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Proinflammatory activation of human macrophages by the serine protease plasmin
Characterization of signaling pathways

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Abbreviations

2ME	2-mercaptoethanol
A2Ct	Annexin A2 C-terminus
A2NP	Annexin A2 N-terminus peptide
A2Nt	Annexin A2 N-terminus
AA2t	Annexin A2 heterotetramer
AB	Acrylamide bisacrylamide
AG490	Alpha-cyano- (3,4-dihydroxy) -N-benzylcinnamide
AKβBA	Acetyl-11-keto-β-boswellic acid
AP-1	Activating protein-1 (transcription factor)
APS	Ammonium persulfate
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CIS-1	Cytokine-inducible SH_2 containing protein
CLC	Cardiotrophin-like cytokine
CNTF	Ciliary neurotrophic factor
CT-1	Cardiotrophin-1
CTA	Committee on thrombolytic agents
DC	Dendritic cells
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
DNTP	Deoxynucleoside triphosphates
DTT	Dithiothreitol
EB	Elution buffer
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FACS	Fluorescence activated cell scan/sorter
FCS	Foetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GB	Gel buffer

GPL	Gp130-like receptor
HEPES	4- (2-hydroxyethyl) piperazine-1-ethanesulfonic acid
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule-1
IEC	Intestinal epithelial cells
IFNγ	Interferon-γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl- β -D-thiogalactoside
JAK	Janus kinases
LB-medium	Luria-bretani medium
LDL	Low-density lipoproteins
LIF	Leukaemia inhibitor Factor
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCP-1	Monocytes chemoattractant protein-1
M-CSF	Macrophage-colony stimulating factor
MHC	Major histocompatibility complex
MIP-3α	Macrophage inflammatory protein-3α
MMLV-RT	Moloney murine leukaemia virus reverse transcriptase
MMP	Matrix metalloproteinases
MPS	Mononuclear phagocyte system
NES	Nuclear export signal
NF-κB	Nuclear factor κ-B
ODN	Oligodeoxynucleotides
Oligo (dT)	Oligo-deoxythymidine
OSM	Oncostatin M
PAb	Polyclonal antibody
PAI	Plasminogen activator inhibitors
PAMPS	Pathogen associated molecular patterns
PAR1	Protease activated receptor 1
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent protein kinase 1

PE	Phycoerythrin
PEG	Polyethylene glycol
Pi3K	Phosphoinositide-3-kinase
PIAS	Protein inhibitor of activated STAT
PKC	Protein kinase C
PMSF	Phenylmethanesulfonyl fluoride
qPCR	Quantitative real time PCR
RNase	Ribonuclease
RT-PCR	Reverse transcription PCR
SB203580	4- (4-fluorophenyl) -2- (4-methylsulfinylphenyl) -5- (4-pyridyl) 1H - imidazole
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SHP-2	Src homology 2 (SH2) domain-containing protein tyrosine phosphatases
SLB	Sample lysis buffer
SOCS	Suppressors of cytokine signaling
STAT	Signal Transducer and Activator of Transcription
TBE	Tris-borate-EDTA buffer
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
TEMED	N, N, N', N'-tetramethylethylenediamine
TF	Tissue factor
TGF-ß	Transforming growth factor-ß,
Th	T helper
TNF-α	Tumor necrosis factor-α
tPA	tissue type plasminogen activators
Tricine	N-tris (hydroxymethyl) methyl glycine
TSR	Template suppression reagent
Tween 20	Polyoxyethylene-20-sorbitan monolaurate
TYK2	Tyrosine kinase 2
U0126	1,4-Diamino-2, 3-dicyano-1, 4-bis (2-aminophenylthio) butadiene
uPA	urinary type plasminogen activators
VCAM	Vascular cell adhesion molecule-1
VPLCK	D-Val-Phe-Lys chloromethyl ketone
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

1. Introduction

1.1. Immune cells

The immune system is the body's defense against infectious organisms and other invaders. Through a series of steps called the immune response**,** the immune system attacks organisms and substances that invade our systems and cause diseases. Disorders of the immune system can be broken down into four main categories: immunodeficiency disorders, autoimmune disorders, allergic disorders and cancers of the immune system. The immune system is made up of a network of cells, tissues, and organs that work together to protect the body. The cells that are part of this defense system are white blood cells, or leukocytes.

Mononuclear phagocytes and dendritic cells (DCs) function as accessory cells in the induction of immune responses. Mononuclear phagocyte system includes bone marrow progenitors, circulating monocytes, tissue macrophages and DC (Sasmono et al. 2003). The differentiation of immune cells is shown in **Figure 1**.

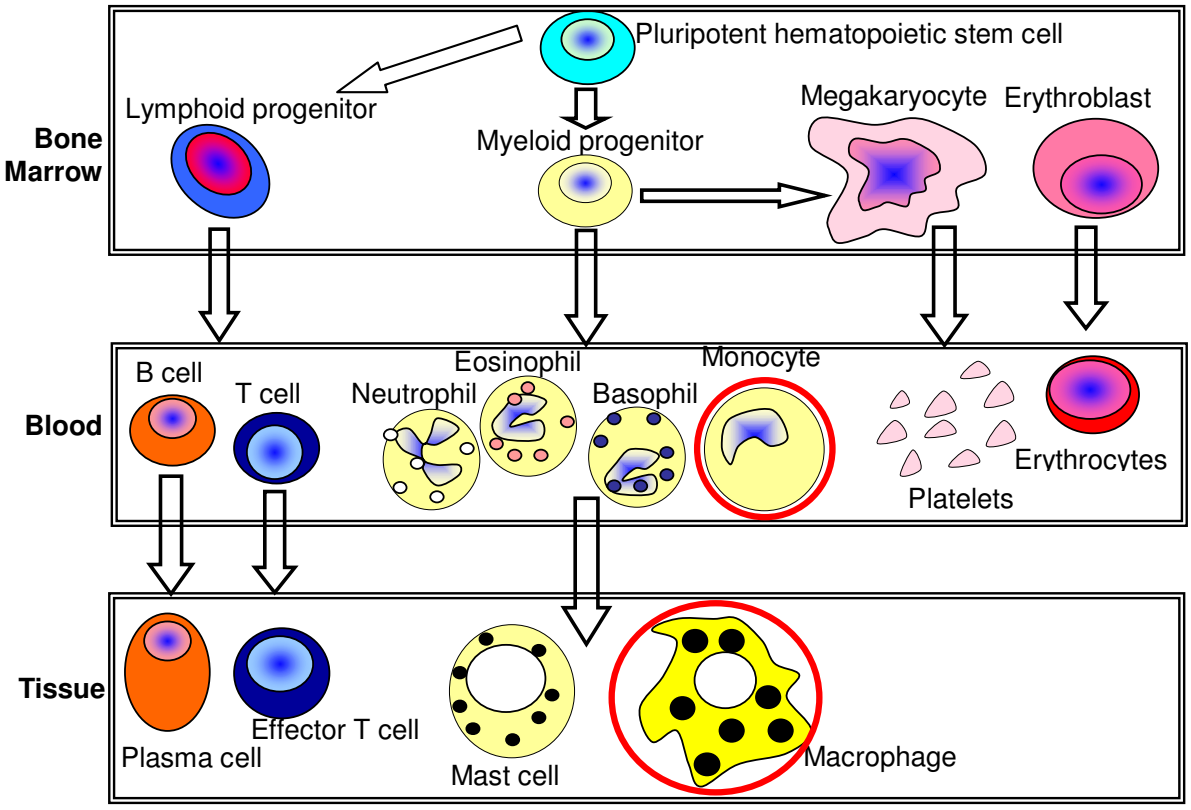


Figure 1. The immune cells are differentiated along 2 pathways: myeloid and lymphoid (Provided by John Schmitz, FDA, www.fda.gov/ohrms/dockets/ac/00/slides/3652s1_02.ppt).

1.1.1. Macrophages and monocytes

Mononuclear phagocytes include monocytes and macrophages. Blood monocytes, 5% of leukocytes, are immature macrophages, circulate in the blood for a few hours, then crawl into tissues, enlarge and differentiate into macrophages or DCs. Macrophages are key players in the immune response that help to destroy bacteria, protozoa, tumor cells and release substances that stimulate other cells of immune system, and they are involved in antigen presentation. Up to date, numerous studies have been performed to understand the role of monocytes and macrophages in the regulation of immune responses (Langermans et al. 1994).

1.1.2. Macrophages and monocytes in the immune response

Macrophages are heterogeneous and versatile bone - marrow derived cells that produce a wide range of mediators and exert a multitude of biological functions. Monocytes/macrophages play a central role in pathogenic inflammatory responses associated with atherosclerosis, restenosis (Ito and Ikeda 2003; Hansson 2005), tumor surveillance (Sunderkotter et al. 1994) and arthritis (Gelderman et al. 2007). Monocytes and macrophages are phagocytes, acting in both non-specific defense as well as specific defense of vertebrate animals. Their role is to phagocytize cellular debris and pathogens either as stationary or mobile cells, and to stimulate lymphocytes and other immune cells to respond to pathogens.

Activated macrophages are major producers of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (Salkowski et al. 1995; Wang et al. 2006; Marble et al. 2007), and play a crucial role in modulating immune responses (Bilyk and Holt 1993; Thepen et al. 1994; Herbein et al. 1995). They can serve as antigen presenting cells, but also directly inhibit antigen presentation by DCs (Bilyk and Holt 1993). T-cell proliferation, phenotype, and ultimately the type of immune responses induced, can distinctly be influenced by macrophages (Thepen et al. 1994; Strickland et al. 1996; Grewe et al. 1998).

Macrophages are specifically polarized by the microenvironment to exert different functional programms. Initial signals from microbes through their pathogen associated molecular patterns (PAMPS) (Schnare et al. 2000) followed by a second signal, such as IFNγ, give rise to “classically activated macrophages”. In early immune responses, IFNγ is produced by NK and NKT-cells, later the main source of IFNγ are antigen-specific Th1-cells (Kethineni et al. 2006). These stimuli generally activate macrophages to produce TNF-α, MCP-1, iNOS, IFNγ and to promote strong IL-12-mediated Th1 responses.

When macrophages are activated in the presence of IL-4, IL-10, TGF-β or glucocorticoids they become “alternatively activated macrophages” being characterized by Dectin-1 expression and supporting Th2-associated immune responses. However, activation of Dectin-1 with the fungal β-glucan triggers a severe autoimmune arthritis in genetically susceptible mice (Yoshitomi et al. 2005). Thus, in inflammatory processes macrophages contribute to both, initiation of inflammation and to its resolution. However, during chronic inflammatory responses, interaction between macrophages and T-cells may lead to a vicious cycle, which by itself is capable of maintaining local inflammation without the necessity of external stimuli (Avice et al. 1998).

1.1.3. The role of macrophages in atherosclerosis

Atherosclerosis is a pathological condition that underlies several important adverse vascular events including coronary artery disease (CAD), stroke, and peripheral arterial disease, responsible for most of the cardiovascular morbidity and mortality in the Western world today. Macrophages are certainly the main source of cytokines in the atherosclerotic plaques (Hansson 2005; Potteaux et al. 2006).

Macrophages express scavenger receptors on their surface, which recognize modified (for instance, oxidized or acetylated) low-density lipoproteins (LDL) (Li et al. 1995). These receptors are involved in lowering the cholesterol levels in the blood, an event that mostly occurs in the liver with the help of Kupffer cells (van Berkel et al. 1991). However, in some cases the uptake of lipid by macrophages may lead to disease. The earliest event in the development of atherosclerosis is the adhesion of circulating monocytes to regions of the lumenal surface of the endothelium of a blood vessel (Lessner et al. 2002). Once macrophages have been recruited, they can secrete chemotactic factors to increase the response (Jessup et al. 2002). Adherent macrophages migrate across the endothelium and reside in the intima. Most of the intimal macrophages accumulate large intracellular deposits of lipid, which locate in cytoplasmic fat droplets, giving the cells a foamy appearance. The increasing amounts of macrophages that accumulate in the lesion around the lipid core eventually develop into atherosclerotic plaques.

1.2. IL-6 family cytokines

1.2.1. IL-6 family cytokines and their receptors

The IL-6-type cytokines IL-6, IL-11, IL-27, LIF (leukaemia inhibitor factor), OSM (oncostatin M), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and

cardiotrophin-like cytokine (CLC) are an important family of mediators involved in the regulation of the acute-phase response to injury and infection. The IL-6-type cytokines bind to plasma membrane receptor complexes containing the common signal transducing receptor chain gp130. These receptors involved in the recognition of the IL-6-type cytokines can be subdivided in the non-signaling α-receptors (IL-6Rα, IL-11Rα, and CNTFRα) and the signaling transducing receptors (gp130, LIFR, and OSMR) (Heinrich et al. 2003).

Since only the complex of a cytokine and its α-receptor efficiently recruits the signaling receptor subunits, IL-6, IL-11 and CNTF first bind specifically to their respective α-receptor subunits. An α-receptor subunit has been also postulated for CT-1 (Robledo et al. 1997), but this putative receptor protein has not been cloned yet, and its existence is questionable. IL-6 and IL-11 are the only IL-6-type cytokines that signal via gp130 homodimers. The remaining IL-6 type cytokines signal via heterodimers of either gp130 and the LIFR (LIF, CNTF, CT-1 and CLC) or gp130 and the OSMR (OSM). Human OSM has the exceptional capability to recruit two different receptor complexes. It forms both, LIFR-gp130 and OSMR-gp130 heterodimers. LIF and OSM directly engage their signaling receptor subunits without requirement for additional α-receptor subunits (**Figure 2**) (Kordula et al. 1998; Senaldi et al. 1999). The latter induces signal transduction involved in the activation of Janus kinases (JAK) family members, leading to the activation of transcription factors of Signal Transducer and Activator of Transcription (STAT) family. Another major signaling pathway for IL-6-type cytokines is the MAPK cascade (Heinrich et al. 2003).

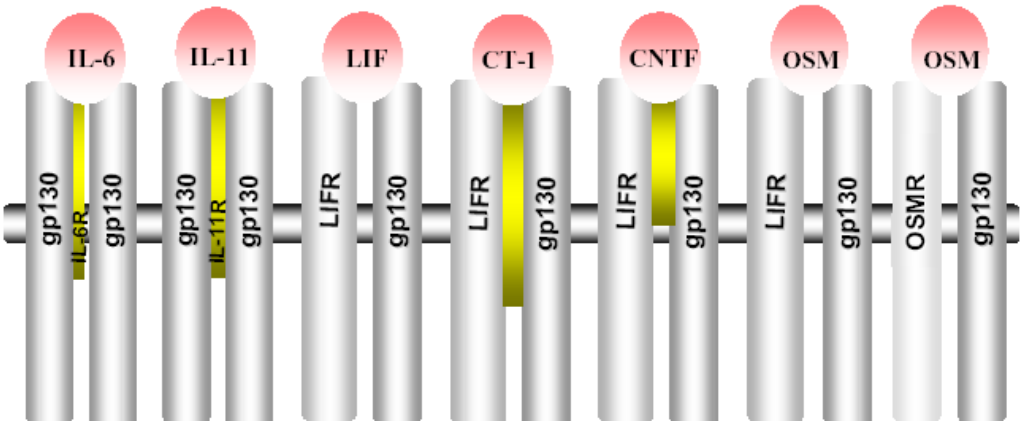


Figure 2. Receptor complexes activated by the IL-6-type cytokines. IL-6-type cytokine receptor complexes signal through the different combinations of the signaling receptor subunits gp130, LIFR and OSMR, with gp130 being used by all the family members.

Recently, a novel cytokine receptor displaying similarities with gp130 signaling receptor has been described. This receptor was designated GPL, or gp130-like receptor, or IL-31Rα, which displays some structural homology with LIFR, OSMR, and gp130 receptors (**Figure 3**) and appears to exist in at least four different isoforms diverging in their C-terminus. Interestingly, analysis of this cytokine receptor in invertebrates indicates that their IL-31Rα closely related to the human IL-31Rα (Diveu et al. 2003). Therefore, it is convenient to study the IL-31Rα function by using invertebrates as a model.

A

gp130	28.46			
LIFR	20.08	22.76		
OSMR	19.68	21.95	36.62	
	IL-31Rα	gp130	LIFR	OSMR

B

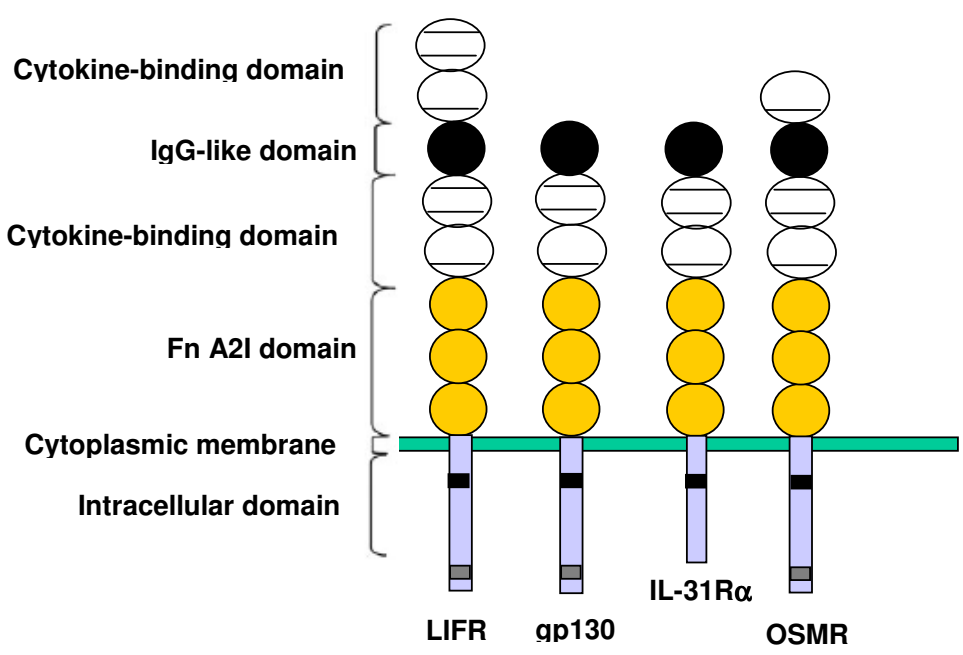


Figure 3. Structures of the IL-6 receptor family. *A*, Identity rates of the receptors activated by the IL-6-type cytokines expressed in percent. Calculations were done with multiple sequence alignment involving the CBD and the three FnA2I domains of each cytokine receptor built with ClustalW. For LIFR, the carboxyl-terminal CBD was used. B, Schematic representation of the receptors activated by the IL-6-type cytokines (Diveu et al. 2003).

IL-6 cytokine receptors play an essential role in blood cell development and function. The role of the most recently identified IL-31Rα is not elucidated yet. High levels of IL-31Rα transcripts were detected in two monocytic human cell lines, THP1 and U937 (Ghilardi et al. 2002). Considerable amounts of IL-31Rα transcripts were also found to be expressed by monocytes and DCs (Diveu et al. 2003). IL-31Rα protein was found to be expressed predominantly on IFNγ-activated human monocytes (Dillon et al. 2004). IL-31Rα expressed on blood cells is a signaling receptor subunit possibly involved in the development and function of monocytes and macrophages (Ghilardi et al. 2002).

IL-31 identified as a ligand for the IL-31Rα receptor, is preferentially produced by T helper type 2 cells (T cells-derived cytokine) and may be involved in promoting skin disorders and in regulating other allergic diseases such as asthma. The cytoplasmic domain of IL-31Rα contains three tyrosine residues (Tyr^{652}, Tyr^{683} and Tyr^{721}) of which the first and the last are believed to recruit STAT1/5 and STAT1/3, respectively (Chattopadhyay et al. 2007) .

