chemotherapy). In the treatment of RA, much lower MTX doses are used that prevent the synthesis of tetrahydrofolate. The most common adverse effects of MTX include mouth ulcers, nausea and dizziness. The precise mechanism of sulphasalazine action is not known. However, it reduces the production of RF and TNF, and down-regulates lymphocytes and neutrophils. Sulphasalazine may cause intestinal problems and nausea. Other widely used DMARDs include hydroxychloroquine, leflunomide, penicillamine, cyclosporine A, azathioprine, and gold compounds (Möttönen *et al.* 2002, O’Dell 2004).

The treatment strategies include the use of one DMARD, a combination of DMARDs or a biological agent. The patients with poor prognosis would greatly benefit from the biologicals. However, these drugs are expensive and may predispose to serious infections, and patients who do not develop significant joint damage do not require them (Morel and Combe 2005). Anti-inflammatory glucocorticoids are also used (O’Dell 2004).

The most promising biologicals are TNF inhibitors etanercept, infliximab and adalimumab (Nash and Florin 2005) (Table 16). The usefulness of TNF as a target is evidenced by several research lines. First, neutralisation of TNF suppresses collagen-induced arthritis and reduces inflammation in immunodeficient arthritis. Furthermore, deregulated TNF expression causes development of chronic arthritis, and TNF levels in the synovial fluid

Table 16. Licensed tumour necrosis factor (TNF) inhibitors.

	Etanercept	Infliximab	Adalimumab
Type	Soluble TNF receptor	TNF antibody	TNF antibody
Construct	Recombinant human fusion protein	Chimeric human-mouse monoclonal antibody	Human monoclonal antibody
Use	In monotherapy or with methotrexate	With methotrexate	In monotherapy or with methotrexate

From Nash and Florin 2005.

correlate with the degree of bone erosions (Kinne *et al.* 2000).

70% of RA patients develop radiological erosions. Factors predicting worse radiological outcome include the SE alleles, high levels of RF and ACPAs, early erosions and radiological score at disease onset. Close to 50% of the patients are unable to work after 10 years of disease (Morel and Combe 2005). The predicted lifespan of RA patients is shortened by 15-20%, and the increased mortality is often caused by infections and renal disease associated with a severe RA. The gastrointestinal problems and hematopoietic malignancies leading to death may be treatment-related (Myllykangas-Luosujärvi *et al.* 1995). The risk of cardiovascular diseases (with subsequent excess mortality) is also increased among RA patients, maybe due to the inflammatory phases of the pathologic cardiovascular events (O'Dell 2004).

AIMS OF THE STUDY

The aim of the study was to examine the innate immune system in inflammatory rheumatic diseases. The specific aims were to study

1. Innate immune function of subjects with a history of ReA focusing on
 - a) monocyte TNF production capacity (Study I)
 - b) neutrophil activation using cellular markers CD11b, CD14 and CD16 (Study II)

2. The prognostic value of innate immune gene alleles:
 - a) *CD14* -159 and *TNF* promoter polymorphisms in acute arthritis (Study III)
 - b) *TLR4* +896 polymorphism in early RA (Study IV)

3. Peripheral blood mononuclear VEGF production in association with the activation of the innate immune system (Study V)

EXPERIMENTAL PROCEDURES

MATERIALS

SUBJECTS

All subjects in studies I-V were native Finns (Table 17). Study V comprised healthy subjects. The study protocols were approved by local ethics committees (the FIN-RACo protocol also by national health authorities), and the subjects gave their informed consent to participate.

Table 17. Characteristics of the subjects in clinical studies (I-IV).

Study	n	Women/ men	Age, years*	Early diagnosis	Follow-up time, years*	Outcome
I, II	45	10/5 10/5 10/5	57 (39-71) 47 (32-64) 53 (43-58)	ReA Healthy (HLA-B27+) Healthy (HLA-B27-)	18 (15-25) - -	Recovery - -
III	141	77/64	49 (30-80)	ReA, n=93 Other SpA, n=13 RA, n=14 Other inflammatory arthritis, n=21	17 (10-38)	Asymptomatic, n=92 Chronic SpA, n=30 AS, n=19 Other SpA, n=11 Chronic RA, n=11 Other autoimmune disease, n=8
IV	169	105/64	47 (20-65)	RA	0.5	Remission, n=77 Nonremission, n=92

* Expressed as median (range). n, number of patients; ReA, reactive arthritis; SpA, spondyloarthritis; RA, rheumatoid arthritis; AS, ankylosing spondylitis.

Studies I and II

Fifteen HLA-B27 positive subjects who had had *Yersinia*-triggered reactive arthritis (Leirisalo *et al.* 1982) with complete recovery (Leirisalo-Repo *et al.* 1996a) were recruited (Table 17). Fifteen HLA-B27 positive and 15 HLA-B27 negative healthy laboratory employees served as reference subjects. None of the subjects had anti-inflammatory medication or signs of infection at the time of donation or during the preceding week. Neither did they have subclinical systemic inflammation, verified by a high sensitivity CRP assay.

Study III

141 subjects were included who had been diagnosed with spondyloarthropathy, rheumatoid arthritis or acute inflammatory arthritis of diverse causes (Leirisalo *et al.* 1982, Valtonen *et al.* 1985) and re-examined for disease outcome (Leirisalo-Repo *et al.* 1996a, Leirisalo-Repo *et al.* 1996b) (Table 17). The most common early diagnosis was ReA, triggered by *Yersinia* in 46, *Chlamydia* in 27, *Salmonella* in 1, *Shigella* in 1 and an unknown microbe in 18 patients.

Study IV

The patients had been diagnosed with early rheumatoid arthritis and participated in the FIN-RACo (FINnish Rheumatoid Arthritis Combination therapy) trial (Möttönen *et al.* 2002). FIN-RACo was a randomised, nation-wide multi-center study in which the efficacy and tolerability of a single DMARD therapy (sulphasalazine with or without the corticosteroid prednisolone) were compared with those of a combination DMARD therapy (sulphasalazine, methotrexate, hydroxychloroquine and prednisolone) in active early

Table 18. Inclusion and exclusion criteria for the FINnish Rheumatoid Arthritis Combination therapy (FIN-RACo) trial.