To analyze the signaling requirements for the IL-6-type receptors some functional chimera receptor complexes (IL-5R/IL-31Rα, or IL-5R/LIFR, or IL-5R/gp130, or IL-5R/ OSMRß) were constructed (**Figure 4**). The COS-7 cell line, which does not express IL-5 receptor, was used as a recipient cell line to express the chimeric receptors. A clear induction of STAT3 and STAT5 phosphorylation upon addition of IL-5 was detected when the cells expressing the chimera receptor containing intracellular portion of IL-31Rα fused to extracellular IL-5R (IL-5R/IL-31Rα) expressed also either the gp130, LIFR or OSMRβ chimera receptors. STAT3/STAT5 activation after addition of IL-5 was also observed when IL-5R/IL-31Rα was expressed alone and formed homodimers (Diveu et al. 2003). Thus, IL-31Rα in either homo- or heterodimeric receptor complex with gp130, LIFR or OSMRβ is sufficient to activate JAK1/STAT3 as well as STAT5. However, the homodimer of IL-31Rα is unable to activate ERK1/2, because of variance to other receptors, it can not recruit SHP-2 tyrosine phosphatase and Shc adaptor protein essential for the ERK1/2 activation (Dreuw et al. 2004).

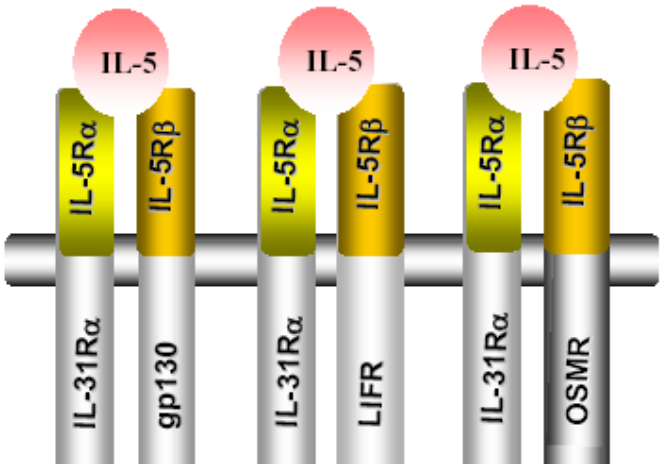


Figure 4. Structures of IL-5 receptor/IL-31Rα chimeras. The chimera receptors encoding the external portions of IL-5 α or β receptors fused to the transmembrane and cytoplasmic parts of IL-31Rα, gp130, LIFR or OSMRβ.

IL-31, the only known ligand for IL-31Rα, signals through a heterodimeric receptor composed of IL-31Rα and OSMRβ, but it does not engage gp130. In transfected BaF3 cells IL-31 signaled via the heterodimeric receptor composed of IL-31Rα and OSMR to activate STAT1, STAT3, STAT5 but not STAT6 (Dillon et al. 2004). In HEK293T cells cotransfected with expression chimeric receptor complex IL-31Rα and OSMRβ IL-31 activates JAK1, JAK2, STAT1/3/5 signaling pathways and Pi3-K/Akt cascade (Diveu et al. 2004; Dreuw et al. 2004). Colorectal cancer derived intestinal epithelial cell lines (IEC) express the functional IL-31Rα receptor complex, and their stimulation with IL-31 leads to phosphorylation of STAT1/3, Akt and ERK MAP kinases (Dambacher et al. 2007).

1.2.2. JAK/STAT signaling pathways

In 1994 it was discovered that IL-6-type cytokines utilize tyrosine kinases of the JAK family and transcription factors of the STAT family as major mediators of signaling transduction (Kordula et al. 1998). JAK and STAT are critical components of many cytokine receptor systems, regulating growth, survival, differentiation and pathogen resistance. JAK autophosphorylation induces a conformational change within itself enabling it to transduce the intracellular signal by further phosphorylation and activation of transcription factors STAT. The activated STAT dissociate from the receptor and form

dimers before translocation to the cell nucleus where they regulate transcription of selected genes (Kisseleva et al. 2002) (**Figure 5**).

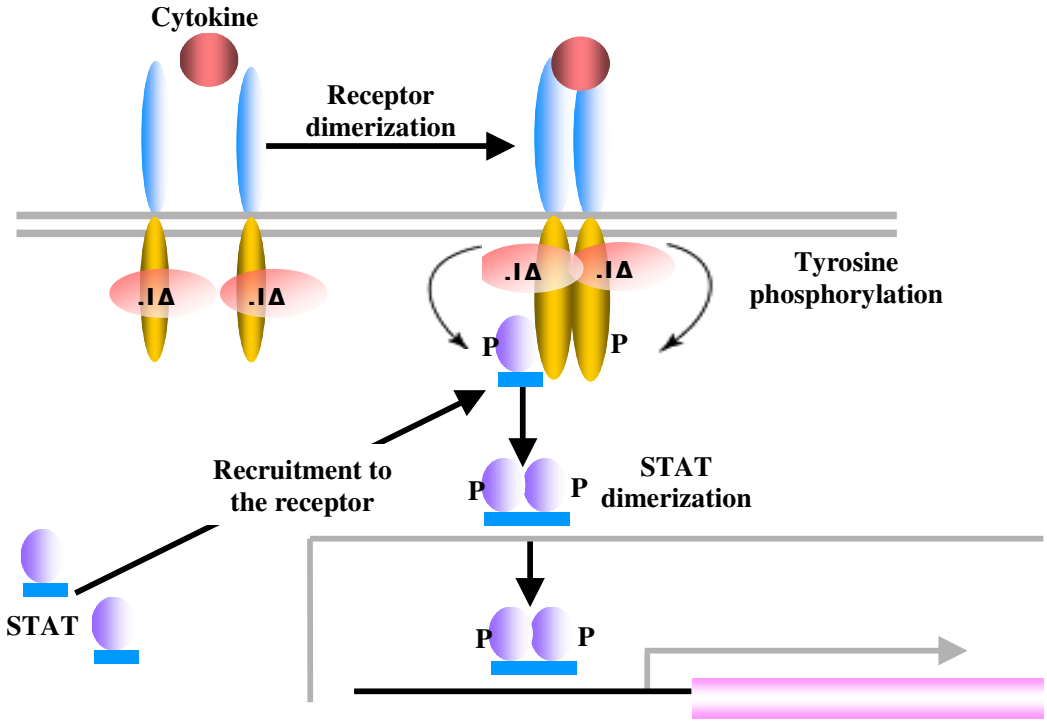


Figure 5. The key steps of the JAK/STAT activation (Ihle 2001).

1.2.2.1. JAK family

The JAK are cytoplasmic tyrosine kinases, which were first implicated in cytokine-stimulated signal pathways. Four members have been identified so far; JAK-1, JAK-2, JAK-3, and TYK-2 (Gual et al. 1998). JAK-1, JAK-2, and TYK-2 are ubiquitous (Firmbach-Kraft et al. 1990), wherease JAK-3 is expressed in T lymphocytes (Johnston et al. 1994; Witthuhn et al. 1994). JAK range from 120-140 kDa in size and have seven defined regions of homology called Janus homology domain 1-7 (JH1-7). The structure of JAK family proteins is shown in **Figure 6**. JH1 is the kinase domain important for the enzymatic activity of the JAK and contains typical features of a tyrosine kinase such as conserved tyrosines necessary for JAK activation (e.g. Tyr^{1038}/Tyr^{1039} in JAK1, Tyr^{1007}/Tyr^{1008} in JAK2, Tyr^{980}/Tyr^{981} in JAK3, and Tyr^{1054}/Tyr^{1055} in Tyk2). Phosphorylation of these dual tyrosines leads to the conformational changes in the JAK protein to facilitate binding of substrate. JH2 is a pseudokinase domain, a domain structurally similar to a tyrosine kinase and is essential for a normal kinase activity yet it lacks enzymatic activity.

This domain may be involved in the regulation of the activity of JH1. The JH3-JH4 domain of JAK shares homology with Src-homology-2 (SH2) domains.

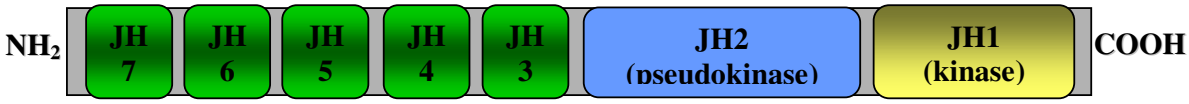


Figure 6. Domain structure of JAK. Comparison of the amino-acid sequences of the JAK kinases shows seven conserved domains. JH - Janus homology domain.

The amino terminal end of JAK (JH4-JH7) is called a FERM domain (short for four-point-one ezrin radixin moesin); this domain is also found in the focal adhesion kinase (FAK) family and is involved in association of JAK with cytokine receptors and/or other kinases (Kisseleva et al. 2002).

The members of the JAK family associate constitutively with a variety of cytokine receptors. Cytokines have critical functions in regulating immune responses. A large number of these factors bind related receptors termed the type Ⅰ and type Ⅱ cytokine receptors (O'Shea et al. 2000). Upon binding of the specific ligand to these receptors, JAK are rapidly activated and their kinase activities induce tyrosine phosphorylation of various effectors and initiate activation of downstream signaling pathways (Verma et al. 2003). The receptors interacting with JAK family members are EPO-R (erythropoietin receptor), IL-6R, IL-2R, IFNγ-R and IFNαβ-R (Gauzzi et al. 1997). In addition to STAT JAK can activate other signaling pathways, i.e. MAP kinases. As JAK are essential elements in cytokine signaling, it has been widely recognized that these kinases might be reasonable targets for the development of novel immunosuppressants (Waldmann and O'Shea 1998). Various JAK-dependent pathways are also involved in malignancies, therefore these pathways gained recently more attention as putative antitumor targets (Nefedova and Gabrilovich 2007).

1.2.2.2. STAT family

STAT family of transcription factors participates in a variety of cellular events: differentiation, proliferation, cell survival, apoptosis and angiogenesis. Transcription factors of STAT family are activated in response to many different cytokines and growth factors by phosphorylation of specific tyrosines leading to the nuclear retention. The unphosphorylated STAT proteins shuttle between the cytosol and the nucleus. Once the

activated transcription factors reach the nucleus, they bind to a consensus DNA-recognition motif called gamma activated sites (GAS) in the promoter region of cytokine-inducible genes and activate the transcription of these genes. The cytokines and growth factor receptors involved in STAT activation are: the interferon (IFN) family, the gp130 family, the γC family and the single chain receptor family activated by cytokines erythropoietin (Epo), growth hormone (GH), prolactin (PRL), and others (Schindler and Strehlow 2000).

The STAT family consists of seven highly homologous STAT members ranging from 750 to 900 amino acids (STAT1, -2, -3, -4, -5A, -5B, and –6). STAT1, STAT3, STAT4, STAT5 and STAT6 can form homodimers. STAT1 and STAT2, STAT1 and STAT3 can form heterodimers. The STAT family domain structure is shown in **Figure 7**. It includes several conserved domains: the SH2 domain through which STAT dimerize upon tyrosine phosphorylation, the DNA binding domain, coiled-coil domains mediating protein-protein interactions, the N-terminal domain interacting with inhibitors (Levy and Darnell 2002).

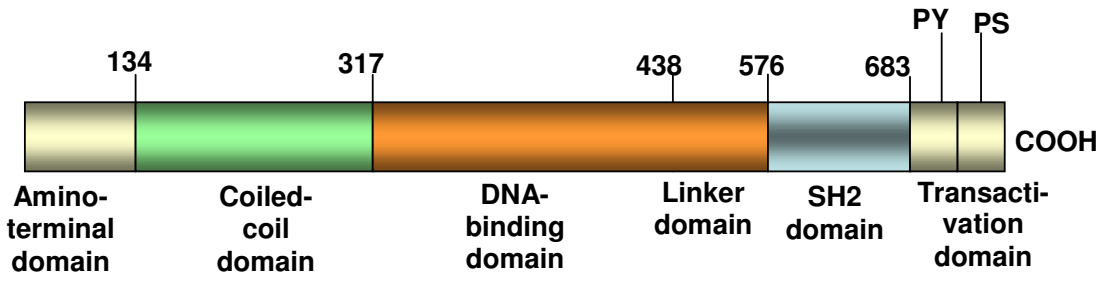


Figure 7. Domains and modification sites of STAT proteins.

STAT activation (especially STAT3 and STAT5) occurs in a large number of cancers. The following **Table 1** lists the activation of STAT in human cancers (Yu and Jove 2004).

Table 1. Activation of STAT3 in human cancers (Levy and Darnell 2002).

Tumor type	Activated STAT
Blood tumors:	
Multiple meyloma	STAT1, STAT3
Leukaemias:	
HTLV-I-dependent	STAT3, STAT5
Erythroleukaemia	STAT1, STAT5
Acute myelogenous leukaemia (AML)	STAT1, STAT3, STAT5
Chronic myelogenous leukaemia (CML)	STAT5
Large granular lymphocyte leukaemia (LGL)	STAT3
Lymphomas:	
EBV-related/Burkitt´s	STAT3
Mycosis fungoides	STAT3
Cutaneous T-cell lymphoma (NHL)	STAT3
Anaplastic large-cell lymphoma (ALCL)	STAT3
Solid tumors:	
Breast cancer	STAT1, STAT3, STAT5
Head and neck cancer	STAT1, STAT3, STAT5
Melanoma	STAT3
Ovarian cancer	STAT3
Lung cancer	STAT3
Pancreatic cancer	STAT3
Prostate cancer	STAT3

1.2.2.3. Regulation of JAK/STAT signaling pathways

The JAK/STAT pathways are essential for the signaling transduction of many cytokines. The signaling pathway modulation includes both negative and positive regulation. Dysregulation of JAK-STAT signaling is associated with various human diseases. Recent

studies have helped to shed some light on the regulatory mechanisms that modify quantity and quality of the signaling responses (Hebenstreit et al. 2005).

More recent studies have determined that STAT signals are downregulated at several points in the signaling cascade including the receptors, and the JAK and STAT molecules themselves.

The well-characterized membrane-spanning phosphatase, CD45, negatively regulates JAK-STAT signaling stimulated by IL-3, IL-4, Epo and IFNγ (Irie-Sasaki et al. 2001). The suppressors of cytokine signaling (SOCS) are a family of STAT target genes that directly antagonize STAT activation, thus establishing an important and classic ′feed loop′ (Kile and Alexander 2001). SOCS-1 is believed to impede signaling through a direct interaction with the JAK activation loop (Yasukawa et al. 1999). SOCS-3 binds JAK2, but fails to block its catalytic activity (Feener et al. 2004). CIS-1 (cytokine-inducible SH_2 containing protein) is believed to antagonize signaling by blocking STAT receptor recruitment (Li et al. 2000). STAT activity can also be negatively regulated through its inhibitor proteins, such as PIAS (protein inhibitor of activated STAT) family, which appears to bind to activated STAT1 dimers, thereby blocking their ability to bind DNA (Liao et al. 2000). Tyrosine kinases, serine kinases and the interacting proteins play an important role in the positive regulation of STAT activation. Reporter gene studies have demonstrated that serine phosphorylation enhances the transcriptional activity of STAT1 and STAT3 (Decker and Kovarik 2000; Saura et al. 2006). Serine phosphorylation is also capable to enhance STAT4 transcriptional activity (O'Shea et al. 2000). For STAT5, serine phosphorylation rather appears to enhance protein stability (Beuvink et al. 2000). Numerous studies have described frequent interactions between STAT and other transcriptional regulators. Thus, STAT1 was shown to interact with NF-κB, Sp1, USF-1, PU.1 and the glucocorticoid receptor (Look et al. 1995; Ohmori and Hamilton 1997; Muhlethaler-Mottet et al. 1998; Aittomaki et al. 2000). STAT3 was shown to interact with Sp1, c-Jun and the glucocorticoid receptor (Schaefer et al. 1997; Cantwell et al. 1998). STAT5 interacts with YY-1, Sp1, C/EBPβ and the glucocorticoid receptor (Meier and Groner 1994; Stocklin et al. 1996; Martino et al. 2001; Wyszomierski and Rosen 2001). These studies provide insight into the ability of STAT to interact with other signaling molecules.

JAK mutations are the major molecular events in human hematological malignancies. A unique somatic mutation in the JAK2 results in the pathologic activation JAK2 kinase, which leads to malignant transformation of hematopoietic progenitors. Several mutations of the JAK3 pseudokinase domain present in some patients with acute megakaryoblastic leukemia, render JAK3 constitutively active (Staerk et al. 2005).

1.3. Plasmin

1.3.1. Structure of plasmin

Plasmin is a protease which is distributed throughout the human body in the form of the zymogen plasminogen (Castellino and Ploplis 2005). The domain structure of human plasminogen is presented in **Figure 8**.

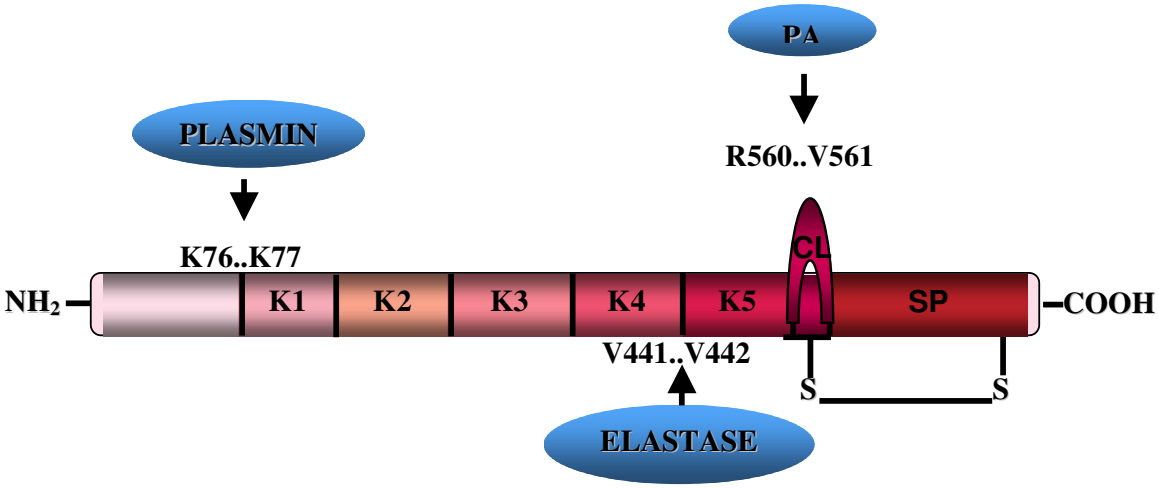


Figure 8. Domain structure of human plasminogen. The structural and functional domains of human plasminogen are shown. The 77- residue activation peptide (AP) is followed by 5 consecutive kringle (K) domains. These regions are also known to fold independently. Similarly, the cleavage (activation) loop (CL) is depicted (residues 543–582). Plasminogen activators (PA) catalyze cleavage at the peptide bond of residues Arg561-Val562, leading to plasmin formation. The functional serine protease (SP) domain (562–791) overlaps with the CL since its amino-terminus begins at residue Val562. The arrows indicate the sites of proteolytic cleavage by plasmin, elastase, and plasminogen activators (Castellino and Ploplis 2005).

The conversion of human plasminogen to plasmin involves cleavage of Arg^{561}-Val^{562} resulting in the generation of Glu-plasmin, which contains an N-terminal heavy (A) chain of 561-amino acids and a disulfide-linked carboxy-terminal light (B) chain of 230-amino acids. The catalytic triad is located in the light chain and consists of His^{603}, Asp^{646}, and Ser^{741}. Another proteolytic reaction that has physiological relevance is the Glu1-plasmin and Lys78-plasmin-catalyzed hydrolysis of the N-terminal 77 amino acids of Glu1-plasminogen or Glu^{1}-plasmin, converting Glu1-plasminogen to Lys^{78}-plasminogen and Glu^{1}-plasmin to Lys^{78}-plasmin. Lys-plasmin has a greater affinity for fibrin than the Glu form (Violand and Castellino 1976). This conversion is catalyzed by a variety of physiological and pathological activators, including urinary type plasminogen activators (uPA), tissue type plasminogen activators (tPA) (Plow et al. 1995).

The COOH-terminal light chain (Mw =26,000 Da) contains the catalytic triad (His^{42}, Asp^{85} and Ser^{180}) as well as the streptokinase binding site. The NH_2-terminal heavy chain ranges in molecular weight from 12,000 to 63,000 depending on the type of plasminogen from which it originated. In the absence of inhibitors, plasmin cleaves the amino-terminal Glu1 to Lys76 peptide from plasmin/plasminogen to yield Lys-plasmin, which has a greater affinity for fibrin than the Glu form. The heavy chain of Lys-plasminogen contains 5 triple loop disulfide bridged regions of internal sequence homology known as kringles. Kringles 1-4 contain the ω-aminocarboxylic acid and fibrin binding sites (Ramakrishnan et al. 1991).

Two immunologically distinct main physiologic plasminogen activators (PA) have been identified: the tPA and the uPA (Collen and Lijnen 1995; Plow et al. 1995). The tPA-mediated plasminogen activation is mainly involved in the dissolution of fibrin in the circulation. uPA binds to a specific cellular receptor (uPAR) resulting in enhanced activation of cell-bound plasminogen. The main role of uPA appears to be the induction of pericellular proteolysis via the degradation of matrix components or via activation of latent proteinases or growth factors. Inhibition of the fibrinolytic system may occur either at the level of PA, by specific plasminogen activator inhibitors (PAI-1 and PAI-2), or at the level of plasmin, mainly by α2-antiplasmin (Nicholl et al. 2006). Aprotinin is a protein-based inhibitor of plasmin that has been used in the clinic to stop bleeding during various surgical procedures, before it has been taken from the market because evidence for the risk associated with aprotinin in cardiac surgery and side effect were published in 2007 (Gonias, 1988; Backer, 2007).

1.3.2. Functions of plasmin

The serine protease plasmin has a broad specificity that is responsible for the breakdown of blood clots, a process called fibrinolysis. The functions of plasmin in the process of fibrinolysis are well known. In the plasminogen deficient mice, the effects of this proteolytic system has been vastly decreased (Collen and Lijnen 1995; Plow et al. 1995). Plasmin itself can directly degrade few components of the ECM such as laminin and fibronectin, whereas other components are degradated by matrix metalloproteinases (MMP), which can be activated by plasmin. Thus, two proteolytic systems, the fibrinolytic (plasminogen/plasmin) and MMP systems can degrade most ECM components (Lijnen 2001). Interactions between the fibrinolytic and matrix metalloproteinase systems are shown in **Figure 9**.

Apart from its fibrinolytic function, plasmin is a potent cell activator. In platelets plasmin induces aggregation and in endothelial cells it promotes biosynthesis of platelet-activating factor and chemotaxis (Shi et al. 1992; Ishii-Watabe et al. 2000).

In addition to the breakdown of blood clots, the plasmin pathway has been purported to play a role in the development of rheumatoid arthritis, angiogenesis and bone remodeling. Plasmin plays an important role during cell invasion and chemotaxis, as well as during tissue remodeling, which are essential during many pathological processes and require degradation of the extracellular matrix (ECM).

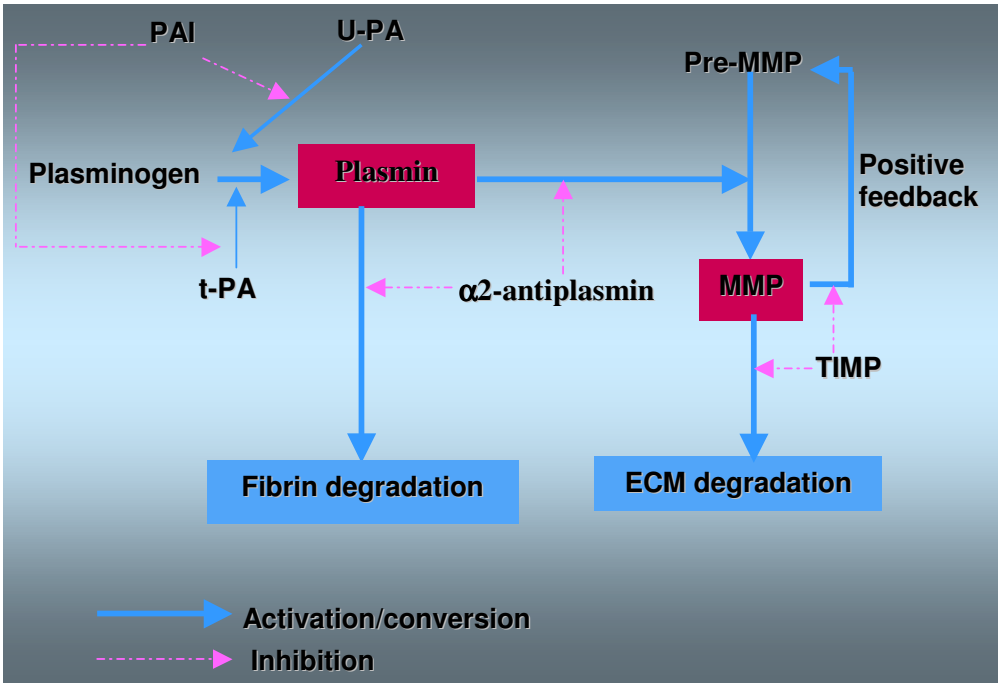


Figure 9. Schematic representation of the interactions between the fibrinolytic and MMP systems. Plasmin, the main active enzyme of the fibrinolytic system, degrades fibrin and can convert latent MMP into the active forms, which in turn degrade ECM. Activation of proMMPs is regulated by positive feedback mechanisms in which some active MMPs can activate other proMMPs. Plasmin-mediated effects are inhibited mainly by α2-antiplasmin, whereas MMP-mediated effects are inhibited by tissue inhibitors (TIMPs). Inhibition can also occur at the level of plasminogen activation by PA inhibitors (mainly by PAI-1) (Lijnen 2001).

1.3.3. Plasmin receptors

Plasminogen-receptors are a diverse group of proteins expressed on a wide array of cell types (Hajjar 1995). Reported receptors include α-enolase, glycoprotein IIIb/IIa complex, the Heymann nephritis antigen, amphoterin, integrin αMβ2, TIP49α, gp330, and annexin

A2, Histone H2B and protease activated receptor 1 (PAR1). Plasmin binding has been shown to be antagonized by lysine and lysine analogues, pointing that plasmin binds to a protein on the cell surface, which exposes a terminal lysine (Syrovets and Simmet 2004)

α-Enolase (45 kDa) has a C-terminal lysine (Miles et al. 1991), and functions as a plasminogen-binding site on a variety of cell types (Lopez-Alemany et al. 1994; Nakajima et al. 1994; Lopez-Alemany et al. 2003; Lopez-Alemany et al. 2003).

Annexin A2 (36 kDa) has been found on the surface of endothelial cells, monocytic cell lines and macrophages (Hajjar et al. 1994; Falcone et al. 2001). Annexin A2 is a member of the annexin superfamily of a calcium-dependent, phospholipid binding proteins. At least 20 members of the annexin family have been described to date, all of which share a conserved core domain (Mw 30-40 kDa), which binds membrane-associated phospholipids. In addition, the annexin proteins contain a variable amino terminal 'tail' domain (MW 3-6 kDa), which is responsible for the specialized functions of the various annexin proteins (Raynal and Pollard 1994; Swairjo and Seaton 1994). Annexin A2 is a multi-functional protein (Raynal and Pollard 1994), which binds acid phospholipids and actin with significant affinity (Merrifield et al. 2001). Annexin A2 is cleaved by chymotrypsin and plasmin into a 33 kDa C-terminal core domain and a 3 kDa N-terminal domain of 27 amino acids. The first 14 residues of the N-terminal domain contain a high affinity binding site for S100A10, a Ca^{2+} binding protein (MW 11 kDa, **Figure 10**) (Waisman 1995). Annexin A2 interacts with S100A10 to form a annexin A2 heterotetramer, AA2t (MacLeod et al. 2003). Because S100A10 has a C-terminal lysine, Waisman and colleagues proposed that it is the S100A10 subunit that imparts the plasminogen binding activity to the AA2t (Waisman 2005).

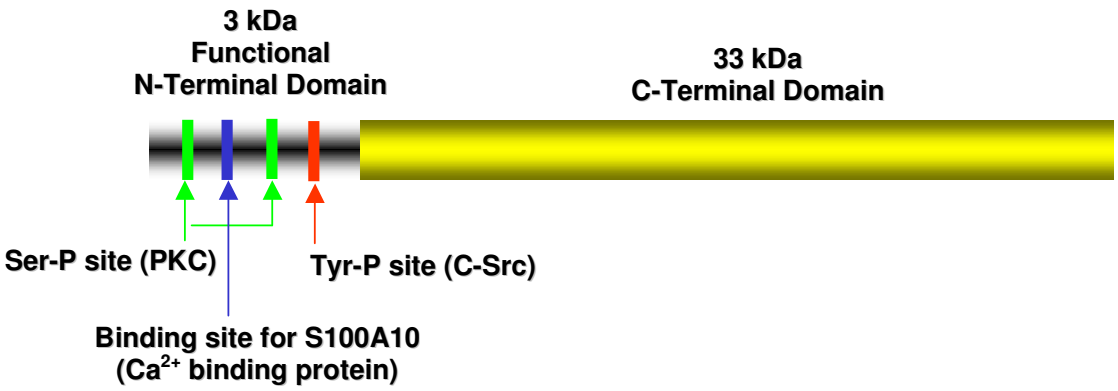


Figure 10. Structure of annexin A2. The known phosphorylation sites in the N-terminal end of the annexin A2 molecule are shown.

Monomeric and heterotetrameric annexin A2 is present on the extracellular surface of endothelial cells and tumor cells, wherein annexin A2 has been described as high affinity binding receptor for plasminogen, tPA and plasmin (Hajjar et al. 1994), or as a high affinity receptor for the extracellular matrix protein tenascin-C (Chung and Erickson 1994). In addition, it has been proposed that the membrane associated annexin A2 functions as a high affinity receptor protein for PG/gastrins (Singh 2007). The S100A10 subunit of AA2t, rather than annexin A2, has also been shown to bind tPA and plasminogen (MacLeod et al. 2003). AA2t is mainly associated with the cytoskeleton (Zokas and Glenney 1987). The monomer is found in the cytoplasm and the nucleus (Kumble and Vishwanatha 1991; Vishwanatha et al. 1992), but it is rapidly exported due to a functional nuclear export signal (NES) sequence that closely overlaps the S100A10 binding region in the annexin A2 N-terminus (Eberhard et al. 2001).

Annexin A2 is encoded by a growth-regulated gene (Vellucci et al. 1995). The serum stimulated up-regulation of the annexin A2 expression is characteristic for early response genes (Keutzer and Hirschhorn 1990). Annexin A2 was found to be necessary for cell proliferation by subtractive hybridization between primary human keratinocytes and a head-and-neck squamous cell carcinoma cell line (Vellucci et al. 1995). Annexin A2 expression is increased in many human cancers, such as primary pancreatic cancers and oxidative stress-induced cancers (Vishwanatha et al. 1993; Mitani et al. 2001). Down-regulation of its expression in HeLa cells, HEK 293 cells, IEC and human colon cancer cells results in the loss of proliferation (Chiang et al. 1999; Singh 2007).

Many physiological roles have been proposed for annexin A2, including signal transduction, membrane fusion, cell adhesion, DNA synthesis and cell proliferation (Singh 2007). AA2t was identified as a receptor for plasmin-induced signaling in human peripheral monocytes, which involved activation of the NF-κB, JAK/STAT and p38 MAPK signaling cascades, leading to a full scale pro-inflammatory response (Laumonnier et al. 2006). It is possible, that binding of plasmin to annexin A2 may mediate activation of a similar cascade of signaling molecules in human macrophages, since during the differentiation of monocytes to macrophages, there was a 2.4- fold increase in annexin A2-specific mRNA, and a 7.9-fold increase in surface annexin A2. The annexin A2-mediated assembly of plasminogen and t-PA on monocytes/macrophages contributes to plasmin generation, matrix remodeling, and directed migration (Brownstein et al. 2001).

Histone H2B (H2B) has been identified as another plasminogen binding protein with a C-terminal lysine on the surface of leukocytes (Herren et al. 2006). Although histones are primarily nuclear proteins, H2B has been found on the surface of human blood monocytes (Holers and Kotzin 1985) and lymphocytes (Watson et al. 1995), and enhanced expression of H2B on stimulated monocytoid cells is associated with a marked upregulation of plasminogen binding (Herren et al. 2006).

PAR1: PAR1 is expressed in almost all tissues: endothelium, intestine, kidneys, in the central nervous system (on astrocytes and neurons), skeletal muscles, bone (osteoclasts), in the cardiovascular system, blood (platelets), epidermis, and the immune system (monocytes, mast cells and T cells) (Macfarlane et al. 2001). Plasmin actually desensitizes PAR1 by cleavage at 3 arginine and lysine residues located at the C-terminal end of the tethered region and N-terminal to the ligand binding sites (Kuliopulos et al. 1999). In fibroblasts the plasmin-induced *CYR61* gene expression has been reported to proceed via cleavage of PAR1 and rapid and transient activation of ERK1/2 (Pendurthi et al. 2002). PAR1 is also important for the plasmin-induced CHO cell migration (Majumdar et al. 2004). Plasmin activated PAR1 and the nicotine-induced place preference and dopamine release were diminished in PAR1-deficient ($PAR1^{-/-}$) mice. It has been suggested that targeting the tPA-plasmin-PAR1 system would provide new therapeutic approaches to the treatment of nicotine dependence (Nagai et al. 2006).
Available evidence indicates that on human monocytes the signaling plasmin receptor is distinct from PARs (Colognato et al. 2003), yet its identity remains obscure.

1.3.4. Plasmin-induced cell activation

A variety of effects of plasmin had been demonstrated on various cell types. Plasmin might be able to exert mitogenic activity in certain cell types (Syrovets and Simmet 2004) Plasmin induces shape changes in platelets by a Ca^{2+}-dependent, Rho kinase-dependent pathway, a pathway inhibited by cyclic AMP (cAMP)-elevating agents (Ishii-Watabe et al. 2000). Plasmin also causes platelet aggregation via interaction with protease-activated receptor 4 (PAR-4) at two separate sites of the receptor: at the traditional thrombin cleavage site and at a currently uncharacterized site (Kunapuli et al. 2003). Plasmin mediates platelet aggregation predominantly through proteolytic cleavage of PAR-4 that ultimately leads to integrin αA2bβ3 activation. Absence of PAR-1 has no effect on the

plasmin-mediated platelet aggregation, whereas absence of functional PAR-4 inhibits this response (Quinton et al. 2004).

Plasmin stimulates the arachidonic acid cascade in endothelial cells, which leads to activation of prostacyclin biosynthesis through a phospholipase C-independent mechanism. In the early phase of stimulation the release of arachidonic acid is Ca^{2+}-dependent and pertussis toxin sensitive, pointing to receptor-mediated signaling. In contrast, the late-phase activation process is G-protein-independent and requires only the catalytic center of plasmin (Chang et al. 1993). More recent studies with this cell type revealed that plasmin but not plasminogen binds to integrin $\alpha v\beta 3$ through its kringle domains and triggers plasmin-induced stress fiber formation and migration of endothelial cells, a mechanism relevant for the process of angiogenesis. This activity of plasmin requires both an interaction with $\alpha v\beta 3$ and catalytic activity of plasmin (Tarui et al. 2002).

Plasmin induces aggregation of neutrophils, which is dependent on lysine binding sites and the active catalytic center (Ryan et al. 1992). It is possible that the adhesion molecule CD18 is involved in the aggregation. CD18 is activated in neutrophils after stimulation with plasmin (Lo et al. 1989). Expression of two types of adhesion molecules on endothelial cells and neutrophils may provide the basis of the CD18-P-selectin-mediated adhesion of neutrophils to endothelial cells. In fact, this process represents the initial phase of the inflammatory reaction (Syrovets and Simmet 2004).

Plasmin has been implicated in the proliferation of hepatocytes in primary culture. The cell proliferation could be inhibited by the lysine analogue tranexamic acid and PASI-535, an active-center-directed inhibitor of plasmin (Akao et al. 2002).

More detailed data on plasmin-induced intracellular signaling have been obtained in experiments with human peripheral blood monocytes. In monocytes, plasmin and structurally similar protein apolipoprotein(a) elicit full-scale proinflammatory activation, including lipid mediator release, chemotaxis (**Figure 11**) and expression of proinflammatory genes (**Figure 12**).

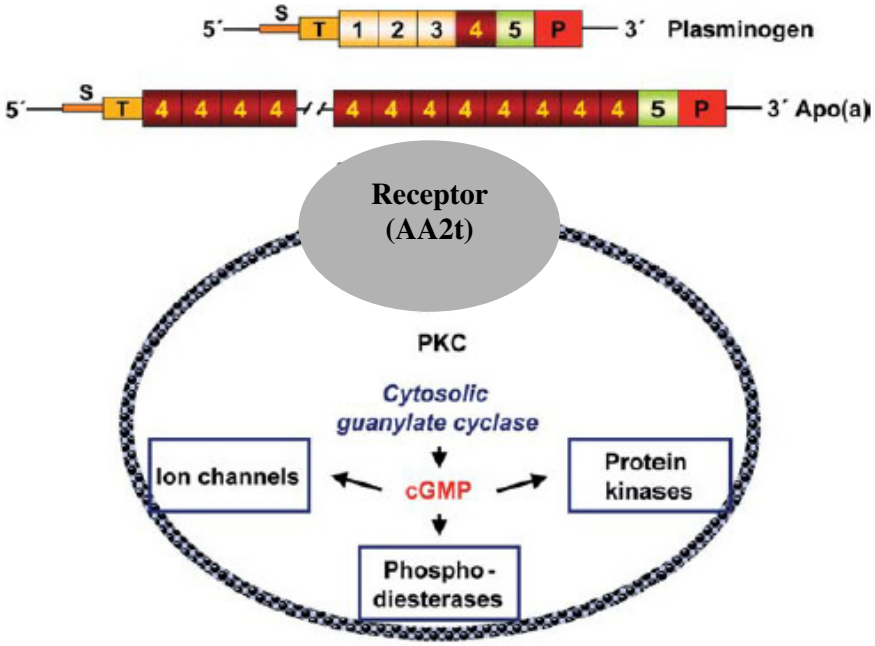


Figure 11. Plasminogen and apolipoprotein(a) induced activation of human monocytes. In human peripheral monocytes plasmin as well as lipoprotein (a) trigger activation of protein kinase C (PKC) and the soluble guanylate cyclase, followed by release of cGMP, which is essential for the plasmin and lipoprotein (a)-induced monocyte chemotaxis (Syrovets and Simmet 2004).