Inclusion criteria

Fulfilment of the classification criteria for rheumatoid arthritis by the American Rheumatism Association (Arnett *et al.* 1988)

Age between 18 and 65 years

Duration of symptoms < 2 years

Active disease with three or more swollen joints, and at least three of the following:

Erythrocyte sedimentation rate \geq 28mm/h

C-reactive protein > 19 mg/l

Morning stiffness \geq 29 minutes

Number of swollen joints > 5

Number of tender joints > 10

Exclusion criteria

Previous DMARD usage

Glucocorticoid therapy within two weeks

Serious comorbidity

History of cancer

Pregnancy or absence of reliable contraception among women at fertile age

DMARD; disease- modifying antirheumatic drug.

rheumatoid arthritis (Table 18). Availability of an applicable blood sample reduced the number of the 195 FIN-RACo participants to 169 (87 receiving single and 82 combination DMARD therapy) in the present study.

BLOOD SAMPLES

Studies I and II

Venous blood samples of 3 ml were collected into Falcon tubes (Becton Dickinson, Lincoln Park, New Jersey, USA) containing pyrogen-free heparin (Lövens, Ballerup, Denmark) 10 IU/ml blood and placed in an ice-water bath. Aliquots were taken for the whole blood culture assay and for flow cytometry, which was performed within ten hours of blood sampling. In study I, an additional venous blood sample of 7 ml was collected into an EDTA(K₃) tube (Vacutainer No 367657, Becton Dickinson), aliquoted into Cryo tubes (Greiner GmbH, Solingen, Germany) and stored at -70°C until the nucleic acid analysis was done within four months.

Study III

In the studies on the outcome of early arthritis (Leirisalo-Repo *et al.* 1996a, Leirisalo-Repo *et al.* 1996b), a 10-ml sample of venous blood had been collected from each patient into a citrate anticoagulant tube and stored at -18°C. Nucleic acid analysis for the present study was done five years later.

Study IV

Venous blood samples obtained during the FIN-RACo trial were stored at -20°C and used for nucleic acid analyses in this study ten years later.

Study V

100-ml venous blood samples were collected from 7 healthy subjects, anticoagulated by EDTA and diluted with an equal volume of 0.9% NaCl. Peripheral blood mononuclear cells (PBMNCs) were isolated using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation, washed once with PBS containing 10% heat-inactivated fetal bovine serum

(Life Technologies, Rockville, Maryland, USA), twice with sterile Dulbecco's modified Eagle's medium (DMEM, from Life Technologies) and resuspended in DMEM. The cells were identified and counted by a Technicon H2 differential cell counter (Bayer AG, Leverkusen, Germany). The PBMNCs were used immediately in serum-free cultures.

METHODS

LABORATORY

Cultures

Whole blood (Studies I and II)

Escherichia coli O111:B4 LPS, phorbol 12-myristate 13-acetate (PMA) and Ca²⁺ ionophore A23187 (all from Sigma, St. Louis, MO, USA) were stored at -20°C as stock solutions (400 µg LPS/ml Dulbecco's phosphate buffered saline (PBS) from Life Technologies Ltd., Paisley, UK; PMA and Ca²⁺ ionophore A23187 both at 5 mM in 99.5% ethanol). LPS was used for receptor-mediated, and PMA and A23187 for non-specific stimulation of leukocytes.

100-µl aliquots of heparinised blood were added into polypropylene tubes (No 352063, Becton Dickinson) or cell culture wells (No 3515, Corning Inc., Corning, New York, USA) pre-supplemented with: 1) 800 µl of RPMI 1640 medium (Life Technologies) and 100 µl of LPS (finally at 1 µg/ml) or 2) 900 µl RPMI and 1 µl of PMA and 1 µl of A23187 (both finally at 5 µM) or 3) 900 µl of RPMI 1640 alone. Two sets of cultures were prepared for both 2-hour and 4-hour incubations, one in test tubes and one in cell culture wells, and incubated at 37°C in 5% CO₂. Polypropylene tubes and cell culture wells were used in the incubations in order to mimic the non-adherent conditions in the circulation and the adherent conditions on the endothelium at the inflamed site, respectively. The culture supernatants were snap-frozen and stored at -70°C. TNF levels were determined within two months. The cell pellet was suspended in 50 µl of ice-cold sterile AB serum (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) and used for flow cytometry.

Serum-free (Study V)

The freshly isolated PBMNCs transferred onto multiwell plates were incubated in 500 μ l of DMEM supplemented with 5 mg/ml bovine serum albumin (Sigma, St. Louis, Missouri, USA), 50 μ g/ml gentamicin (Life Technologies) and various concentrations of endotoxin from *S. typhimurium* (Sigma) and/or recombinant human interferon- α 2b (PeproTech, London, UK) at 37°C in 5% CO₂ for 24 h. The protein synthesis was blocked using 1 mM cycloheximide (Sigma).