Like endotoxic LPS, plasmin induces expression of several cytokines such as TNF-α, IL-1α and β, the chemokine MCP-1, as well as, TF and CD40 in human monocytes (Syrovets et al. 2001; Burysek et al. 2002). The cytokines and TF belong to the so-called ´early-immediate genes´, which are rapidly expressed upon activation of transcription factors such as NF-κB and AP-1 (Bierhaus et al. 1995; Oeth et al. 1997; Foletta et al. 1998). Plasmin activates several signaling cascades that cooperate in the expression of proinflammatory genes. Within minutes plasmin induces phosphorylation and thereby activation of MAPK kinase 3/6, followed by p38 MAPK. In addition, plasmin triggers JAK/STAT signaling pathway. Among 4 known JAK, JAK1, JAK2, JAK3 and TYK2, only JAK1 was phosphorylated after plasmin treatment. Activated p38 and JAK1 phosphorylated the transcription factors STAT1 and STAT3 on serine and tyrosine residues, respectively. This double phosphorylation of STAT proteins leads to their translocation to the nucleus and binding to DNA containing STAT response elements. As a result of p38 activation, another transcription factor, AP-1, essential for the expression of cytokine and TF genes, was activated. Activated AP-1 contained phosphorylated ATF-2 and c-Jun. Plasmin-mediated activation of p38 MAPK and JAK/STAT pathways but not

of ERK1/2 was indispensable for the expression of MCP-1 and CD40 (Burysek et al. 2002)

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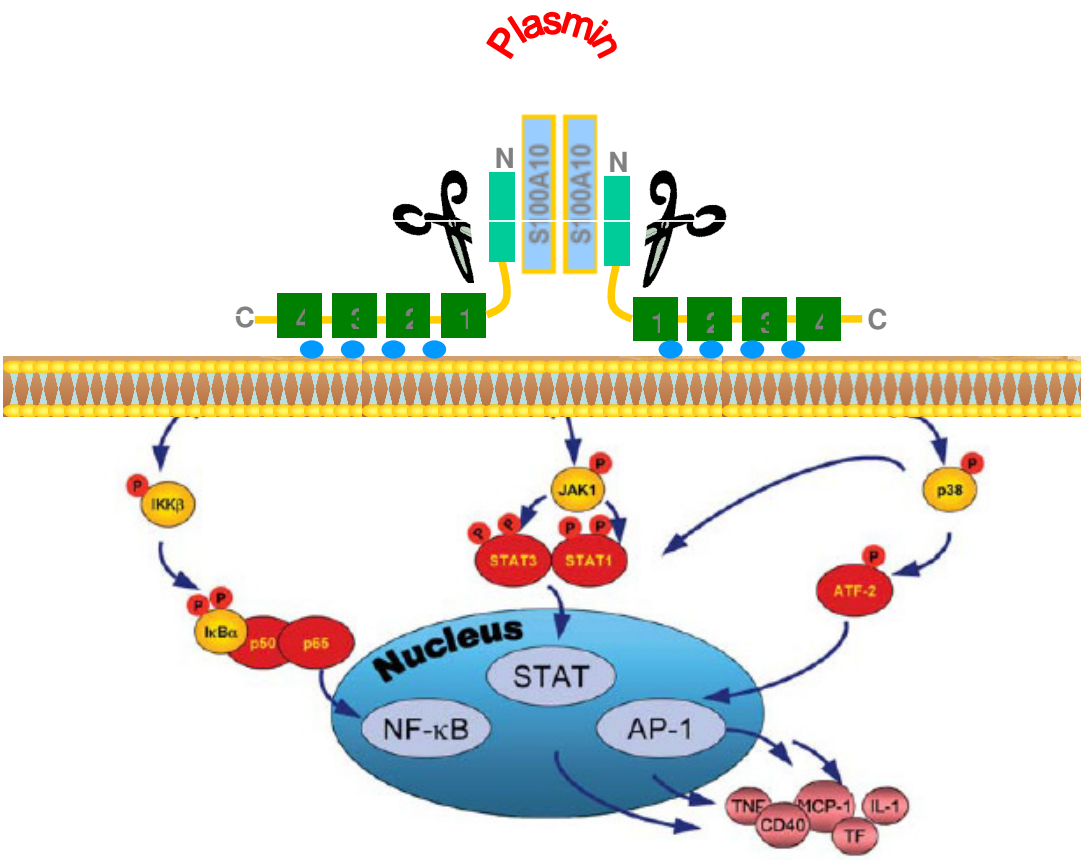


Figure 12. Plasmin-mediated activation of human monocytes. Plasmin induces phosphorylation/activation of NF-κB, JAK/STAT and p38/AP-1 pathways, leading to the expression of TNF-α, IL-1α, IL-1β, MCP-1, CD40 and tissue factor genes (Syrovets and Simmet 2004).

1.4. Plasmin in human diseases

The plasmin/plasminogen system has been implicated in many inflammatory diseases such as atherosclerosis, arthritis, and tumor cell invasiveness and metastasis (Syrovets and Simmet 2004).

1.4.1 A role for plasmin in atherosclerosis

Atherosclerotic lesions begin with the recruitment of inflammatory cells such as monocytes and T-cells to the endothelial wall. The vascular endothelial cells are

stimulated primarily by oxidized low density lipoproteins (oxLDL) and pro-inflammatory cytokines to express leukocyte adhesion molecules including E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Hansson 2001). These cells-surface proteins are upregulated during inflammation, which is central to all stages of atherosclerosis (Hansson 2005; Tedgui and Mallat 2006).

Contact activation takes place in any type of chronic inflammation including atherosclerosis and is invariably associated with the generation of plasmin (Kaplan and Silverberg 1987; Syrovets and Simmet 2004). Consistently, in human atherosclerotic lesions fibrinolytic activity is upregulated and positively correlates with the severity of coronary lesions (Nicholl et al. 2006). In addition, studies in plasminogen knockout mice indicated that plasmin/plasminogen alters the vascular response to injury. During remodeling, plasmin/plasminogen is essential for this compensatory response (Syrovets and Simmet 2004; Nicholl et al. 2006). Plasmin is a pathophysiologically significant activator of pro-MMPs in vivo and may also have implications in aortic-wall destruction by controlling Plau gene function (Carmeliet et al. 1997).

The excessive inflammatory and immune responses driven by proinflammatory cytokines such as IL-6 and TNF-α are responsible for cardiovascular events associated with atherosclerosis, Macrophages are the main source of cytokines in atherosclerotic plaques (Tedgui and Mallat 2006). Exogenously applied, IL-6 enhances fatty lesions in mouse models, and in apolipoprotein E/IL-6 double-knockout mice a reduced recruitment of inflammatory cells into the atherosclerotic plaque was observed. In addition, IL-6 is crucial for the acute phase response and IL-6 plasma levels appear to be predictive of future coronary artery disease (Tedgui and Mallat 2006).

TNF-α is a pleiotropic cytokine exerting both inflammatory and cell death modulatory activity, and is thought to play a role in the pathogenesis of atherosclerosis (Boesten et al. 2005). It has been identified in all stages of atherosclerosis, from early intima thickening to established occlusive atherosclerosis (Haddy et al. 2003; Ponthieux et al. 2004). TNF-α can upregulate the expression of cell surface adhesion proteins and augment inflammation (Hansson 2001). Consistantly, inhibition of TNF-α decreased the progression of atherosclerosis in apoE knockout mice (Branen et al. 2004).

1.4.2. Plasmin and cancer

Numerous data collected for various types of solid cancer including cancers of the breast, gastrointestinal and urological tracts, lung, brain, ovary, and cervix demonstrate a strong clinical relevance for the plasminogen system in predicting disease recurrence and

survival in cancer patients (Reuning et al. 1998; Muehlenweg et al. 2001). Plasmin generated at the surface of tumor cells is considered as a key event in tumor invasion and metastasis. Elevated levels of uPA, uPAR and PAI-1 appear to be markers of poor prognosis in colorectal, breast and other tumors. Plasmin plays an important role in neoangiogenesis and cancer invasion and metastasis. Angiostatin, an internal fragment of plasminogen, has been reported to inhibit human tumor growth and metastasis (Sharma and Sharma 2007). However, a number of experiments with plasminogen-deficient mice have shown that tumors also rely on other proteolytic pathways for growth and metastasis (Syrovets and Simmet 2004).
The detailed understanding of these processes might allow the pinpointing of therapeutic targets to fight tumor progression and metastasis.

1.5. Aim of the study

Plasmin initiates signaling leading to inflammatory cytokine release in monocytes. The mechanism of monocyte activation by plasmin is believed to include binding of plasmin to the annexin A2 heterotetramer and cleavage of annexin A2. Cleavage of annexin A2 impairs its interaction with S100A10 leading to dissociation of the annexin A2 heterotetramer complex. This event initiates downstream signaling and changes functional responses of monocytes. Yet, it is not known sofar whether plasmin is able to activate human macrophages. The aim of my study is to investigate whether plasmin activates human macrophages, and elucidate the molecular mechanisms of the plasmin-induced receptor-mediated transcellular signaling.

2. Materials and Methods

2.1. Antibodies

Mouse-anti-annexin A2 mAb

Mouse-anti-S100A10 mAb

Mouse-anti-CD14 mAb

Mouse-anti-CD47 mAb

Mouse-anti-CD71 mAb

Above antibodies were from Pharmingen, BD Biosciences (San Diego, CA, USA)

Rabbit-anti-phospho-JAK1 pAb

Rabbit-anti-phospho-TYK2 pAb

Mouse-anti-phospho-STAT3- (Tyr^{705}) mAb

Rabbit-anti-phospho-p38 pAb

Rabbit-anti-p38 pAb

Mouse-anti-phospho-ERK1/2 mAb

Rabbit-anti-ERK2 pAb

Mouse-anti-phospho-SAPK/JNK (Thr^{183}/Tyr^{185}) mAb

Rabbit-anti- SAPK/JNK mAb

Rabbit-anti-phospho-Akt - (Tyr^{326})

Rabbit-anti-phospho-PDK1- (Ser^{241}) pAb

Mouse-anti-phospho-IκBα ($Ser^{32/36}$) mAb

Above antibodies were from Cell Signaling (Beverly, MA, USA)

Rabbit-anti-phospho-JAK1 pAb

Rabbit-anti-JAK1 pAb

Rabbit-anti-phospho-JAK2

Mouse-anti-JAK2 mAb

Mouse-anti-gp130 mAb

Above antibodies were from Biosource (Camarillo, CA, USA)

Goat-anti-human IL-31Rα pAb (R&D Systems, Minneapolis, MN)

HRP-conjugated anti-mouse antibody (Sigma-Aldrich, Steinheim, Germany)

HRP-conjugated anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

HRP-conjugated donkey anti-mouse or anti-rabbit $F(ab)_2$ (DAKO Cytomation, Hamburg, Germany)

HRP-conjugated rabbit anti-goat $F(ab)_2$ (Pierce, Rockford, IL, USA)

Mouse and rabbit IgG (Dianova, Hamburg, Germany)
Mouse IgG (Sigma-Aldrich, Steinheim, Germany)
Mouse-anti-actin mAb (Chemicon International, Temecula, NY, USA)
Mouse-anti-CD11c mAb and mouse-anti-CD68 mAb (Dako Cytomation, Hamburg, Germany)
Mouse-anti-CD14 mAb (Dianova, Hamburg, Germany)
Mouse-anti-IL-31Rα mAb and rabbit-anti-IL-31Rα pAb (kindly provided by Dr. H. Gascan (Angers, France)).
Mouse-anti-LexA mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
Mouse-anti-phosphotyrosine mAb (clone 4G10) and rabbit-anti-gp130 pAb (Upstate Biotechnology, Lake Placid, NY, USA)
Mouse-anti-S100A10 mAb (RDI, Flanders, NJ, USA)
PE-conjugated donkey anti-rabbit or anti-mouse $F(ab)_2$ (Dianova, Hamburg, Germany)
Rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
Rabbit-anti-annexin A2 pAb (Biodesign, Saco, Maine, USA)
Rabbit-anti-LIFR pAb and rabbit-anti-OSMR pAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
Rabbit-anti-STAT-3 (Ser^{727}) mAb (Epitomics, Burlingame, CA, USA)

2.2. Chemicals and kits

2ME (Sigma-Aldrich, Steinheim, Germany)
A/G plus agarose (Santa Cruz, CA, USA)
A2NP (1-27) peptide and scrambled peptide (Thermo Hybaid, Ulm, Germany)
Agarose (Invitrogen, Karlsruhe, Germany)
AKβBA (B. Büchele, Inst. Pharmacology of Natural Products and Clinical Pharmacology, Ulm University, Germany)
Ampicillin (Invitrogen, Karlsruhe, Germany)
Aprotinin (Bayer, Berlin, Germany)
BCA protein assay reagent kit (Pierce, Rochford, IL, USA)
BSA endotoxin-free (Sigma-Aldrich, Steinheim, Germany)
Chloramphenicol (Sigma-Aldrich, Steinheim, Germany)
Dithiothreitol (DTT) (USBiological, Swampscott, MA, USA)
DMSO (Sigma-Aldrich, Steinheim, Germany)
dNTPs (Invitrogen, Karlsruhe, Germany)
Dulbecco's modified eagle's medium (DMEM) (Gibco, Karlsruhe, Germany)
E. coli, KC8 electrocompetent cells (BD Biosciences, San Diego, CA)

EDTA (Titriplex A2I) (Merck KG, Darmstadt, Germany)

ELISA Kits (MCP-1, TNF-α, IL-6 and MIP-3α) (R&D Systems, Abingdon, UK)

FCS - foetal calf serum (Seromed, Berlin, Germany)

Film developer and film fixative (Kodak Industries, Chalon, France)

Glutaraldehyde solution (Sigma-Aldrich, Steinheim, Germany)

Glutathione-Sepharose 4B (GSH) (Amersham Bioscience, Freiburg, Germany)

Histopaque-1077 and Percoll (Sigma-Aldrich, Steinheim, Germany)

Human α-thrombin (Enzyme Research Laboratories, Swansea, UK)

LPS from *E. coli* serotype 055:B5 (Sigma-Aldrich, Steinheim, Germany)

M-CSF (Biovision, Mountain View, CA)

Micro BCA assay kit (Pierce, Rochford, IL, USA)

M-MLV (Moloney-murine leukaemia virus) reverse transcriptase (Invitrogen, Karlsruhe, Germany)

NaCl 0.9% (Diaco, Karlsruhe, Germany)

ODN (annexin A2, S100A10, gp130 and IL-31Rα) (Thermo Hybaid, Ulm, Germany)

Oligo $(dT)_{12-18}$ (Amersham Biosciences, Freiburg, Germany)

PBS (1x or 10x) w/o Ca^{2+}, Mg^{2+} (Gibco, Karlsruhe, Germany)

Penicillin/streptomycin (Invitrogen, Karlsruhe, Germany)

Plasmid purification kit (Qiagen, Hilden, Germany)

Plasmin (Fluka, Deisenhofen, Germany)

Plasmin Lot 2004-07L (Athens, Georgia, USA)

QIAquick gel extraction kit (Qiagen, Hilden, Germany)

QIAquick PCR purification kit (Qiagen, Hilden, Germany)

RPMI 1640 medium (Gibco, Karlsruhe, Germany)

SCH79797, PAR1 inhibitor (Tocris Bioscience)

Skim milk powder, blotting grade (Carl Roth, Karlsruhe, Germany)

Super signal west pico chemiluminiscent substrate (Pierce, Rochford, IL, USA)

Taq DNA polymerase recombinant (Invitrogen, Karlsruhe, Germany)

TransAM ELISA kits for STAT and NF-κB family transcription factors (Active Motif, Carlsbad, CA, USA)

Trizol reagent (Invitrogen, Karlsruhe, Germany)

Tween 20 (Merck, Darmstadt, Germany)

VPLCK, a catalytic inhibitor of plasmin, JAK inhibitor AG490, p38 inhibitor SB203580 and U0126, ERK1/2 inhibitor (Calbiochem, La Jolla, CA, USA)

2.3. Cell preparation and cultures

Monocytes, macrophages and neutrophils

Monocyte-derived human macrophages were differentiated from buffy coats for 7 days with 15 ng/ml M-CSF (Colognato et al. 2003). Monocytes and neutrophils were isolated by Percoll gradient centrifugation (Laumonnier et al. 2006). Preparation of monocytes with 94% or more $CD14^+$ cells was used. Contaminating cells were lymphocytes (2-6%). Monocytes were generally incubated in lysine-free RPMI 1640. Macrophages were cultured in RPMI 1640 (Invitrogen, Karlsruhe, Germany), supplemented with 10% FCS. The cells were stimulated with plasmin in lysine-free RPMI 1640.

2.4. Analysis of mRNA expression

2.4.1. Reverse transcription and polymerase chain reaction analysis (RT-PCR)

2.4.1.1. Buffers for reverse transcription reaction and PCR

5x First-strand buffer

250 mM Tris-HCl (pH 8.3)

375 mM KCl

15 mM $MgCl_2$

0.1 M DTT

Taq polymerase 10x PCR buffer

200 mM Tris-HCl (pH 8.4)

500 mM KCl

50 mM $MgCl_2$

2.4.1.2. Buffers for agarose gel electrophoresis

10x TBE (Tris -borate-EDTA) buffer

880 mM Tris - HCl (pH 8.3)

880 mM boric acid

25 mM EDTA

10x TAE (Tris -acetate -EDTA) buffer

400 mM Tris-HCl (pH 8.3)

1.1% (v/v) acetic acid

10 mM EDTA

TE buffer

10 mM Tris-HCl (pH 7.5)

1 mM EDTA

Loading solution

50% glycerol

0.1% bromphenol blue

0.1% xylene cyanol

2.4.1.3. Methods

RNA was extracted using TRIzol-reagent (Invitrogen) as described in the manufacturer's protocol. For cDNA synthesis, 1 μg RNA was transcribed using M-MLV reverse transcriptase-reagent (Invitrogen), and analyzed by semi-quantitative RT-PCR. PCR was performed in the linear range of amplification; GAPDH served as internal standard (Syrovets et al. 2005). The transcripts were identified by direct automated sequencing (Genetic Analyzer; Applied Biosystems). All PCRs shown are representative of at least three independent experiments. The following primers were used:

Table 2. Primers for PCR

Gene	Forward	Reverse	Ta (°C)	Cycles	Product size (bp)
IL-6	tacatcctcgacggcatctca	agttgtcatgtcctgcagcca	52	28	399
TNF-α	cagagggaagagttccccag	ccttggtctggtaggagacg	65	33	325
MIP-3α	tgcaaccagttctctgcatc	acaggggaactctcagagca	60	25	300
IL-31Rα all	gttttgggcatcaaacgaat	gttctggagtgactggagcc	56	35	259
IL-31Rα long	tggagtccctgaaacgaaag	ttagacttctcccttggtgtgc	55	35	757
IL-31Rα short	gaccaaggtggagaacattgg	cttcaggtttagcttatcttggg	59	35	467
Gp130	catgctttgggtggaatggac	catcaacaggaagttggtccc	58	35	325
LIFR	cctcatcttagatgtgtctc	ttctcctcacctggcattac	55	35	358
OSMRβ	gctgatgccagccacttctggaaa	agcacatcctgggtatggtcacac	69	35	487
GAPDH	ccacccatggcaaattccatggca	tctagacggcaggtcaggtccacc	55	21	650

2.4.2. Quantitative real time PCR (qPCR)

2.4.2.1. Buffers and solution for qPCR

QuantiTest SYBR green qPCR master mix (Qiagen, Hilden, Germany)

HotStart Taq plus DNA polymerase

QuantiTest SYBR green RT-PCR buffer

DNTP mix (dATP, dCTP, dGTP, dTTP)

RNase-free water (UltraPure quality, PCR-grade)

Table 3. qPCR reaction setup

Component (µl)	96-well block		
2x QuantiTest SYBR green PCR Master Mix (µl)	12.5	10	5
Primer A (1µM)	2.5	2	1
Primer B (1µM)	2.5	2	1
cDNA (µl)	2.5	2	1
RNase-free water (µl)	5	4	2
Total reaction volume (µl)	25	20	10

2.4.2.2. Methods

Two-step RT-PCR was performed using the QuantiTest SYBR green PCR kit (Qiagen) as described in the handbook. The reaction conditions are given in Table 4. All amplification reactions were performed in duplicate. The ABI Prism 7300 sequence detection system (ABI 7300) was used for real-time PCR analysis. At the end of each PCR run, the data were automatically analyzed by the system and amplification plots were obtained. The delta-delta CT or delta CT method was used to calculate the real-time PCR results (amount of target = $2^{-\Delta\Delta CT}$).