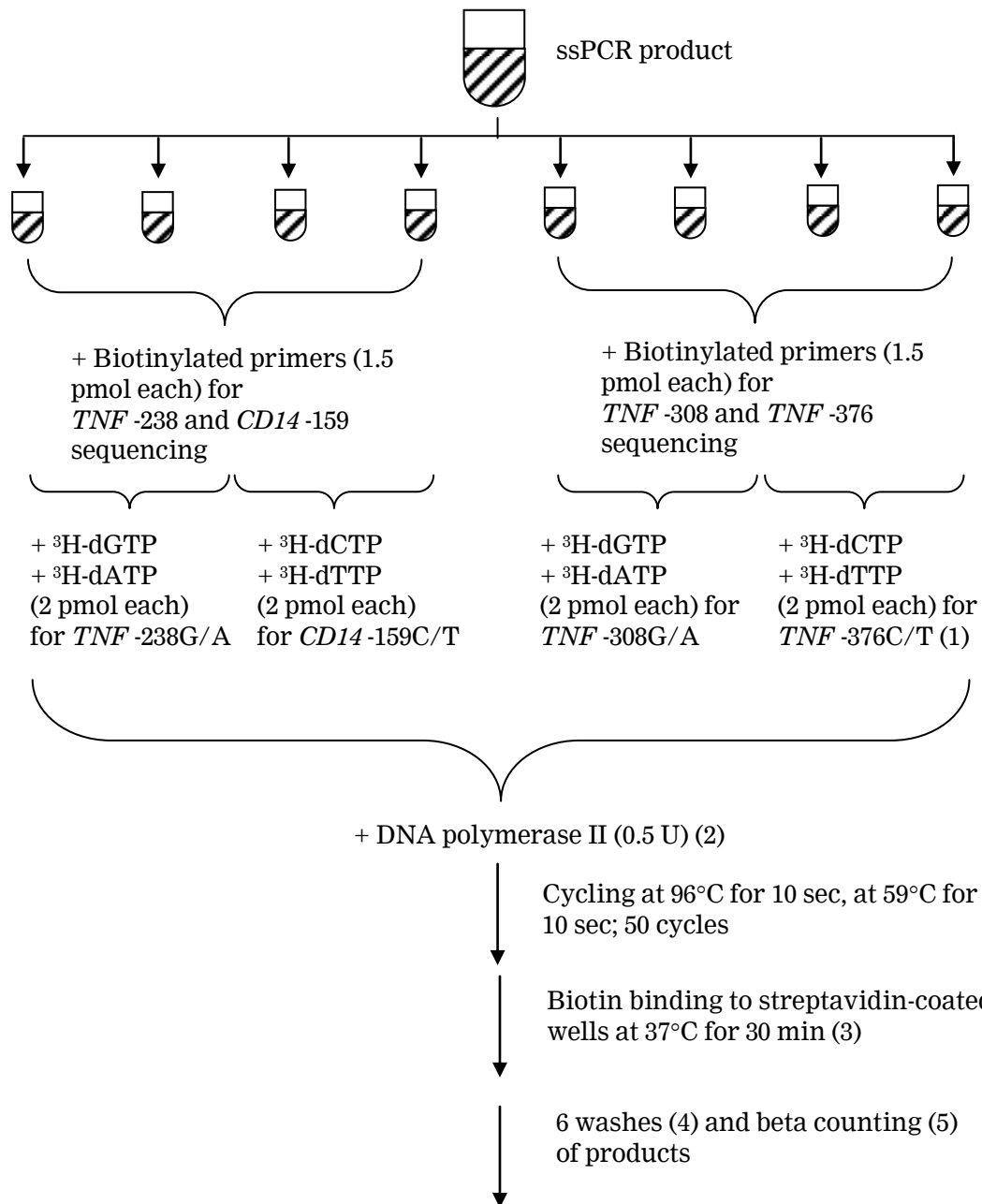
Nucleic acid analyses

CD14 and TNF promoter SNPs (Studies I and III)

DNA was isolated using QIAamp DNA Blood Midi Kit (QIAGEN, Hilden, Germany). Fragments containing the target single nucleotide polymorphisms (SNPs) were amplified by multiplex polymerase chain reaction (PCR). Thereby, 5 μ l of sample DNA (5 ng/ μ l) were added to 20- μ l mixtures on a 96-well PCR plate (Thermo-Fast 96, ABGene, Epsom, Surrey, United Kingdom) that contained 12.5 pmols of each of the multiplex PCR primers (Table 19), dNTPs at 200 μ M (Pharmacia Biotech, Uppsala, Sweden) and 0.5 U of AmpliTaq Gold polymerase (Perkin Elmer, Boston, Massachusetts, USA) in DyNAzyme buffer (Finnzymes, Espoo, Finland). After initial denaturation (at 96°C for 7 min), 35 amplification cycles (at 96°C for 1 min, 60°C for 1 min, and 72°C for 2 min) were run in a thermal cycler (MJ Research DNA Engine 200). Unreacted dNTPs and primers were removed by treating the amplification products with 5 μ l of SAP-EXO mix (1 U of shrimp alkaline phosphatase and 5 U of exonuclease I, both from USB, Cleveland, Ohio, USA) at 37°C for 30 min, after which the enzymes were inactivated at 80°C for 15 min.

The PCR products were diluted 1:1 (v/v) in DyNAzyme buffer (Finnzymes), divided into 8 aliquots, 5 μ l each, on new 96 or 384 PCR plates (Thermo-Fast 96 or 384). The plates had been prealiquoted with reagents for the SNP determination by an automated cycle minisequencing technique. (Figure 8, Table 19)

Figure 8. Cycle minisequencing procedure.



The minisequencing result of the ratio R of counts per minute from wild type allele wells/polymorphic allele wells:

R < 0.1 = homozygous for the polymorphic allele

0.5 < R < 2.5 = heterozygous for the wild type/polymorphic allele

R > 10 = homozygous for the wild type allele

(1) ³H-labelled nucleotides, specific activity 69 Ci/mmol for dATP, 53 Ci/mmol for dCTP, 32 Ci/mmol for dGTP and 96 Ci/mmol for dTTP (Amersham Life Science, Little Chalfont, Buckinghamshire, UK); (2) DNA polymerase II (Finnzymes); (3) streptavidin-coated ScintiStrips (Wallac, Turku, Finland) containing 0.1% Tween 20 in PBS, 50 mM NaOH for 5 min; (4) MiniTent buffer (Helsinki University Central Hospital, Helsinki, Finland); (5) beta counter (Microbeta, Wallac).

Table 19. Primer and probe sequences.

Multiplex PCR primersFor fragment containing *CD14* -159 (Studies I and III)

5'-CCT GGA AAT ATT GCA ATG AAG GAT G-3'

5'-CCA GGA GAC ACA GAA CCC TAG ATG C-3'

For fragment containing *TNF* -238, -308, and -376 (Studies I and III)

5'-CCT CAA GGA CTC AGC TTT CTG AAG-3'

5'-AAG TTG GGG ACA CAC AAG CAT C-3'

For fragment containing *TLR4* +896 (Study IV)

5'-TGA CCA TTG AAG AAT TCC GAT TAG CA-3'

5'-ACA CTC ACC AGG GAA AAT GAA GAA-3'

Cycle minisequencing primers (Studies I and III)For *CD14* -159 determination

5'-Biotin-GCA GAA TCC TTC CTG TTA CGG-3'

For *TNF* -238 determination

5'-Biotin-AGA AGA CCC CCC TCG GAA TC-3'