Table 4. qPCR amplification conditions

Step	Time	Temperature
PCR initial activation step	5 min	95
Denaturation (°C)	10 s	95
Combined annealing/extension (°C)	60 s	60
Number of cycles	35-40	

2.5. Analysis of protein expression

2.5.1. Western blotting

2.5.1.1. Buffers and solutions

3x GB (Gel buffer)

3 M Tris-HCl (pH 8.45)

0.3 % SDS

Cathode buffer

100 mM Tris-HCl (pH 8.25)

100 mM Tricine

0.1 % SDS

Anode buffer

100 mM Tris-HCl (pH 8.9)

Acrylamide/Bisacrylamide

30 % acrylamide

1.5% bisacrylamide

3x SLB (Sample lysis buffer)

150 mM Tris-HCl (pH 7.0)

12 % SDS

6 % -β-mercaptoethanol

30 % glycerol

0.05 % Coomassie brilliant blue

TBS (Tris buffered saline)

0.1 M NaCl

0.01 M Tris-HCl (pH 7.6)

TBST

TBS + 0.1% Tween 20

Blotting buffer

250 mM glycine

25 mM Tris-HCl (pH 8.3)

10 % methanol

Blocking buffers

5 % Milk in TBST or

5 % BSA in TBST

Stripping solution

62.5 mM Tris-HCl (pH 6.7)

2 % SDS

100 mM β-mercaptoethanol

Lysis buffer

Buffer 1: ($CaCl_2$)

50 mM Hepes (pH 7.5)

150 mM NaCl

1 % Triton
1.5 mM $CaCl_2$
1 mM DTT
0.1 % NP40
1 mM PMSF

Buffer 2: (RIPA)
50 mM Tris-HCl (pH 7.4)
150 mM NaCl
0.1 % SDS
0.25 mM EDTA
1 % Triton
1 % Deoxycholic acid

Phosphatase inhibitors (100x)
50 mM NaF
25 mM β-glycerolphosphate
1 mM Na_3VO_4 (orthovanadate)

Protease inhibitor cocktail set III (1:100) (Calbiochem, La Jolla, CA, USA)

2.5.1.2. Materials

Electrophoresis apparatus (BioRad Laboratory Munich, Germany)
PVDF membrane and nitrocellulose membrane (Amersham Biosciences Freiburg, Germany)
Sonorex RK 100 SH (Bandelin Berlin, Germany)
Hyperfilm (Amersham Biosciences, Freiburg, Germany)
Solution detection ECL (Amersham Biosciences Freiburg, Germany)
Rainbow protein marker RPN800 (Amersham Biosciences Freiburg, Germany)
BCA protein assay reagent kit (Pierce Rockford, USA)

2.5.1.3. Methods

Preparation of whole cell lysates was done with lysis buffer. Determination of the protein content was performed by using the BCA protein assay reagent kit. Equal amounts of protein were separated based on molecular size using 8%, 10%, 12% or 15% polyacrylamide SDS-PAGE as described in a standard protocol (Current Protocols in

Molecular Biology, John *Wiley* & Sons). Samples were transferred onto a PVDF or nitrocellulose membrane for 1 h or 40 min. To reduce unspecific protein binding, membranes were blocked for 1 h in blocking buffer. Detection of specific protein was conducted using the indicated primary antibodies after the membranes were washed with TBST. After probing with primary antibody, the membranes were washed and probed with secondary antibodies for 45 min at 22°C. The proteins were visualized using enhanced chemiluminescence detection reagent system ECL and subsequent exposure to Hyperfilm. For all Western blots analysis actin was used as a control for loading equal amounts of protein. The Western blots shown are representative of at least three independent experiments.

2.5.2. Analysis of STAT and NF-κB transcription factor activation

2.5.2.1. Buffers and solutions

Extraction buffer A (Cell-lysis buffer):

10 mM Hepes (pH 7.9)

1.5 mM $MgCl_2$

10 mM KCl

0.5 mM DTT

0.1 % Igepal CA630 (Sigma)

Phosphatase and protease inhibitors (1:100)

Buffer B (Extraction buffer):

20 mM Hepes (pH 7.9)

1.5 mM $MgCl_2$

0.5 mM DTT

0.2 mM EDTA

25% Glycerol

Phosphatase and protease inhibitors (1:100)

2.5.2.2. Methods

Human macrophages were stimulated with 0.43 CTA U/ml plasmin for 15 min (STAT) or 1 h (NF-κB), and nuclear extracts were prepared as described previously (Burysek et al. 2002; Syrovets et al. 2005). Activation of STAT1α, 3, 5A, 5B and p65, c-Rel, RelB were determined in 5 μg nuclear extract with DNA-binding TransAM ELISAs for STAT and NF-

κB family transcription factors. Data are expressed as amount of corresponding transcription factor in treated cells compared to unstimulated controls using 3-4 different nuclear extract preparations each.

2.5.3. Flow cytometry

2.5.3.1. Buffers for FACs analysis

Staining buffer (Kogi et al. 2002)
11 mM Hepes (pH 7.4)
137 mM NaCl
4 mM KCl
11 mM Glucose

Fixation buffer
4% Paraformaldehyde in PBS (pH 7.2)

2.5.3.2. Methods

Fluorescent-activated cell sorting is a method for sorting a suspension of cells, one cell at a time, based upon specific light scattering and fluorescence characteristics of each cell. This method allows analysis of the cell surface fluorescence in order to check the expression of antigens, without breaking the cell membrane. Cells were analyzed as described (Laumonnier et al. 2006). The cells were incubated in FACs buffer (PBS with 0.5% BSA). Primary antibody was added so that the final concentration was 10 µg/ml, and incubated for 1 h on ice. For washing, 1 ml of FACs buffer was added and the cells were centrifuged at 400 g for 5 min at 4°C. Secondary antibody, conjugated with phycoerythrin (PE), was added in 1:100 dilution and incubated for 1 h in the dark at 4°C. The cells were washed again and fixed with paraformaldehyde dissolved in PBS, so that the final concentration of paraformaldehyde was 2%. When the fluorescence was measured on the same day, cells were not fixed. Samples were analysed for antigen expression using a FACScan (BD Biosciences, San Jose, USA).

2.5.4. Enzyme-linked immunoSorbent assay (ELISA)

Macrophages or monocytes incubated in RPMI 1640 without lysine were stimulated with plasmin, LPS, A2NP or other stimuli for the indicated time. The supernatants were

recovered and the concentration of cytokines was measured with ELISAs (R&D Systems). A 96-well plate was coated with 100 µl of 2 µg/ml capture antibody diluted in PBS for overnight at RT. After washing with washing buffer (PBS/0.05%Tween 20), the plate was blocked by addition of 300 µl of reagent diluent (PBS/1% BSA) for 1 h, washed again. 100 µl of samples and standard were added in reagent diluent for 2 h incubation at RT, followed by washing. Then 100 µl detection antibody for 2 h was added and, after washing, a 100 µl streptavidin-HRP (1:200 dilted in reagent diluent) for 20 min. After washing, 100 µl of substrate solution containing peroxide was added (ratio: Substrate A: Substrate B =1:1) for 20 min. Finally, the reaction was stopped by addition of 50 µl stop solution (2 N H_2SO_4). The optical density of each well was determined using a microplate reader set to 450 nM.

2.6. Inhibition of gene expression by antisense ODN

For in vitro knockdown of annexin A2 and S100A10, gp130 and IL-31Rα, 10 µM of phosphorothioate-modified oligodeoxynucleotides (ODN) (Thermo Hybaid, Ulm, Germany) were added to macrophage or monocytes cultures every 24 h for a total of 48 h as described (Laumonnier et al. 2006).

The following media were used for ODN experiment:

Dulbecco's modified eagle medium (DMEM) containing:

10% FCS

100 U/ml penicillin

100 µg/ml streptomycin

RPMI 1640 without lysine:

10.07 g/l RPMI 1640-powder (Applichem, Darmstadt, Germany)

2 g/l $NaHCO_3$ (Merck, Darmstadt, Germany)

2 mM L-glutamine (Invitrogen, Karlsruhe, Germany)

100 U /100 µg/ml Penicillin /Streptomycin (Invitrogen, Karlsruhe, Germany)

Dissolve in Ampuwa till 1l (Fresenius Kabi, Bad Homburg, Germany)

Steriled by using 90 mm bottle top filter - 500 ml (Nalgene, New York, USA)

The following ODNs against different genes were used:

Antisense annexin A2: 5'-cttttagctcagaagcatcata-3'

Sense annexin A2: 5'-tatgacgcttctgagctaaaag-3'

Antisense S100A10: 5'-gagatggcattttggtgtg-3'
Sense S100A10: 5'-cacaccaaaatgccatctc-3'
Antisense gp130: 5'- ccttaggaatagtaaa-3'
Sense gp130: 5'-tttactattcctaagg-3'
Antisense IL-31Rα: 5'-caagtgcaggttaaatt-3'
Sense IL-31Rα: 5'-aatttaacctgcacttc-3'

2.7. Immunofluorescence microscopy

Monocytes or macrophages ($2x10^6$) resuspended in 800 µl RPMI were permitted to adhere on chamber slides (BD Biosciences) for 30 min, then they were fixed with paraformaldehyde and stained with Hoechst 33342 (1 µg/ml, a DNA marker), rabbit-anti-gp130, mouse-anti-IL-31Rα, rabbit anti-annexin A2 and mouse anti-S100A10 Abs. The antibodies were visualized with anti-mouse Cy5-labeled $F(ab)_2$ and anti-rabbit FITC-labeled $F(ab)_2$ and analyzed with a Diaphot fluorescence microscope (Nikon).

2.8. Equipment

Centrifuges
Sigma 4K15 (Sigma laboratory centrifuges, Osterode im Harz, Germany)
Routina 48RS (Hettich Zentrifugen, Tuttlingen, Germany)
Eppendorf centrifuge 5415 (Eppendorf, Hamburg, Germany)
Spectrophotometer
Gene quant pro RNA/DNA calculator (Amersham, Freiburg, Germany)
Cell culture incubator:
Queue (Nunc, Wiesbaden, Germany)
Electrophoresis apparatus
Mini protean 3 electrophoresis cell (BioRad Laboratories, Munich, Germany)
Agagel mini and maxi (Biometra, Goettingen, Germany)
Microplate reader
Dynatech MR7000 (Dynex Technologies, Berlin, Germany)
PCR machines
GeneAmp PCR system 2400 PE (Applied Biosystems, Foster City, CA, USA)
GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA)
Luminometer
Plate lumina (Started Biomedicine Systems AG, Birkenfeld, Germany)
Sonicator

Sonopuls GM70 (Bandelin Electronics, Berlin, Germany)

Sonorex RK 100 SH (Bandelin, Berlin, Germany)

Semidry protein transfer apparatus

TransBlot SD (BioRad)

Fluorescence activated cell sorter (FACS)

FACScan (BD Biosciences, San Jose, USA)

Microscope

Olympus CK2 (Olympus, Japan)

Flourescent microscope (Nikon)

DNA sequencer

ABI PRISM 310 gene analyzer (Applied Biosystems, Foster City, CA, USA)

Scanner for autoradiography

FLA-3000 (Fuji Film, Düsseldorf, Germany)

Vacuum centrifuge

SpeedVac plus SC210A (Thermo Savant, NY, USA)

UV transilluminator

Herolab E.A.S.Y. 429 K (Herolab, Wiesloch, Germany)

Vortex genie (Bender & Hobein, Bruchsal, Germany)

Microwelle (Simens, Germany)

2.9. Statistical Analysis

Values shown represent mean ± SEM where applicable. Statistical significances were calculated with the Newman-Keuls test. Differences were considered significant for $P<0.05$.

3. Results

3.1. The serine protease plasmin triggers proinflammatory gene expression in human monocyte-derived macrophages

3.1.1. Phenotypic characterization of macrophages

In attempt to verify the purity and differentiation of human monocyte-derived macrophages, a set of cell surface markers was used to analyze their expression on cell surface. CD14 functions as a LPS receptor, it is a marker specific for monocytes. CD14 is expressed on macrophages to a lower extend than on monocytes (Jacob et al. 2001). CD68 is a marker specific for macrophages, and is expressed at higher levels by macrophages than monocytes (Gottfried et al. 2008). CD71 is a specific macrophage marker as its expression is very high in macrophages compared to monocytes (Laumonnier et al. 2006). Additionally, CD11c is predominantly expressed on monocytes and all monocyte-derived cells, granulocytes and NK cells. It has the function to bind fibrinogen and represents a part of the complement receptor type 4 (Sadhu et al. 2007). The combination of these specific markers indicated that the differentiation process from monocytes to macrophages was complete (**Figure 15A**).

To further confirm the phenotype of human monocyte-derived macrophages, a phase-contrast microscopy was used to compare the morphology of monocytes and macrophages. A distinguishable phenotype between monocytes and macrophages was observed (**Figure 15B**). Additionally, the expression of CD71 was compared in monocytes and human monocyte-derived macrophages by Western blotting. As expected, the expression of CD71 is almost undetectable in monocytes compared to macrophages (**Figure 15C**). Collectively, these data indicate that monocytes had completely differentiated into macrophages after 7 days of culture.

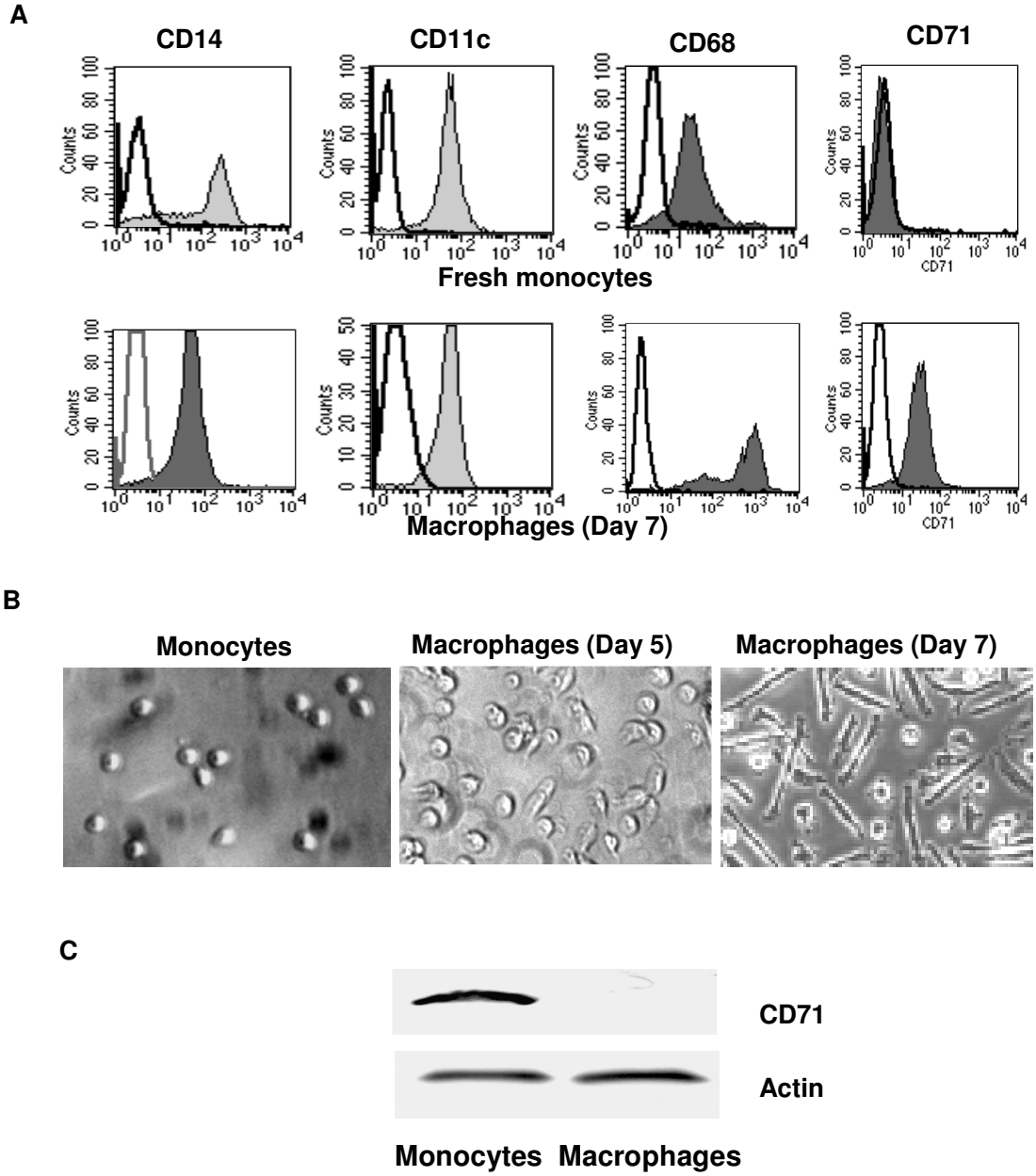


Figure 15. Phenotypic characterization of monocytes and macrophages. A, Phenotypic characterisation by FACs analysis. Monocytes were cultivated for 7 days in the presence of M-CSF (15 ng/ml). Fresh monocytes and macrophages were incubated for 1 h with primary antibodies (CD14, CD11c, CD68 and CD71). For the analysis of CD68, the cells were fixed and permeabilized with 0.1% Triton X100. After incubation 1 h with the corresponding phycoerythrin-labeled $F(ab)_2$ the cells were analyzed by flow cytometry. Empty peaks - isotype controls. B, Morphology of monocytes and macrophages. Photographs of fresh monocytes and macrophages cultured in M-CSF (15 ng/ml) for 5 days or 7 days were then taken by phase-contrast microscopy (original magnification. x20). C, Phenotypic characterisation by Western blotting with specific marker CD71. Whole cell lysates from peripheral blood monocytes on day 0 or from macrophages on day 7 were examined by immunoblotting with anti-CD71. Actin served as a loading control. Results shown are representative of at least 3 independent experiments.

3.1.2. Human macrophages express the annexin A2 heterotetramer

The macrophage phenotype was confirmed by FACS, showing reduction of the monocyte-specific surface marker CD14, increased expression of CD68, and exposure of CD71.

The annexin A2 heterotetramer has previously been identified as a receptor transducing the plasmin-mediated signaling in monocytes (Laumonnier et al. 2006). In this study the expression of the annexin A2 heterotetramer was investigated in human macrophages. Immunoblots of whole cell lysates revealed that macrophages in comparison to monocytes exhibit an increased expression of both subunits of the annexin A2 heterotetramer, annexin A2 and S100A10 (143 ± 8% and 230 ± 6% compared to monocytes, respectively). By contrast, polymorphonuclear neutrophils, which do not respond to plasmin (Syrovets et al. 1997), do not express annexin A2 (**Figure 16A**). In addition, the expression of the respective subunits the annexin A2 heterotetramer on the macrophage surface was confirmed by FACS (**Figure 16B**) and fluorescence microscopy (**Figure 16C**).

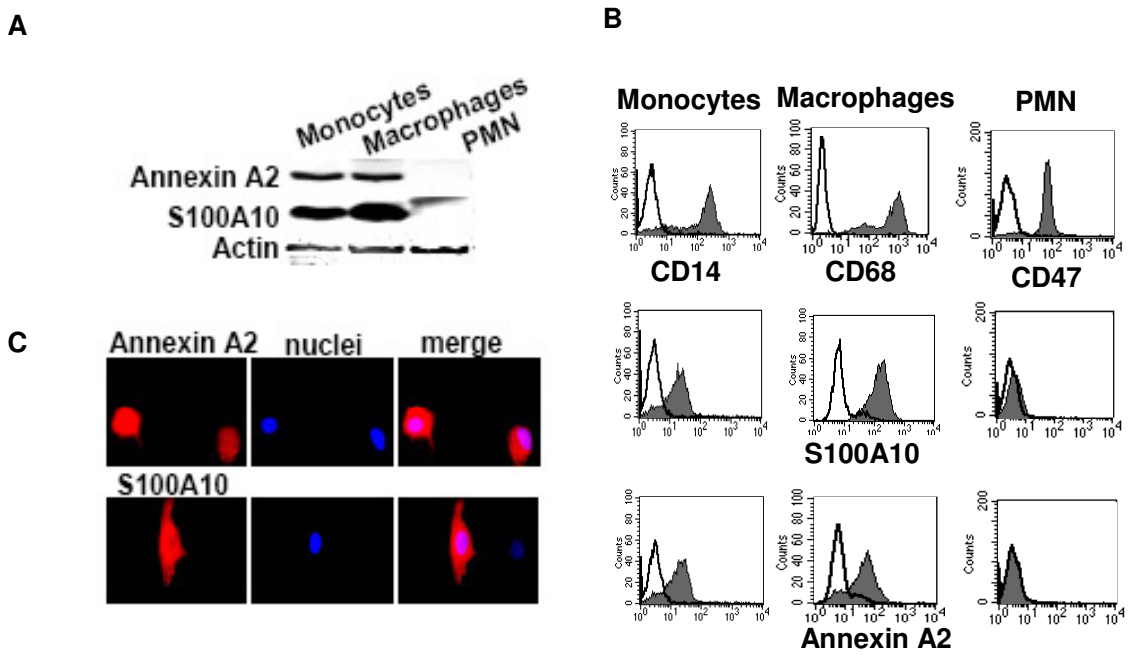


Figure 16. Human macrophages express S100A10 and annexin A2. A, Western immunoblot of annexin A2 and S100A10 in whole cell lysates. Lysates from monocytes, macrophages and neutrophils were separated and blotted onto PVDF membrane. Actin-loading control. B, Surface expression of S100A10 and annexin A2. The cells were incubated with antibodies against CD14 (monocytes), CD68 (macrophages), CD47

(neutrophils), and S100A10 as well as annexin A2. For the analysis of CD68, the cells were fixed and permeabilized with 0.1% Triton X100. After incubation with the corresponding phycoerythrin-labeled $F(ab)_2$, the cells were analyzed by flow cytometry. Empty peaks - isotype controls. C, Immunofluorescence microscopy. Macrophages were fixed and stained with mouse antibodies against annexin A2 or S100A10 followed by staining with anti-mouse Cy5-labeled $F(ab)_2$ (red). Nuclei were counterstained with Hoechst 33258 (blue). Cells were analyzed by fluorescence microscopy. Results are representative of three independent experiments.

Previously, it was demonstrated that plasmin is able to cleave annexin A2 on the monocyte membrane, leading to generation of an annexin A2 fragment of about 33-34 kDa, whereas catalytically-blocked plasmin is unable to generate the cleaved annexin A2 fragment (Laumonnier et al. 2006). Therefore, in this study it was investigated if plasmin is able to cleave annexin A2 on the membrane of macrophages too. Stimulation of macrophages with plasmin led to cleavage of ≈ 6% of annexin A2 as detected in whole cell lysates, yielding a proteolytic fragment of ≈ 33 kDa, whereas S100A10 remained unaffected (**Figure 17A**). This cleavage is dependent on the proteolytic activity of plasmin because exposure of macrophages to catalytically inactivated plasmin failed to produce the fragment (**Figure 17B**). These data suggest that macrophages share with monocytes the annexin A2 heterotetramer as signaling plasmin receptor.

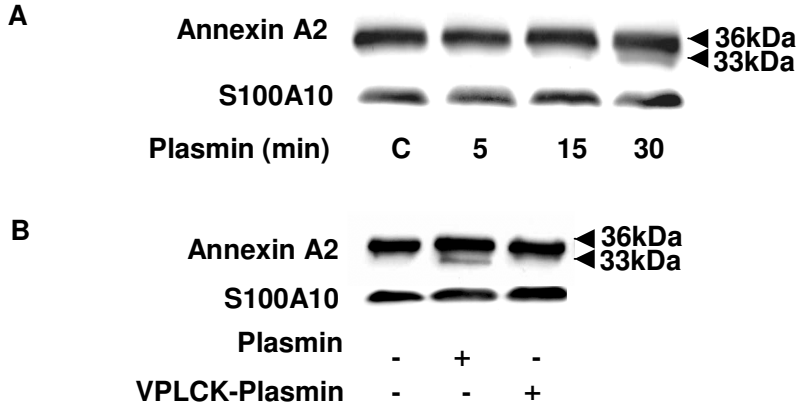


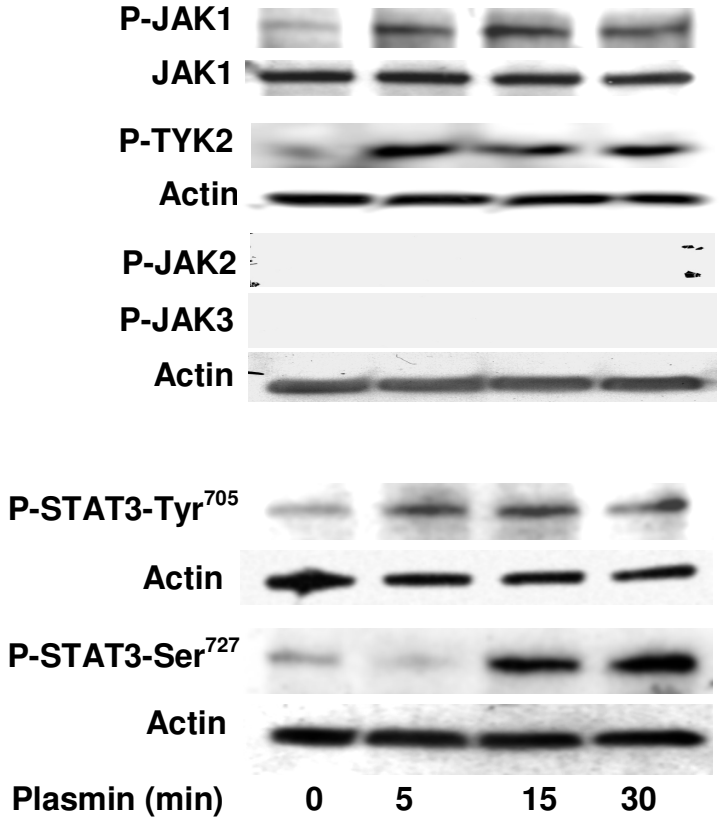
Figure 17. Annexin A2 is a substrate for plasmin. A, Macrophages were stimulated with 0.43 CTA U/ml plasmin for the indicated time (0 min-5 min-15 min-30 min). B, Macrophages were stimulated for 30 min either with 0.43 CTA U/ml plasmin or the equivalent amount of catalytically inactivated plasmin. After treatment, cells were lysed, proteins separated and visualized by immunoblotting with antibodies against annexin A2 and S100A10. VPLCK-plasmin-catalytically inactivated plasmin. Results shown are representative of at least 3 independent experiments.

3.1.3. Plasmin triggers signaling in macrophages

Plasmin may activate multiple signaling pathways. In monocytes it activates the JAK1/STAT, the p38 MAPK (Burysek et al. 2002) and the NF-κB pathway (Syrovets et al. 2001). To explore the activation of JAK kinases, the antibodies against phosphorylated JAK1, JAK2, JAK3 and TYK2 were used in Western immunoblotting of macrophage whole cell lysates (Figure 2A).

Stimulation of macrophages with 0.43 CTA U/ml plasmin triggered tyrosine phosphorylation of JAK1 and TYK2 within 5 min reaching a maximum after 15 min. By contrast, plasmin induced phosphorylation neither of JAK2 nor JAK3. As a consequence of the JAK1/TYK2 activation, phosphorylation of STAT3 on Tyr^{705} was observed within 5 min (**Figure 18A,B**). Transcriptionally active STAT3 requires both Tyr and Ser phosphorylation (Burysek et al. 2002). Indeed, plasmin triggered also STAT3 phosphorylation on Ser^{727} (**Figure 18A,B**).

A



B

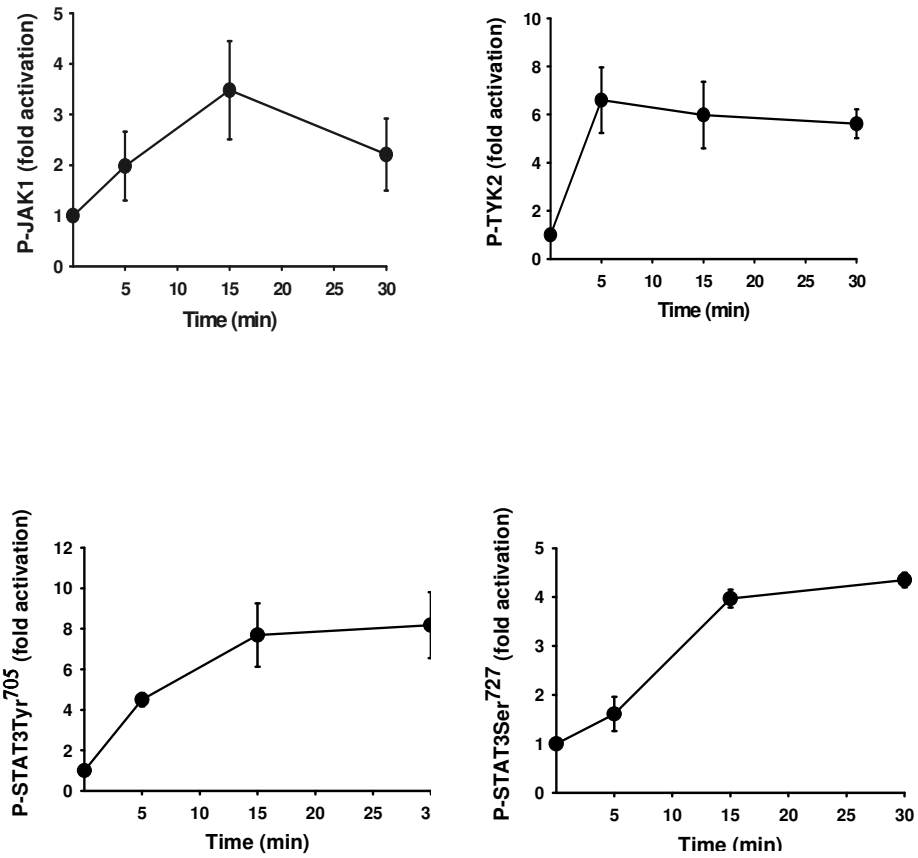


Figure 18. Plasmin triggers activation of JAK/STAT3 signaling pathways. Macrophages ($1x10^6$/assay) were stimulated with plasmin (0.43 CTA U/ml) in serum-free RPMI 1640 for the indicated time (0 min-5 min-15 min-30 min). Whole cell lysates were separated and analyzed by Western blotting (A) and densitometric scanning of Western immunoblots (B). Time-dependent activation of JAK/STAT3. Analysis of phospho-JAK1 (P-JAK1), phospho-JAK2 (P-JAK2), phospho-JAK3 (P-JAK3), phospho-TYK2 (P-TYK2), phospho-STAT3-Tyr^{705} and phospho-STAT3-Ser^{727}. JAK1 and actin served as loading controls. All results are mean ± SEM of at least 3 independent experiments.

In addition to STAT3 activation, plasmin elicits phosphorylation of p38 MAPK after 5 min of stimulation with a marked increase after 15 min (**Figure 19**). Plasmin also activates ERK1/2 within 5 min with the maximum reached after 15 min. During the whole observation time, we did not detect any activation of SAPK/JNK, the third family member of the MAP kinases.

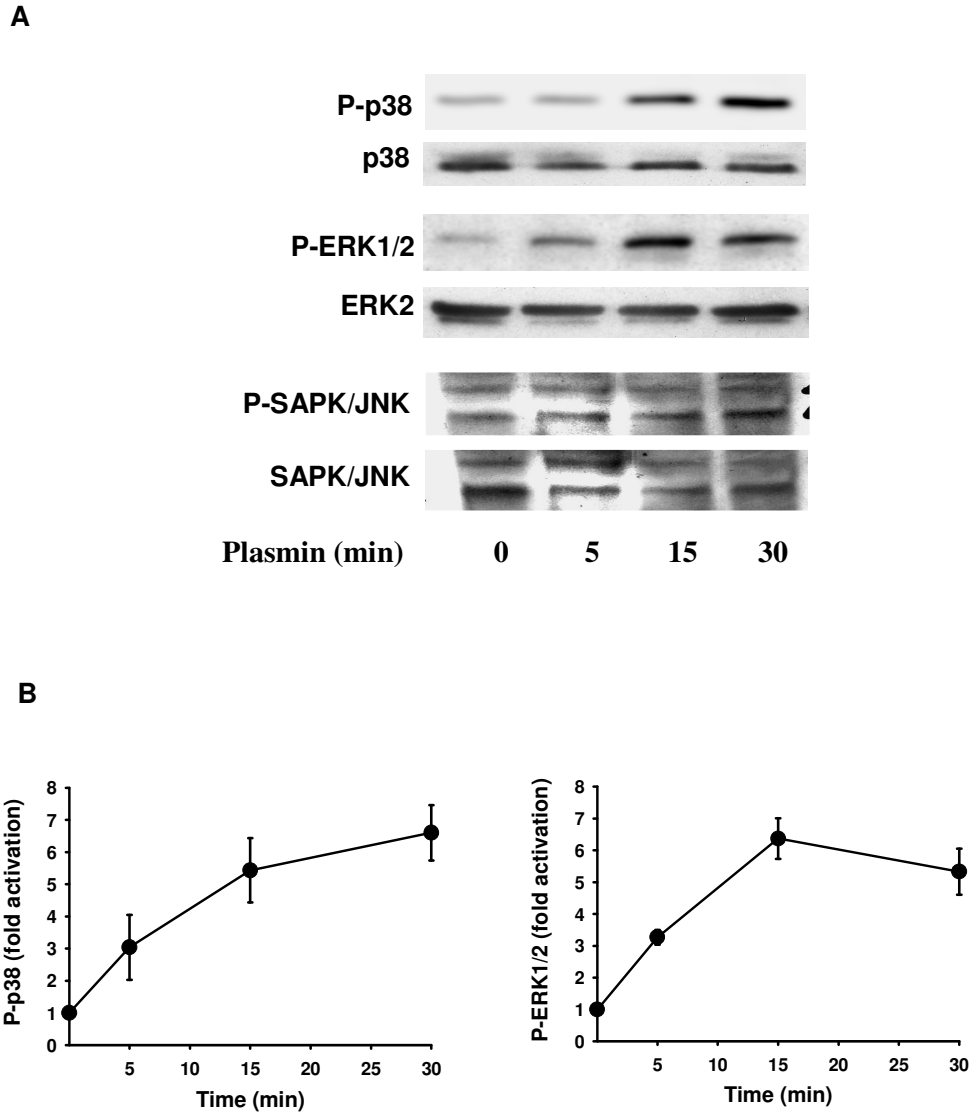


Figure 19. Plasmin triggers activation of MAPK signaling pathways. Macrophages ($1x10^6$/assay) were stimulated with plasmin (0.43 CTA U/ml) in serum-free RPMI 1640 for the indicated time (0 min-5 min-15 min-30 min). Whole cell lysates were separated and analyzed by Western blotting (A) and densitometric scanning of Western immunoblots (B). Time-dependent activation of MAPK. Analysis of phospho-p38, phospho-ERK1/2 and phospho-SAPK/JNK, p38, ERK2 or SAPK/JNK served as loading control. All results are mean ± SEM of at least 3 independent experiments.

As plasmin may activate NF-κB signaling (Syrovets et al. 2001), the phosphorylation state of the NF-κB inhibitor IκBα was investigated by immunoblotting. Indeed, plasmin induced phosphorylation of IκBα, targeting the inhibitor to proteosomal degradation allowing nuclear translocation of NF-κB.

Akt kinase is an upstream effector of NF-κB signaling (Ozes et al. 1999). Therefore, we analyzed Akt activation by immunoblotting. Plasmin induced a time-dependent phosphorylation of Akt at Ser^{473} that is required for the full activation of Akt (Alessi et al. 1996) (**Figure 20**).

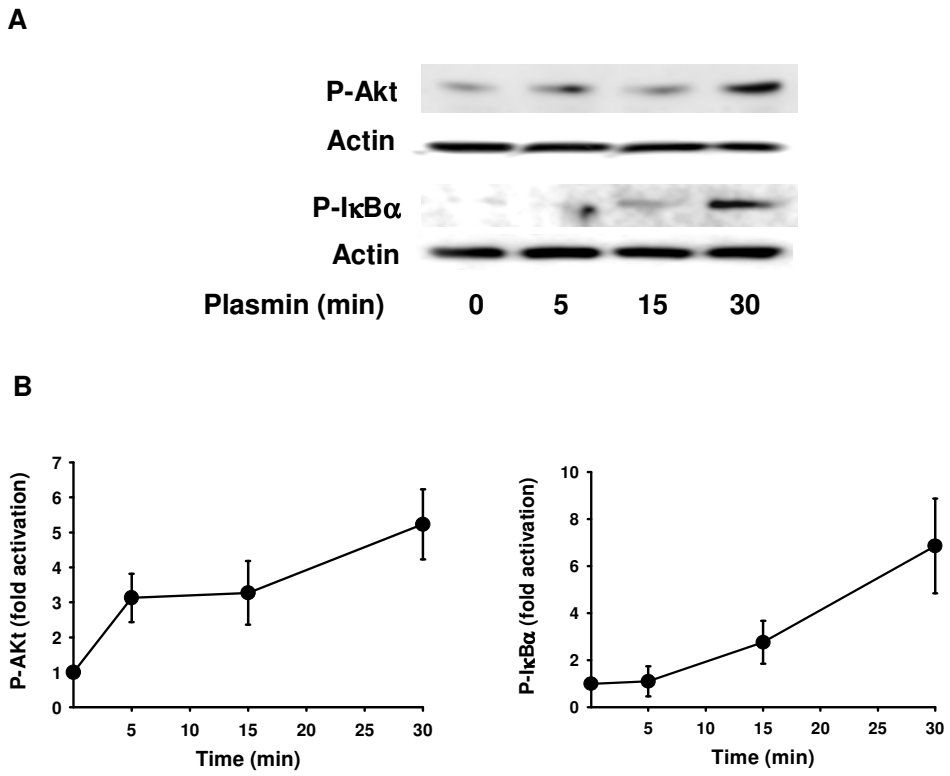


Figure 20. Plasmin triggers activation of NF-κB signaling pathways. Macrophages ($1x10^6$/assay) were stimulated with plasmin (0.43 CTA U/ml) in serum-free RPMI 1640 for the indicated time (0 min-5 min-15 min-30 min). Whole cell lysates were separated and analyzed by Western blotting (A) and densitometric scanning of Western immunoblots (B). Time-dependent activation of the NF-κB pathway. Analysis of phospho-Akt-Ser^{473} and phospho-IκBα from whole cell lysates from macrophages stimulated with plasmin. All results are mean ± SEM of at least 3 independent experiments.

These data show that in macrophages plasmin triggers several signaling pathways, in particular JAK1/STAT3, p38 and ERK1/2 MAPK, and NF-κB.

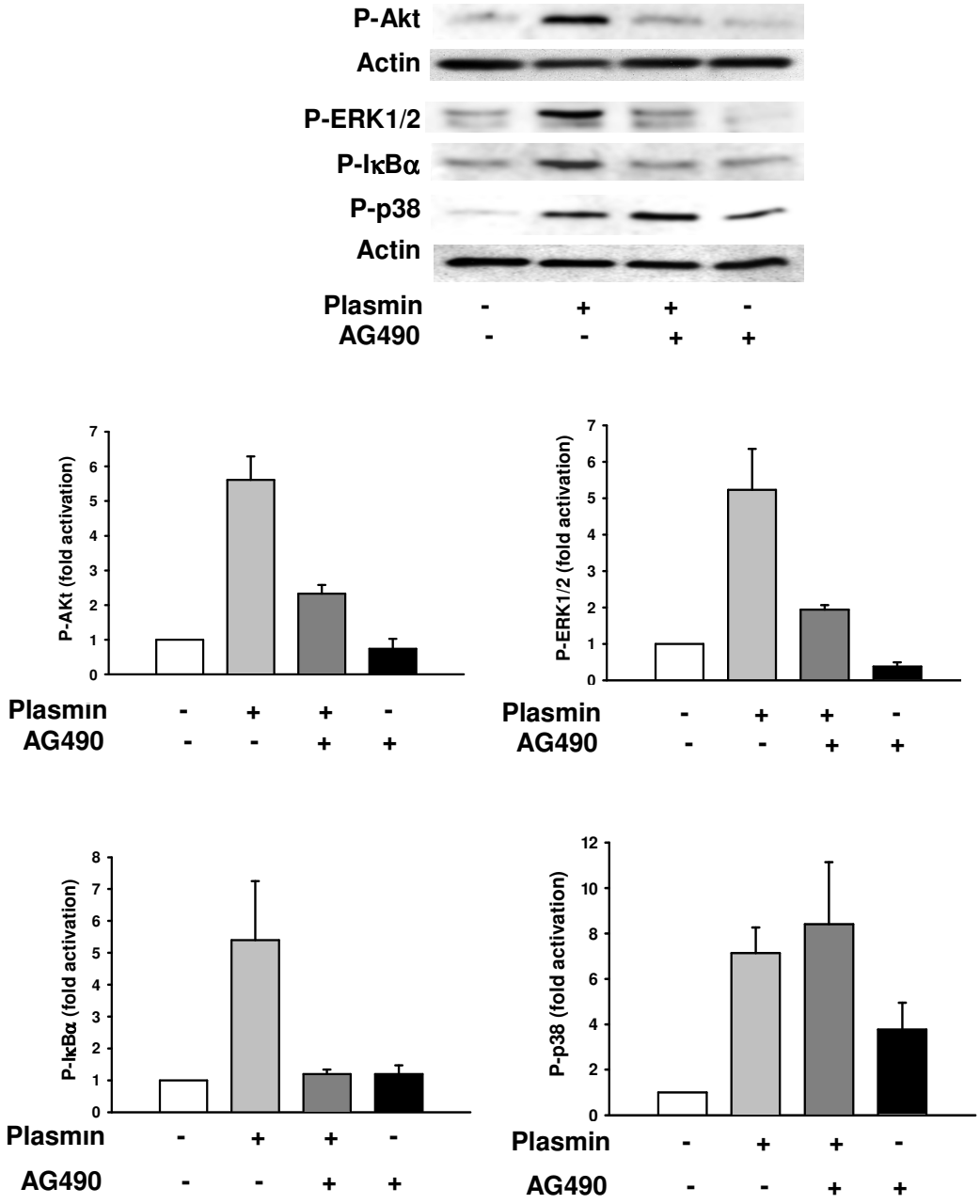


Figure 21. Effects of the JAK inhibitor, AG490, on the plasmin-induced MAPK and NF-κB signaling pathways. Cells pretreated with 50 μM AG490 for 30 min were stimulated with 0.43 CTA U/ml plasmin for 30 min. Whole cell lysates were separated and analyzed by Western blotting. The blots were densitometrically scanned. Analysis of phospho-ERK1/2, phospho-Akt-Ser^{473}, phospho-IκBα, and phospho-p38. Non-phosphorylated proteins and actin served as loading controls. All results are mean ± SEM of at least 3 independent experiments.