For *TNF* -308 determination

5'-Biotin-CAA TAG GTT TTG AGG GGC ATG-3'

For *TNF* -376 determination

5'-Biotin-GGT CTG TGG TCT TCC TTC TAA-3'

Allele-specific hybridisation (Study IV)Probes for *TLR4* +896 determination

FAM-5'-CCT CGA TGG TAT TAT T-3'

VIC-5'-TAC CTC GAT GAT ATT ATT-3'

RT-PCR primers (Study V)

For VEGF mRNA determination

5'-AAGCCATCCTGTGTGCCCTGATG-3'

5'-TCCTTCCTCCT-GCCCGGCTCAC-3'

TLR4 +896 SNP

DNA was isolated using QIAamp DNA Blood Mini Kit (QIAGEN). The A/G polymorphism at +896 of the *TLR4* gene was analysed by an allele-specific hybridisation method (TaqMan). Amplification was performed in an ABI Prism Sequence Detector 7000 (Applied Biosystems, Foster City, California, USA) using 100-well plates. The final volume was 5 µl containing 0.8 µl of DNA solution at a concentration of 5 ng/µl, 0.1 µl of each primer (100 pmol/µl, Table

19), 0.1 μl of each probe (100 pmol/ μl) (Table 19), 2.5 μl of TaqMan Universal PCR Master Mix (Perkin-Elmer), and 1.3 μl of distilled water. The PCR conditions were: initial holding at 50°C for 2 min, denaturation at 95°C for 10 min, and 40 cycles of denaturation (95°C for 15 s), annealing, and extension in one step (60°C for 60 s). Each assay included controls for the wild type and mutations.

Northern blotting (Study V)

The RNA was extracted from isolated PBMNCs using RNeasy RNA extraction kit (QIAGEN), denaturated in loading dye (Ambion, Austin, Texas, USA), size-fractionated on a 1% agarose gel by electrophoresis, blotted onto a nylon membrane and fixed by UV cross-linking. The VEGF probe, a 549-bp fragment of cloned human VEGF121 cDNA, was labeled using BrightStar Psoralen-Biotin kit (Ambion). The RNA membranes were hybridised with 0.3 ng/ml of the probe in ULTRAhyb solution (Ambion) at 60°C overnight. The probe was detected using BrightStar BioDetect kit (Ambion), the membranes were exposed to autoradiographic films (Hyperfilm ECL, Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) and the signals were quantified densitometrically by NIH Image software. To normalise the VEGF mRNA levels, the blots were hybridised with a 1.1-kb human glyceraldehyde-3-phosphate mRNA probe (Clontech, Palo Alto, California, USA).

Isoform-specific RT-PCR (Study V)

To reveal the expression of mRNAs encoding the four VEGF isoforms, primers specific for exons 3 and 8 were used (Table 19). The 50- μl RT-PCR reaction contained 1.0 μg of total RNA, the VEGF-specific primer pair, 40 U of human RNase inhibitor (Ambion) and components of One-Step RT-PCR kit (QIAGEN).

After a 30-min reverse transcription reaction at 50°C and initial denaturation (at 95°C for 15 min), the amplification cycle of denaturation (at 94°C for 1 min), annealing (lowering the temperature gradually from 72°C to 67°C during the first five cycles) and extension (at 72°C for 1 min) was run 35

times. The PCR products were electrophoresed on a 2% agarose gel, stained with 0.5% ethidium bromide and visualised under UV light. A 100-bp DNA ladder (New England BioLabs, Beverly, Massachusetts, USA) was used as a molecular weight marker.

Immunoassays

TNF (Study I)

The cell culture supernatants were thawed and diluted 1:5 in TNF Sample Diluent (Diagnostic Products, Los Angeles, CA, USA). The TNF levels were measured by Immulite, a chemiluminescent immunoassay analyser (Diagnostic Products). The detection limit was 4 pg/ml. The intra-assay variation was 9% and the interassay variation 12%. The concentrations were corrected for dilution and background.

VEGF (Study V)

The serum-free culture medium was collected and centrifuged (250 g) to remove debris. The concentrations of VEGF polypeptides were determined using Quantikine Human VEGF Immunoassay, a quantitative sandwich enzyme technique (R&D Systems, Minneapolis, Minnesota, USA).

Immunostaining (Studies I and II)

Two 25- μ l aliquots of both heparinised blood and cell pellet suspended in AB serum were placed in polystyrene tubes (No 2058, Becton Dickinson) and stained with pretitrated amounts of FITC-anti-CD11b (mouse mAb IgG1, clone BEAR 1 from Immunotech, Marseille, France) and either RPE-anti-CD14 (mAb IgG2a, clone Tük4 from DAKO A/S, Glostrup, Denmark) and PC5-anti-CD16 (mAb IgG1, clone 3G8 from Immunotech) or irrelevant mouse mAbs PC5-IgG1 (Clone 679.1Mc7 from Immunotech) and RPE-IgG2a (clone DAK-GO5 from DAKO A/S).

After staining for 20 min in dark at 0°C, erythrocytes were lysed by 1:10 diluted ice-cold FACS lysing solution (Becton Dickinson, San Jose, CA, USA), cells were separated by centrifugation (1400 rpm), the supernatant was

removed and the cells were resuspended in 2 ml of FACS lysing solution at room temperature. The leukocytes were pelleted as before, resuspended in 1% formalin at 0°C and kept in dark on ice. Flow cytometric data acquisition was performed within 4 hours.

Flow cytometry (Studies I and II)

A FACSort flow cytometer (Becton Dickinson) and CellQuest software were used for data acquisition. QuantiBRITE PE bead standards (Becton Dickinson) were run weekly. The data were analysed using QuantiCalc software (Verity Software House, Topsham, ME, USA). First, CD11b-positive events (monocytes, neutrophils, some lymphocyte populations) were delineated in side scatter/CD11b (FITC intensity) dot plot by the region R1 (Figure 9A). Second, side scatter/forward scatter dot plot was created from R1, and R2 was set to delineate the monocyte population for Study I (Figure 9B). At least 1×10^3 monocytes i.e. events co-locating in R1 and R2 were collected.

For Study II, neutrophil CD11b levels, expressed as relative fluorescence units (RFUs), were obtained by plotting CD11b against CD16 (PC5 intensity) of the collected events. Monocyte contamination was excluded by electronic gating based on the high CD16 expression on neutrophils compared to that of monocytes. According to the manufacturer, the number of PE-linked anti-CD14 molecules equals to each cell's CD14 antibody-binding capacity (ABC). ABC medians for monocytes (Study I) and neutrophils (Study II) were determined by creating a fluorescence intensity plot CD14 (PE) vs. CD16 (PC5) (Figure 9C). CD16 levels were given as RFUs (Study II).

DATA ANALYSIS

Study I

The differences in TNF levels obtained in different culture conditions were compared using Wilcoxon signed ranks test. Differences between the three subject groups were evaluated by Kruskal-Wallis test, and in *post hoc* analysis by Mann-Whitney U test. When testing for linear trend in the subject

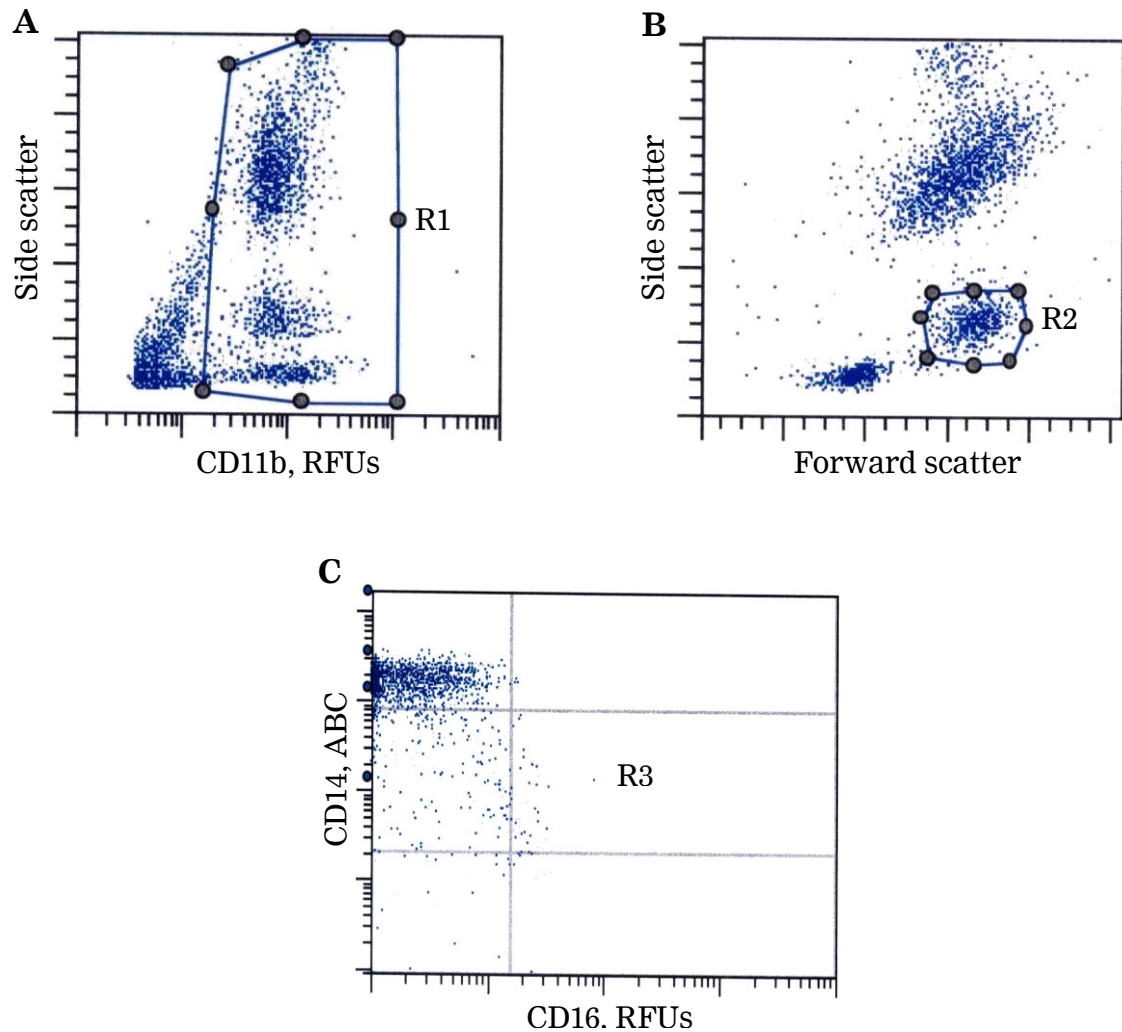


Figure 9. Analysis of CD14 and CD16 surface markers on CD11b positive monocytes. Peripheral blood leukocytes were triple-labelled with fluorescent mAbs. A, CD11b positive cells were delineated by R1; B, monocytes were delineated by R2; C, dot plot was developed from monocytes (cells co-locating in R1 and R2), the median CD14 expression and the proportion of CD14+CD16+ monocytes (R3) were calculated. ABC, antibody-binding capacity; RFU, relative fluorescence unit.

groups having 0, 1 or 2 risk factors, Jonckheere-Terpstra test was used. The *CD14*-159 minor allele prevalences were calculated using Fisher's exact test.

Study II

The differences in CD11b expression in different culture conditions were compared by Friedman test, or in case of two different conditions only, by Wilcoxon signed ranks test. The three subject groups were compared using Jonckheere-Terpstra test.

Study III

Differences in *TNF* -308G/A and *CD14* -159C/T allele frequencies between the patient groups were calculated by χ -square test.

Study IV

The treatment response in the *TLR4* +896AA and AG/GG groups, presented by DAS28 values, was evaluated using generalising estimating equation models with exchangeable correlation structure. DAS28 remission was estimated by multivariate logistic regression with bootstrap estimate of variance.

Study V

Wilcoxon signed ranks test was used when comparing the secretion of VEGF in different culture conditions.