To address the role of JAK1 and TYK2 activation in the regulation of downstream targets such as the Akt1, ERK1/2, IκBα and p38 MAPK, we used the JAK inhibitor AG490 (**Figure 21**). At 50 μM, AG490 inhibits JAK1 but does not inhibit TYK2 (Zhang et al. 2000) . Immunoblots of whole cell lysates from macrophages pretreated for 30 min with AG490 and stimulated with plasmin for 30 min revealed that the inhibitor impairs the phosphorylation of ERK1/2, Akt1, and the subsequent phosphorylation of IκBα, but not that of p38 MAPK (**Figure 21**) suggesting that both ERK1/2 and Akt, but not the p38 MAPK, are directly dependent on JAK1 activation.

3.1.4. Plasmin induces nuclear translocation of STAT and NF-κB transcription factors in macrophages

To define the profile of the plasmin-induced nuclear translocation of STAT and NF-κB proteins, we analyzed nuclear extracts from plasmin-stimulated or control macrophages with ELISAs for the respective transcription factors. Plasmin triggered activation and nuclear translocation of STAT3, but not of STAT1, STAT5A, or STAT5B (**Figure 22A**). Similarly, plasmin-stimulated macrophages exhibit binding of p65, but not of RelB, nor c-Rel (**Figure 22B**). Thus, stimulation of macrophages with plasmin yields nuclear translocation of transcriptionally active STAT3 and p65 transcription factors.

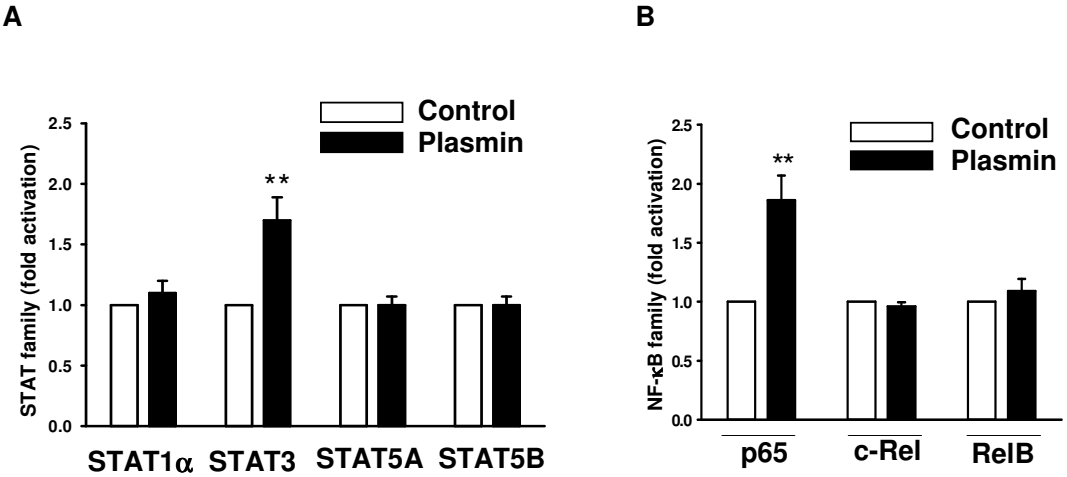


Figure 22. Plasmin induces nuclear translocation of STAT3 and NF-κB transcription factors. A, STAT-1α, 3, 5A and 5B activation were assayed with the STAT family TransAM. Nuclear extracts were prepared from macrophages stimulated with plasmin for 15 min. B, p65, c-Rel and RelB activation were assayed with the NF-κB family TransAM. Nuclear extracts were from macrophages stimulated with plasmin for 1 h. The results

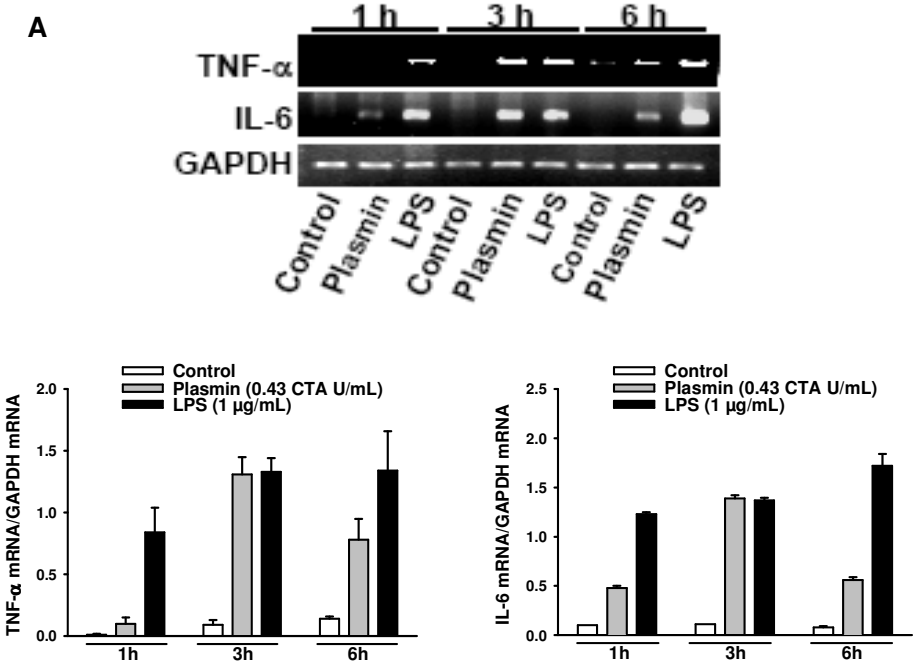
represent the mean ± SEM fold activation of translocated transcription factors compared to control cells of 3-4 independent experiments. ** $P<0.01$ vs control.

3.1.5. Plasmin elicits expression of proinflammatory cytokines in macrophages

Since activated STAT3 and p65 can induce gene expression, the ability of plasmin to elicit expression of proinflammatory cytokines, which are controlled by these transcription factors, was analyzed.

Plasmin (0.43 CTA U/ml) induced a time-dependent increase of TNF-α and IL-6 mRNA (**Figure 23A**). Stimulation with LPS (1 µg/ml), a positive control, induced the expression of both TNF-α and IL-6 mRNA earlier than plasmin, with TNF-α and IL-6 mRNA detectable already 1 h after stimulation. The expression of TNF-α and IL-6 mRNA in macrophages stimulated with plasmin for 3 h was also concentration-dependent (**Figure 23B**). Although 0.043 CTA U/ml plasmin increased already TNF-α and IL-6 mRNA, the optimum response was reached at 0.43 CTA U/ml. At 1.43 CTA U/ml plasmin, there was almost no further increase in the expression levels of both cytokines.

The proteolytic activity of plasmin was shown to be indispensable for the activation of signaling pathways in monocytes (Syrovets et al. 1997; Laumonnier et al. 2006). Therefore, it's role in TNF-α and IL-6 expression was investigated. Catalytically inactivated plasmin failed to stimulate the expression of TNF-α and IL-6 mRNA (**Figure 23C**). Thus, activation of the proinflammatory cytokines by plasmin in macrophages requires proteolytically active plasmin.



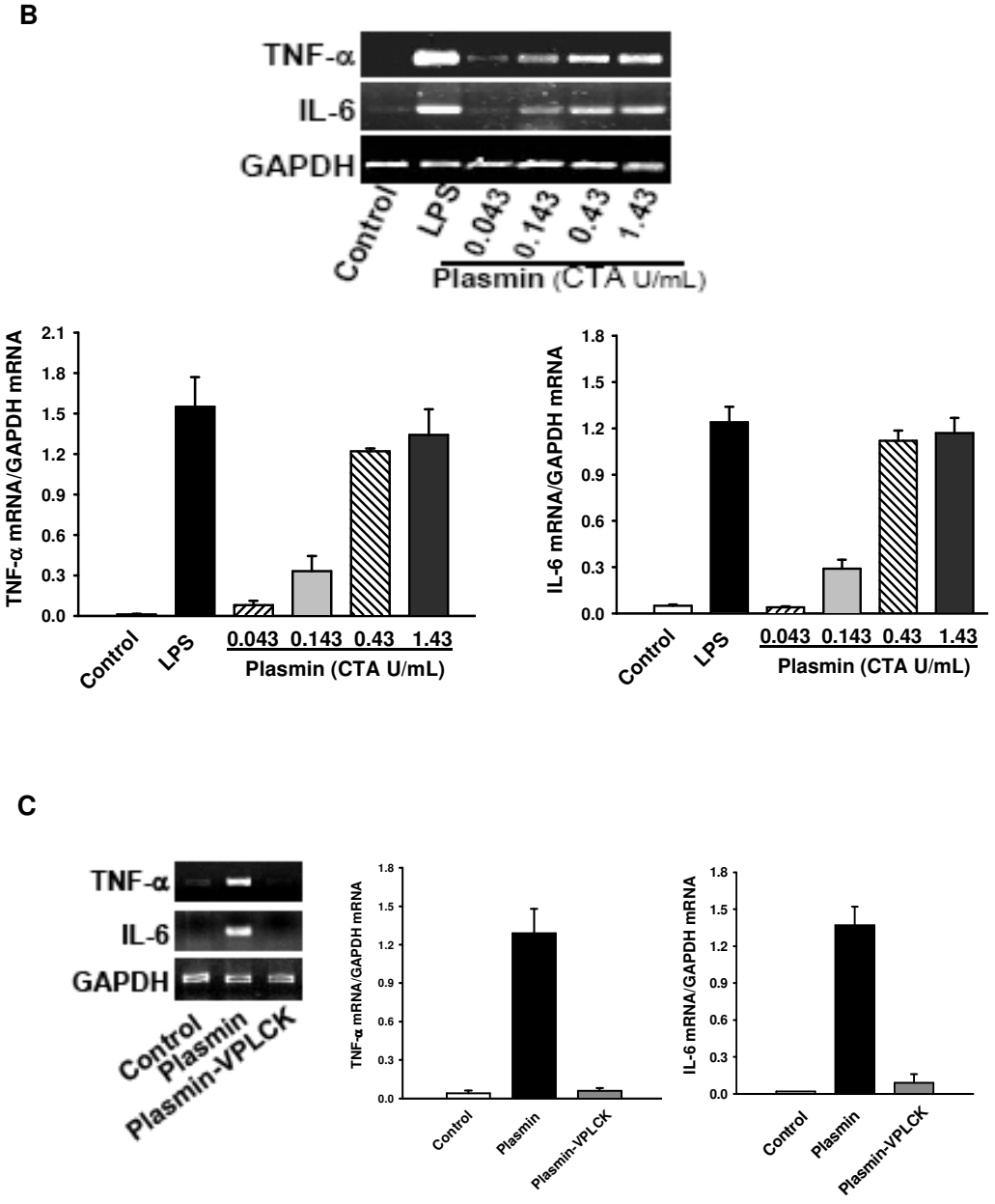


Figure 23. Plasmin triggers time- and concentration-dependent expression of TNF-α and IL-6 genes. A, Time-dependent and B, concentration-dependent stimulation of the TNF-α and IL-6 mRNA expression. Macrophages were stimulated with plasmin (0.43 CTA U/ml) or LPS (1 μg/ml) for the indicated times (1 h-3 h-6 h or 3 h) and the mRNA levels were analyzed by RT-PCR and densitometry. C, The gene expression depends on the proteolytic activity of plasmin. Macrophages were stimulated for 3 h with plasmin (0.43 CTA U/ml) or with equivalent amounts of catalytically inactivated plasmin; TNF-α and IL-6 mRNA expressions were analyzed by RT-PCR and densitometry. GAPDH served as loading control. In each case, results are means ± SEM of 3 independent experiments.

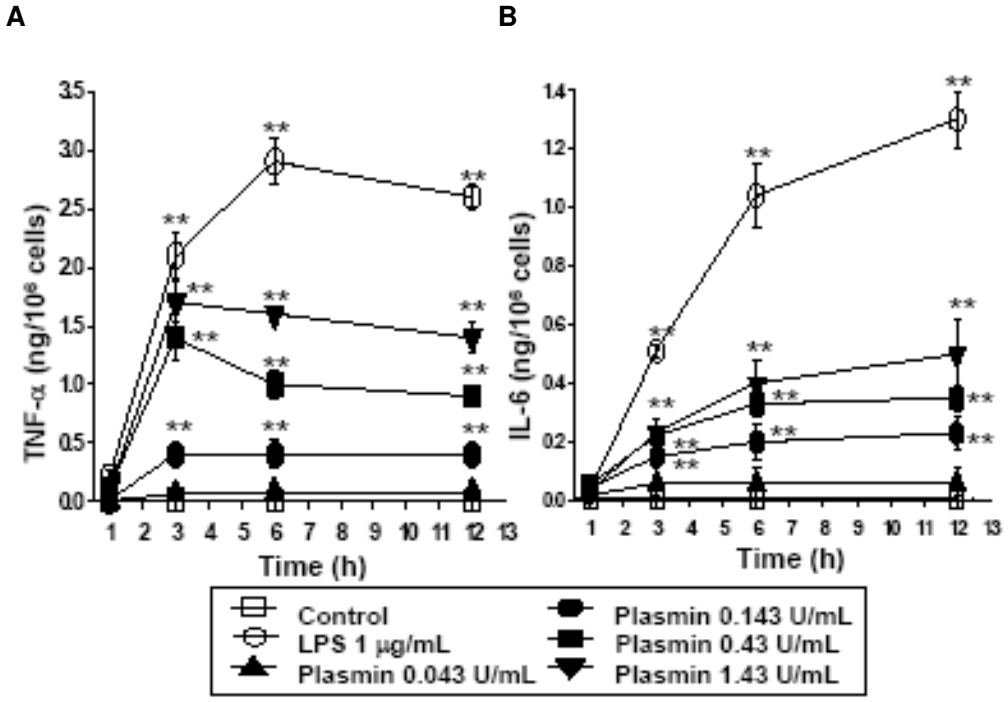


Figure 24. Plasmin induces a time- and concentration-dependent release of TNF-α and IL-6. A, Release of TNF-α from plasmin-stimulated macrophages. B, Release of IL-6 from plasmin-stimulated macrophages. Macrophages were stimulated with plasmin or the positive control LPS (1 µg/ml) for the indicated time. The supernatants were recovered and the concentrations of TNF-α and IL-6 were measured by ELISA. Data are presented as the mean ± SEM of 4 independent experiments. ** $P<0.01$ vs control.

Similar to LPS (1 µg/ml), plasmin induced a time- and concentration-dependent release of TNF-α and IL-6 from human macrophages (**Figure 24A,B**). The cytokines became detectable as early as 3 h after stimulation. The release of TNF-α reached a maximum within 3 h after plasmin and within 6 h after LPS stimulation. Compared to TNF-α, the time-course of IL-6 release appeared somewhat delayed, starting after 3 h, but continuing up to 6-12 h. Generally, LPS triggered a more rapid and extensive release of cytokines, indicating differences between the LPS- and plasmin-mediated activation of macrophages.

These data show that plasmin triggers expression and release of proinflammatory cytokines such as TNF-α and IL-6 in macrophages with a somewhat lower potency than the standard stimulus LPS.

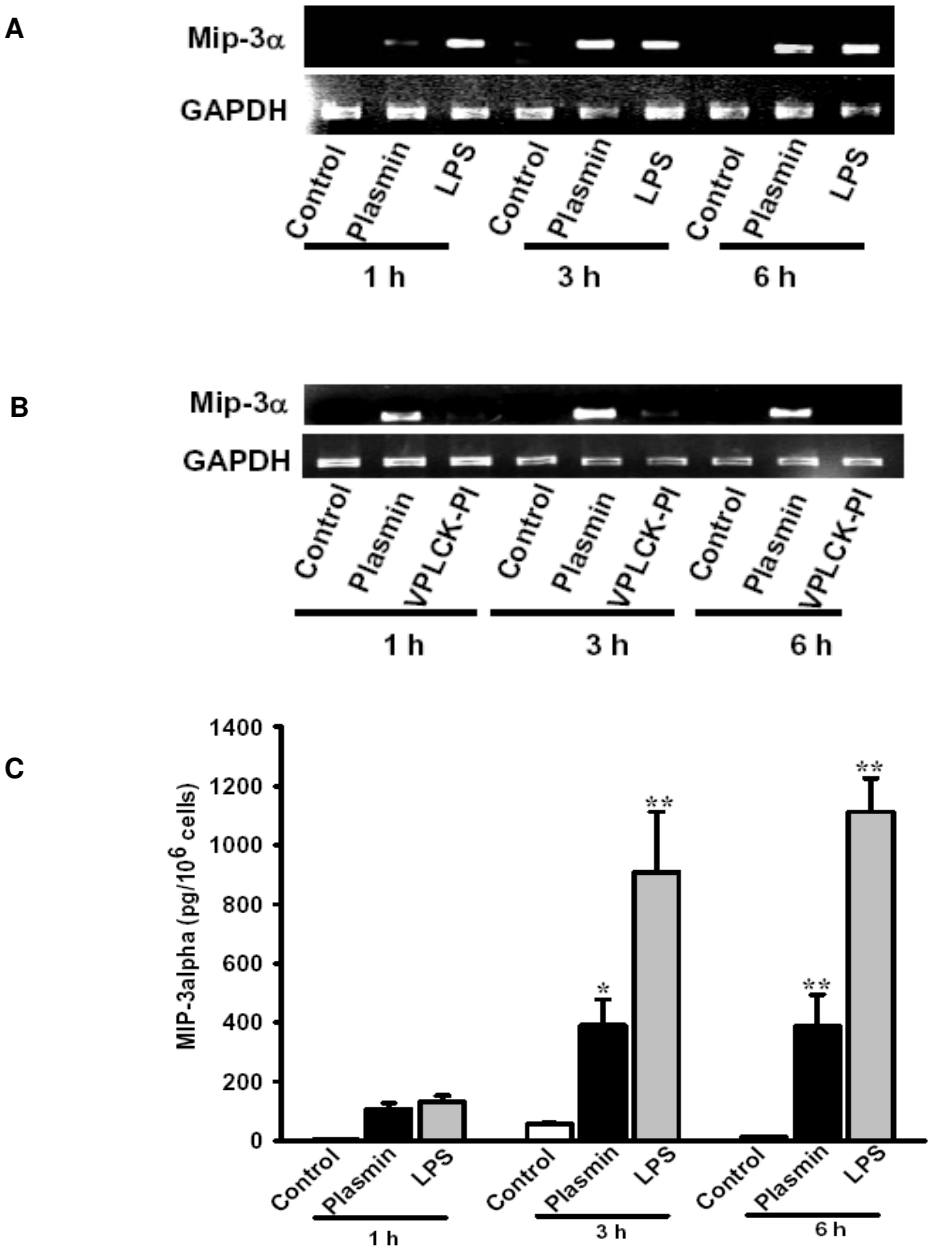


Figure 25. Plasmin induces MIP-3α expression in macrophages. A, Time-dependent expression of MIP-3α mRNA. B, MIP-3α expression depends on the proteolytic activity of plasmin. Macrophages were stimulated with plasmin (0.43 CTA U/ml) or LPS (1 μg/ml) or with equivalent amounts of catalytically inactivated plasmin for the indicated times and the mRNA levels were analyzed by RT-PCR. VPLCK-plasmin-catalytically inactivated plasmin. GAPDH served as loading control. A&B, results are one of 3 independent experiments. C, The supernatants were recovered and the concentrations of MIP-3α were measured by ELISA. Data are presented as the mean ± SEM of 3 independent experiments. **P<0.01 vs control.