RESULTS AND DISCUSSION

INFLAMMATORY PHENOTYPE

PHAGOCYTE RESPONSIVENESS

TNF secretion by whole blood monocytes of subjects with previous ReA (Study I)

In adherent cultures, whole blood monocytes from HLA-B27 positive healthy subjects with previous *Yersinia* arthritis (B27+ReA+) released more TNF in response to non-specific stimulation of the protein kinase C pathway (stimulated by PMA and Ca²⁺ ionophore) than those of HLA-B27 positive reference subjects (B27+ReA-) did. The difference was significant both at 2 and 4 hours of incubation (Study I: Figure 2A and B) and not evident in non-adherent cultures. Further, when the PMA- and Ca²⁺ ionophore -stimulated adherent and non-adherent conditions were compared, the 2-hour TNF levels were significantly higher in the former in all subject groups (B27+ReA+, B27+ReA- and B27-ReA-), but the difference was significantly higher in B27+ReA+ group than in the reference groups. On the other hand, in non-adherent cultures the induction of TNF production was even slower (i.e. the kinetic ratio TNF at 4 hours / TNF at 2 hours was higher) in the B27+ReA+ samples than in those of the reference subjects (with the absolute TNF levels being comparable between the groups) (Study I: Figure 2C). Hence, the elevated TNF levels in the B27+ReA+ group appeared to be associated with adherence.

These results may indicate that monocytes that have the capacity to produce high amounts of TNF in response to non-specific stimulation on adhering to the synovial vessels could promote acute joint inflammation. It is noteworthy that low TNF secretion by blood mononuclear cells in response to phytohaemagglutinin has been reported to correlate with chronicity of arthritis (Westendorp *et al.* 1995). Hence, it is possible that a vigorous rapid TNF response of adhered phagocytes provokes clinical joint inflammation and, on the other hand, helps to overcome the disease more quickly. Also,

considering that circulating monocytes continuously adhere to the endothelium and extravasate to become tissue macrophages, increased response to non-specific stimuli thereby may contribute to the initiation of inflammation. Consistent with the significance of adhesion in our results, the proportion of circulating CD14⁺CD16⁺ monocytes, which have high capacity of TNF production, was not elevated in the samples of the B27⁺ReA⁺ subjects.

The molecular basis of the observations may include some aberration(s) in adhesion-associated intracellular signalling. Interestingly, signalling through the cell-surface integrin CD11b/CD18, the main mediator of monocyte adhesion to the endothelium and cell culture well surfaces (Wallis *et al.* 1985, Springer and Anderson 1986), is known to enhance TNF production (Dackiw *et al.* 1996) and monocyte survival (Nakamura *et al.* 1998).

TNF secretion by whole blood monocytes of healthy HLA-B27 negative reference subjects (Study I)

When the basic guidelines of TNF production kinetics were evaluated by excluding the effect of previous ReA and the SpA susceptibility gene HLA-B27, significant differences between adherent and non-adherent conditions appeared on LPS stimulation (but not on PMA- and Ca²⁺ ionophore-stimulation) (Study I: Figure 1). The TNF levels of the adherent samples of the HLA-B27 negative group remained stable after two hours of culture (Study I: Figure 1A), whereas in non-adherent conditions they increased thereafter (Study I: Figure 1B). This may suggest that in the presence of bacterial LPS, adhesion-mediated intracellular signalling for TNF production turns repressive as the response advances. If exerted *in vivo*, the results could mean that the more restrained TNF production of adhered phagocytes may be biologically meaningful and aimed at protecting endothelial cells from undue exposure to TNF.

CD11b expression by whole blood neutrophils of subjects with previous ReA (Study II)

When stimulated by LPS (as 100 pg/ml, 10 ng/ml or 1 µg/ml) in non-adherent conditions, or without LPS supplement in adherent conditions,

neutrophils of the B27+ReA+ subjects showed significantly higher surface expression of the activation marker CD11b than those of the B27-ReA- subjects did (Study II: Figure 1). *In vivo*, the sources of LPS are ubiquitous, and LPS can occur in healthy subjects as high as 1 ng/ml in the portal venous blood. Minor trauma in the intestine, for example, may allow the leakage of LPS from the gut into the circulation. Bacterial antigens including LPS may also persist in the peripheral blood cells of *Yersinia* patients who develop ReA (Granfors *et al.* 1998).

The differences in CD11b expression might derive from aberrations in LPS signalling pathways, e.g. functional *TLR4* polymorphisms, since TLR4-mediated signalling leads to up-regulation of CD11b on neutrophils (Zhou *et al.* 2005), or at the level of CD11b/CD18. Also, because LPS-stimulated TNF production did not differ between the subject groups, signalling triggered by TNF is not likely to explain the differences in CD11b levels. Priming, defined by enhanced oxygen radical production, has been detected in neutrophils of subjects with previous *Yersinia* arthritis (Ristola 1990), and may also be associated with the elevated CD11b levels. Constitutive CD11b expression, the levels of CD14 and CD16, or CRP levels did not differ between the subject groups, excluding the possibility that an ongoing cryptic infection was the reason for our finding.

The results suggest that neutrophils of the B27+ReA+ subjects can be very responsive when encountering circulating LPS or an adherent surface, which could be associated with the development of ReA. The response may be highly meaningful because neutrophils are abundant, comprising 70% of peripheral blood leukocytes in adults.

Altogether, the molecular mechanisms of the observed adhesion-associated phenomena in TNF production and CD11b expression are subject to our further studies.

ANGIOGENESIS AND TISSUE EDEMA

VEGF production by PBMNCs of healthy subjects (Study V)

VEGF acts as an angiogenesis-promoting mitogen for endothelial cells and strongly increases vascular permeability. It has a dual role both in healing of tissue damage and pathophysiology, both of which include angiogenesis. Our results showed that LPS as 20 pg/ml-2 ng/ml, which cover physiological LPS levels, increase *de novo* VEGF production of PBMNCs in a dose-dependent manner (Study V: Figure 2). The expression pattern of the four VEGF mRNA sizes of the stimulated cells was comparable to that of non-stimulated cells. IFN- α , a cytokine with immunoregulatory capabilities, inhibited both non-stimulated (Study V: Figure 4) and LPS-stimulated (Study V: Figure 5) VEGF production of PBMNCs.

Tissue edema attributed to increased vascular permeability is associated to local and systemic inflammation and various malignancies. Also, angiogenesis involving VEGF occurs early in the chronic stage of rheumatoid arthritis as the synovial hyperplasia leads to pannus formation. The level of joint damage correlates with the level of serum VEGF (Paleolog 2002). Our results suggest that the release of VEGF from circulating and tissue-migrating LPS-activated PBMNCs is a mechanism that could promote edema and progression to the chronic stage of RA. As PBMNCs are long-living cells, they may provide a long-lasting source of VEGF.

IFN- α is likely to modulate and oppose the effects of early pro-inflammatory cytokines. It may reduce the TNF response to LPS thus preventing damage caused by innate immunity, and systemic consequences (Amadori 2007). In the joints, the protective effect of IFN- α may be partly implemented by up-regulating functional TNF and IL-1 antagonists (sTNFR and IL-1RA) (Wong et al. 2003). Possibly, our finding that IFN- α inhibits non-stimulated and LPS-induced VEGF synthesis may represent another means of preventing (joint) damage that is attributed to innate responses. It may partly explain the clinical efficacy of IFN- α , e.g. decrease of joint inflammation in RA (Shiozawa *et al.* 1992) and regression of cancer (Lindner *et al.* 1997).

Despite of the accumulating data of the predominantly anti-inflammatory effects of IFN- α , this cytokine has also been reported to induce arthritic manifestations (Ioannou and Isenberg 2000). It is becoming clear that the effects of IFN- α are dependent of its concentration, target tissue, way of administration and the progression of the disease (Brassard *et al.* 2003, Amadori 2007). Hence, the dependence must be studied in detail in order to evaluate the overall clinical usefulness of IFN- α in the treatment of inflammatory conditions such as rheumatoid arthritis (Wong *et al.* 2003).

GENETIC FACTORS

CD14-159C/T, TNF-308G/A AND PATIENTS WITH ARTHRITIS

TNF production (Study I)

The LPS-stimulated TNF production of whole blood monocytes was *CD14-159T* allele dose-dependent (Study I: Figure 3), which is in agreement with a previous study (Eng *et al.* 2004). The frequency of the T allele in our study was 40%, which is also consistent with other studies, e.g. those involving ethnic groups in India (Sharma *et al.* 2004) and Germany (de la Fontaine *et al.* 2005).

In the past when antimicrobial therapies were not available, carriage of alleles that promote high TNF production on encountering pathogens may have provided the host with a significant selective advantage. Since then, the environment and hygiene conditions have changed especially in the developed countries, giving different kind of stimulation to the immune system (Aguillón *et al.* 2006). The alleles associated with high TNF production are possibly advantageous in some of the new challenges. Allergies, for example, may represent diseases against which the *CD14 -159T* allele is protective (Sharma *et al.* 2004).

Susceptibility

Acute disease (Studies I and III)

The frequency of the minor alleles *CD14* -159T (Studies I and III) and *TNF* -308A (Study III: Table II) did not differ between the acute arthritis and reference groups, suggesting that these SNPs do not affect the susceptibility of acute arthritis. Regarding our result of the PMA- and Ca²⁺ ionophore-induced TNF production capacity of the B27+ReA+ monocytes and the fact that CD14 belongs to the LPS receptor complex, the conclusion may be that the monocyte response to non-specific stimuli and adherence is more potently associated with acute arthritis than the *CD14* -159 polymorphism-associated LPS-induced increase in TNF production is.

Chronic disease (Study III)

All female patients who developed chronic SpA during the follow-up time had the *CD14* -159T allele and none of them had the *TNF* -308A allele, a phenomenon not seen in male patients (Study III: Table II). The finding is in concordance with studies suggesting that female sex hormones as well as the absence of *TNF* -308A may depress effective responses in inflammation: TNF production may be decreased by estrogen (Srivastava *et al.* 1999) and IL-1 production by estradiol and progesteron (Morishita *et al.* 1999), whereas *TNF* -308A has been associated with high TNF production (Wilson *et al.* 1997, Louis *et al.* 1998, Abraham and Kroeger 1999) and protection of AS (McGarry *et al.* 1999).

The molecular background of *CD14* -159T involvement seems more confusing, as this allele has been reported to increase CD14 expression (Le Van *et al.* 2001) and, in this study, to promote TNF production. It is possible that increased shedding of mCD14 occurs on prolonged LPS stimulation to prevent excessive monocyte activation, since sCD14 facilitates LPS clearance (Miyake 2004). Indeed, -159TT homozygotes have been described to have increased sCD14 levels (Baldini *et al.* 1999). It may also be that besides TNF production, other responses originating at the LPS receptor complex, e.g. IFN-

β induction (Pålsson-McDermott and O'Neill 2004), may dictate the development of the chronic disease. Finally, -159T has been associated with increased risk of chronic inflammatory bowel disease, i.e. Crohn's disease (Klein *et al.* 2002) and ulcerative colitis (Obana *et al.* 2002), raising the possibility that LPS from the gut may also be involved in the pathogenesis of chronic SpA in patients with the T allele.

Owing to the low number of the females who developed chronic SpA (n=6), the finding must only be taken as very preliminary. However, if confirmed in larger studies, *CD14* -159T and concomitant absence of *TNF* -308A may represent a susceptibility profile of developing chronic SpA in females.

EFFECT OF *HLA-B27* (STUDIES I AND II)

HLA-B27 is a well-known risk allele in spondyloarthropathies, although its role in the pathogenesis has not been explained yet. In Study I, TNF production of adhesion- and LPS-stimulated whole blood samples of the B27+ReA+ group was higher than those of the B27-ReA- but not the B27+ReA- group. However, the TNF production capacity studied by the strong non-specific stimulation (as described above), was associated with previous ReA but not with the B27 genotype. This suggests that enhanced LPS receptor-mediated response and B27 positivity are independent but additive factors that may operate in the pathogenesis of ReA. This idea is further supported by the results from Study II, in which the neutrophil CD11b levels exhibited a trend: B27+ReA+ > B27+ReA- > B27-ReA- (Figure 1 in Study II). Recent findings suggest that B27 polypeptides are prone to misfold and subsequently accumulate in the endoplasmic reticulum (ER), thus launching ER stress responses, which in turn can alter cellular homeostasis (Penttinen *et al.* 2004). Thereby, the proteins that receive essential information, like CD11b/CD18, may be regulated differently in terms of their synthesis (Zhou *et al.* 2005) or mobilisation to the cell surface in vesicles or granules (Sengelov *et al.* 1993).

***TLR4* +896A/G AND PATIENTS WITH EARLY RA**

Treatment response (Study IV)

It is often difficult to predict if an individual RA patient will respond successfully to the chosen treatment strategy. Hence, discovering biochemical and genetic markers that significantly affect the treatment response would be highly useful. It is known that the maintenance of optimal working capacity can be predicted when a satisfying treatment response is achieved during the first 6 months of treatment (Puolakka *et al.* 2005). In our study, of the patients with early RA who had been treated with a single DMARD (started with sulphasalazine) for 6 months, patients having the *TLR4* minor allele +896G achieved remission less often (Study IV: Figure 1) and required more intra-articular corticosteroid injections than patients homozygous for the wild-type A allele did. Among patients treated with a combination of DMARDs (sulphasalazine, methotrexate and hydroxychloroquine) and prednisolone, there was no difference in the treatment response between the genotypes studied. Thus, *TLR4* +896A/G (causing the amino acid substitution Asp299Gly in TLR4) may be a good candidate in distinguishing the patients who would get advantage from the combination treatment of RA.

Microbes as well as autoantigens have been suggested to be involved in the pathogenesis of RA (Arend 2001, Oliver and Silman 2006). As the Asp299Gly change reduces negative charge and possibly disrupts the secondary structure of the extracellular domain of TLR4 (Arbour *et al.* 2000), our present findings may be explained by altered interaction of TLR4 with LPS, endogenous ligands (such as those released from injured joints) or the other components of the LPS receptor complex. This could be an aberration that a single DMARD is not able to overcome. It is also possible that the minor allele disrupts the transport of TLR4 to the cell surface. Of interest, *TLR4* substitutions have been associated with better resistance to localised LPS-induced inflammation, but also with greater susceptibility to systemic responses (Arbour *et al.* 2000), such as those occurring in RA.

CONCLUSIONS

Elevated TNF production by monocytes in response to adherent conditions or non-specific stimulation (Study I), and increased CD11b expression on neutrophils in response to adherent conditions or LPS (Study II) may contribute to the inflammatory pathogenesis of reactive arthritis (Figure 10).

CD14 -159T and *TNF* -308A (Study III) do not affect the susceptibility of acute arthritis. -159T increases LPS-stimulated TNF production in an allele dose -dependent manner (Study I). The concomitant presence of -159T and absence of -308A may predispose to the development of chronic spondyloarthropathy in females (Study III).

TLR4 +896G can impair treatment response to a single DMARD in recent-onset rheumatoid arthritis, suggesting that individuals with this allele should receive early combination DMARD therapy (Study IV).

LPS induces VEGF production by peripheral blood mononuclear cells, which could be one mechanism causing edema and angiogenesis in rheumatoid joints. The ability of IFN- α to oppose this effect may contribute to the anti-inflammatory potency of IFN- α (Study V).

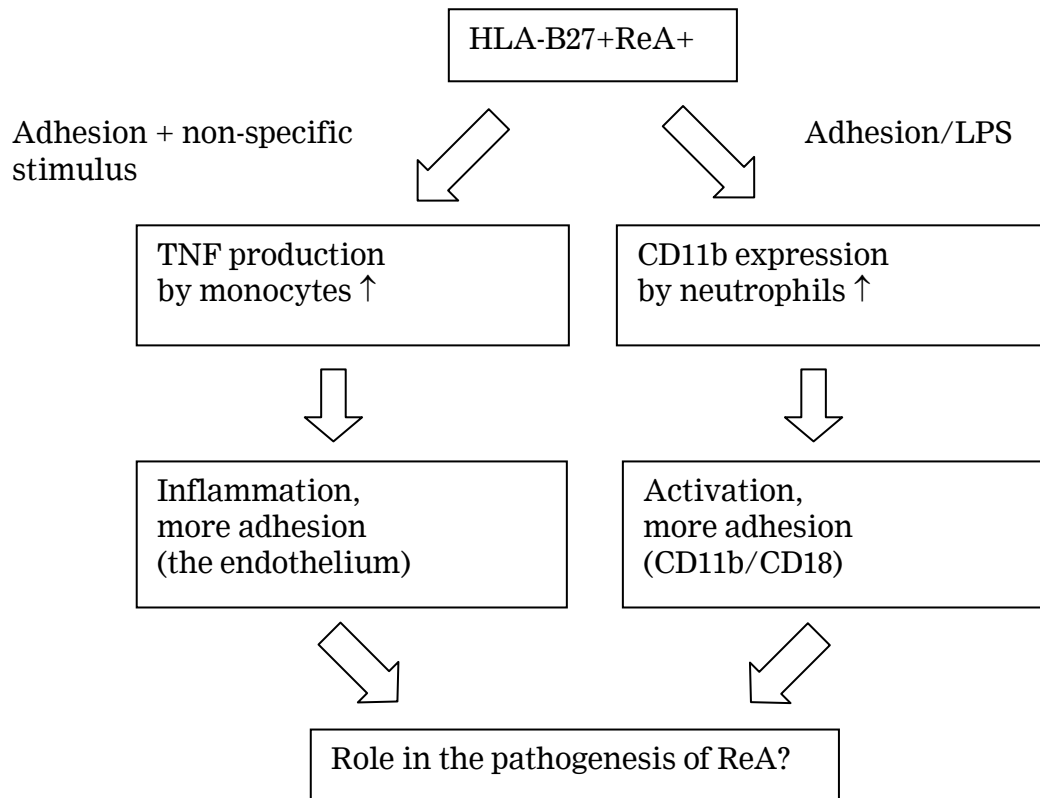


Figure 10. Schematic presentation of how innate immune factors could be associated with reactive arthritis (ReA). In the present study, HLA-B27 positive subjects with previous ReA showed high TNF production by blood monocytes in response to adherent conditions and non-specific stimulation, and high expression of the activation marker CD11b on blood neutrophils in response to adhesion or LPS. TNF is a crucial pro-inflammatory cytokine that e.g. increases the expression of adhesion molecules on the endothelium, thus promoting the recruitment of circulating leukocytes to inflammatory foci. CD11b is part of the adhesion molecule CD11b/CD18 on the phagocyte surface.

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