We also investigated if plasmin (0.43 CTA U/ml) induces increase of MIP-3α mRNA (**Figure 25A**). Similar to TNF-α and IL-6 expression, a positive control LPS (1 μg/ml) induced a rapid expression of MIP-3α mRNA detectable already 1 h after stimulation. Plasmin induced MIP-3α expression 3 h after treatment. The role of the proteolytic activity of plasmin in the MIP-3α expression was also examined. Catalytically inactivated plasmin failed to stimulate the expression of MIP-3α mRNA (**Figure 25B**). Thus, activation of the proinflammatory cytokines by plasmin in macrophages requires proteolytically active plasmin. Finally, expression of MIP-3α protein by ELISA (**Figure 25C**) was further analyzed. Similarly to LPS (1 μg/ml), plasmin induced a time-dependent release of MIP-3α from human macrophages. The cytokine was detectable as early as 1-3 h after stimulation. The release of MIP-3α reached a maximum within 3 h after plasmin and within 6 h after LPS stimulation.

Thus, in addition to proinflammatory TNF-α and IL-6, plasmin induced expression of chemotactic MIP-3α.

3.1.6. Plasmin-induced activation of JAK1, p38, ERK1/2, and NF-κB is indispensable for the cytokine expression in macrophages

To gain evidence for the role of the identified signaling pathways in the plasmin-induced release of TNF-α, IL-6 and MIP-3α, macrophages were pretreated with inhibitors for JAK1 (AG490), p38 MAPK (SB203580), the MAPK/ERK kinase pathway (U0126) and an inhibitor of NF-κB (acetyl-11-keto-β-boswellic acid, AKβBA) (Syrovets et al. 2005) before stimulation with plasmin. The supernatants were collected at 3 h (TNF-α), 6 h (IL-6) and 3 h (MIP-3α) after plasmin (0.43 CTA U/ml) stimulation and analyzed by ELISA. Pretreatment of macrophages with AG490, SB203580 and AKβBA for 30 min inhibited the plasmin-induced TNF-α release by 62.5 ± 6.0%, 74.6 ± 5.8% and 69.6 ± 13.7% (P<0.01), respectively. In contrast, inhibition of ERK1/2 by the MEK inhibitor U0126 10 μM did not affect the plasmin-mediated expression of TNF-α (**Figure 26A**).

Similar to the TNF-α production, pretreatment of macrophages with AG490 and SB203580 significantly decreased the plasmin-induced release of IL-6 by 76.2 ± 10.9% and 79.3 ± 10.5%, respectively (**Figure 26B**, P<0.05). Not unexpected, the plasmin-induced activation of the NF-κB was essential for the expression of IL-6 too, because it was inhibited by AKβBA by 65.8 ± 11.4% (P<0.05). Activation of ERK1/2 was indispensable to the plasmin-induced release of IL-6, because the MEK inhibitor U0126

reduced its release by 86.2 ± 5.3% (*P*<0.01, **Figure 26B**). Differently, the plasmin-mediated expression of MIP-3α was impaired only by p38 MAPK inhibitor SB203580 (**Figure 26C**).

These data point to the pivotal roles of the JAK1/STAT3, MAPK and NF-κB signaling pathways in the plasmin-mediated expression of TNF-α, IL-6 and MIP-3α in human macrophages.

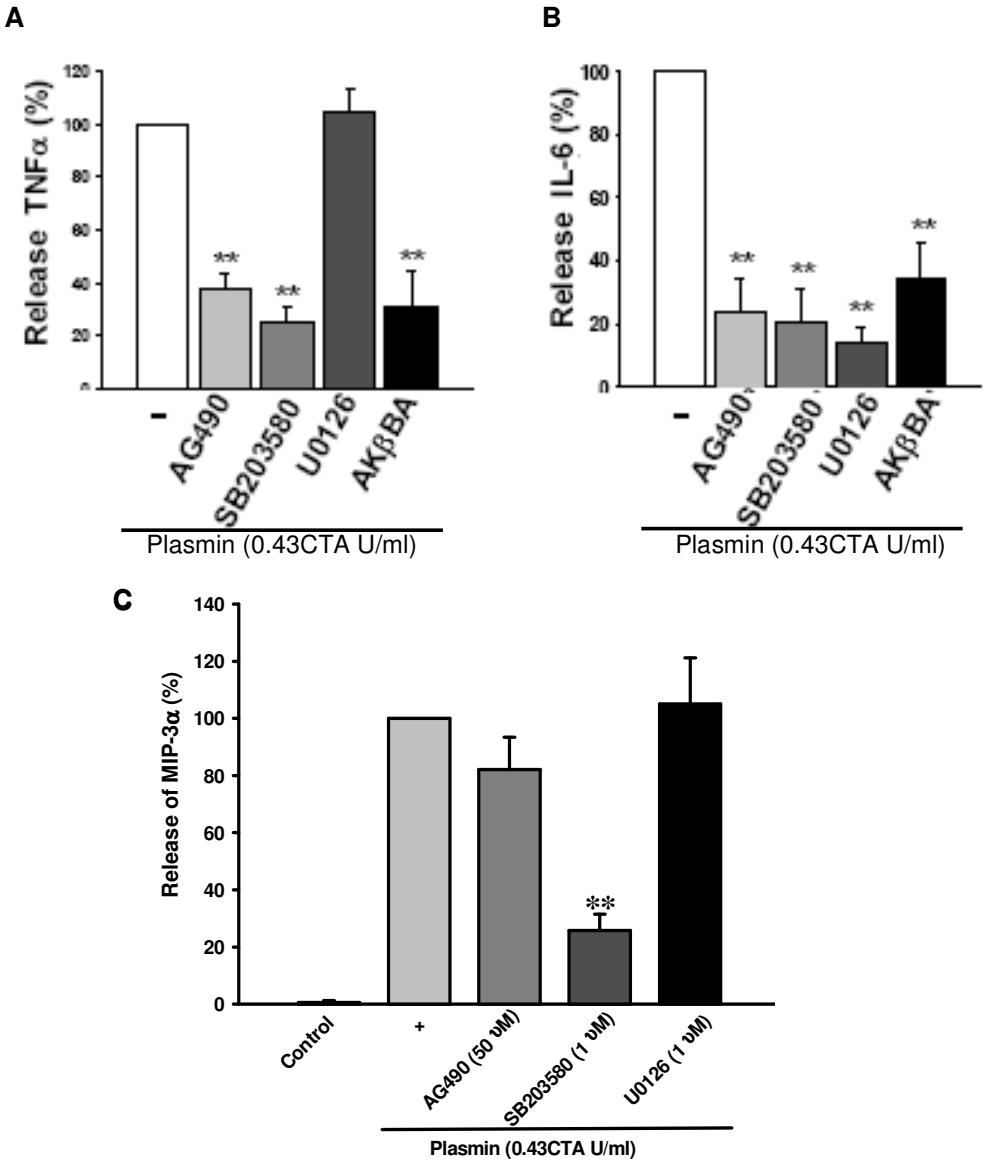


Figure 26. Plasmin induces release of TNF-α and IL-6, which requires activation of JAK1, p38 MAPK, ERK1/2 MAPK and NF-κB. Plasmin-induced release of MIP-3α requires activation of p38 MAPK. A, Effects of pharmacological inhibitors on the TNF-α release. B, Effects of pharmacological inhibitors on the IL-6 release. C, Effects of pharmacological inhibitors on the MIP-3α release. Macrophages preincubated with the indicated inhibitors for 30 min were stimulated with plasmin (0.43 CTA U/ml); Expression of TNF-α, IL-6 and MIP-3α was analyzed by ELISA after 3 h or 6 h. AG490

50 μM (JAK1/2 inhibitor), SB203580 1 μM (p38 MAPK inhibitor), U0126 10 μM (MEK inhibitor) and AKβBA 10 μM (NF-κB inhibitor). Control cells were treated with solvent. The data represent the mean ± SEM of 3 (TNF-α, MIP-3α) or 8 (IL-6) independent experiments. ** P<0.01 vs control.

3.1.7. The annexin A2 heterotetramer is a plasmin receptor in macrophages

The role of the annexin A2 heterotetramer in the plasmin-induced expression of cytokines in macrophages was studied by antisense ODN experiments. Treatment of the cells for 48 h with antisense ODN directed against the annexin A2 or the S100A10 proteins induced a marked decrease of annexin A2 or S100A10 protein expression, which was not observed in untreated macrophages or cells treated with the control sense ODN (**Figure 27A**). Consistent with the notion that annexin A2 stabilizes S100A10 (Choi et al. 2003; MacLeod et al. 2003), down-regulation of annexin A2 concomitantly decreased the expression of S100A10. By contrast, reduction of the S100A10 expression did not affect the expression of annexin A2 (**Figure 27A,B**).

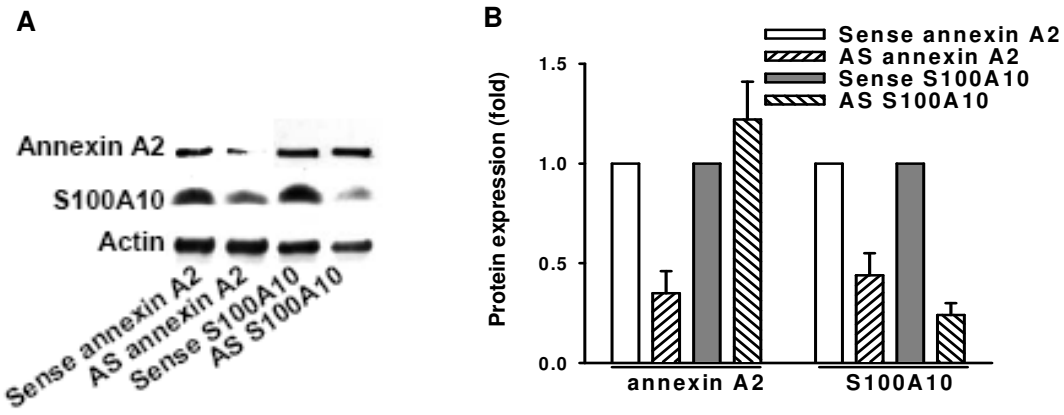


Figure 27. Specific antisense ODNs decrease the expression of annexin A2 and S100A10. A, Macrophages were treated for 48 h with 10 μM of the corresponding ODN (sense or antisense). After 12 h recovery in RPMI 1640 without lysine, the cells were lysed, the proteins were separated and analyzed by immunoblotting, and B, Densitometric scanning of immunoblots. Actin served as loading control. The protein expression in sense ODN-treated samples was set to one. The data represent the mean percentage ± SEM of 4 independent experiments.

Pretreatment of macrophages with annexin A2 antisense ODN profoundly inhibited the TNF-α and IL-6 release by 85.8 ± 2.8% and a 60.8 ±1 0.5%, respectively (P<0.01, **Figure 28A,B**). Similarly, pretreatment with S100A10 antisense ODN resulted in significant inhibition of TNF-α and IL-6 release by 73.3 ± 15.5% and 64.0 ± 7.5%, respectively

(*P*<0.01, **Figure 28A,B**). Treatment of macrophages with ODN without plasmin stimulation had no effect neither on the TNF-α nor the IL-6 release (**Figure 28A,B**).

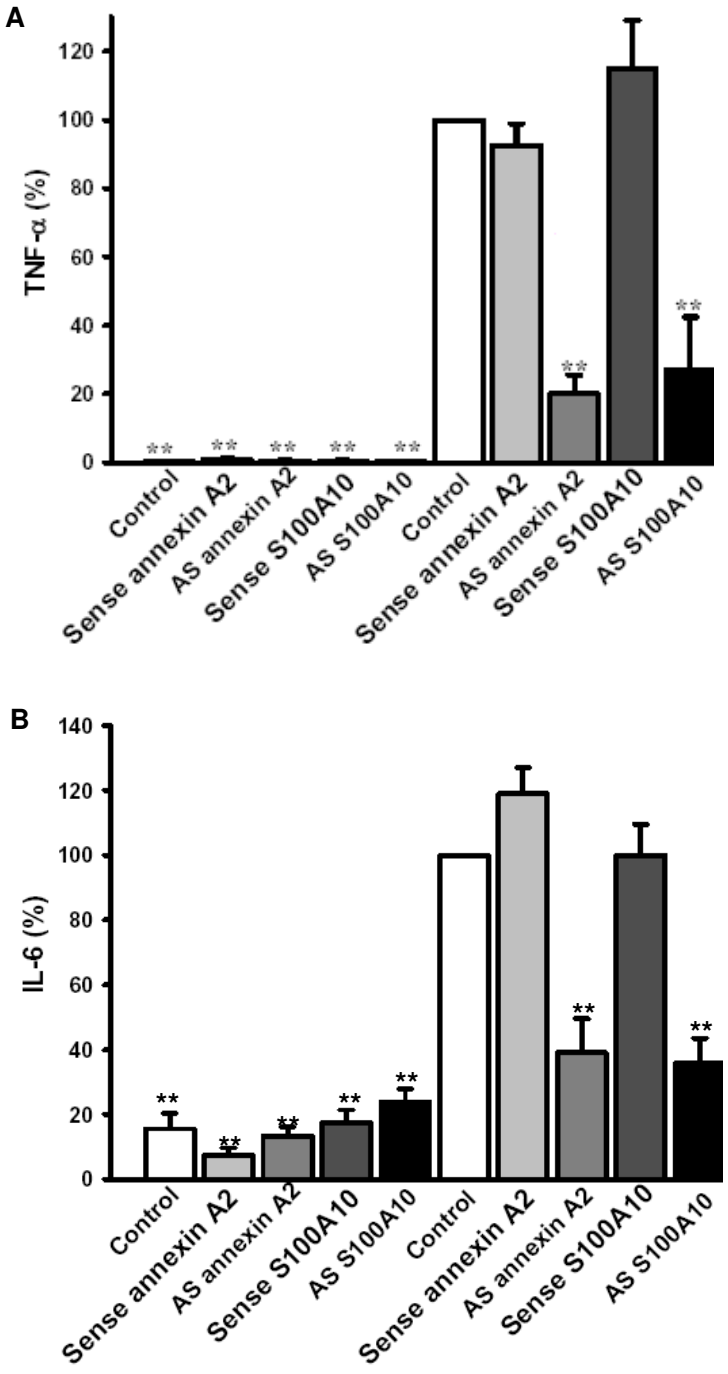


Figure 28. The annexin A2 heterotetramer is indispensable for the plasmin-stimulated release of cytokines by the macrophages. A, Down-regulation of the annexin A2 and S100A10 expression by antisense ODN inhibits the plasmin-induced release of TNF-α. B, Down-regulation of the annexin A2 and S100A10 expression by antisense ODN inhibits the plasmin-induced release of IL-6. Macrophages (controls or treated with sense or antisense ODN) were stimulated with or without plasmin (0.43 CTA

U/ml) for 3 h and 6 h for TNF-α and IL-6 release, respectively. Supernatants were recovered and TNF-α and IL-6 were measured by ELISA. The data represent the mean percentage ± SEM of 4 independent experiments. ** $P<0.01$ vs controls.

These data imply that plasmin stimulates human macrophages through the annexin A2 heterotetramer.

3.1.8. Summary of plasmin-mediated activation of human macrophages

The plasmin-mediated macrophage activation as revealed in the present study is summarized below (**Figure 29**). Plasmin, but not catalytically inactivated plasmin, induces expression of the cytokines such as TNF-α, IL-6 and MIP-3α in human monocyte-derived macrophages. The plasmin-induced signaling utilizes the annexin A2 heterotetramer as receptor that triggers downstream signaling via JAK/STAT, Akt-dependent NF-κB activation as well as ERK1/2 and the p38 MAPK leading to proinflammatory gene expression that has been implicated in the initiation and maintenance of chronic inflammation.

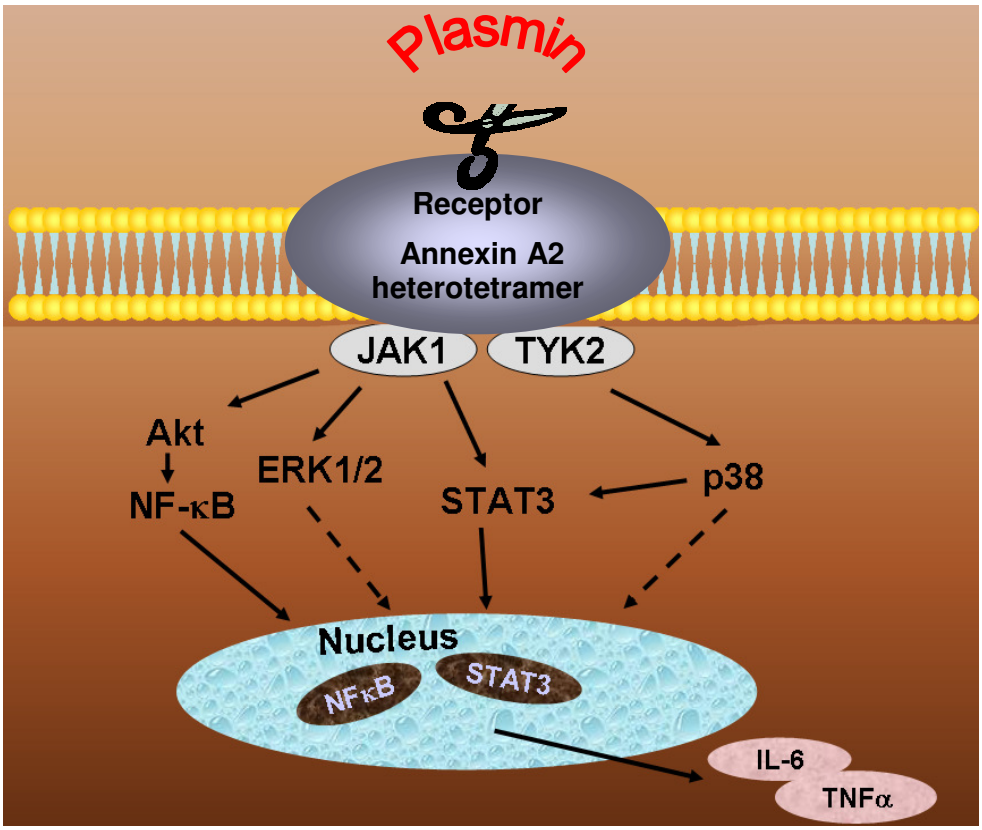


Figure 29. Schematic representation of plasmin-induced signaling in human macrophages.

3.2. A role for the annexin A2 N-terminal peptide (A2NP) in the plasmin-induced activation of human peripheral monocytes

3.2.1. A2NP-induced signaling is similar to that of plasmin

As mentioned above, the annexin A2 heterotetramer composed of annexin A2 and S100A10 has been identified as a plasmin receptor on human monocytes (Laumonnier et al. 2006) and, as defined in the present study, macrophages (Li et al. 2007). On the other hand, it has been demonstrated that full length annexin A2 is a soluble activator of macrophages (Swisher et al. 2007). This study investigated the mechanisms of plasmin-induced signaling in monocytes, which is initiated by the proteolytic cleavage of the annexin A2 subunit of the receptor, followed by the dissociation of the heterotetramer complex.

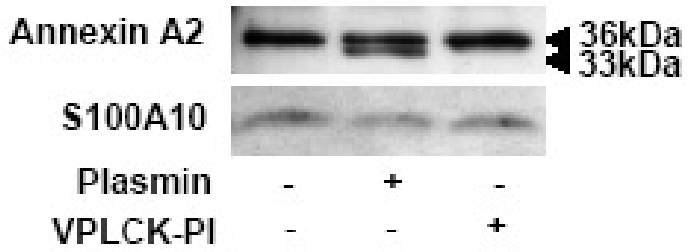


Figure 30. Plasmin cleaves annexin A2 on the surface of monocytes. Monocytes were stimulated for 30 min either with 0.43 CTA U/ml plasmin or the equivalent amount of catalytically inactivated plasmin. After treatment, cells were lysed, proteins separated and visualized by immunoblotting with antibodies against annexin A2 and S100A10. Results shown are representative of at least 3 independent experiments. VPLCK-plasmin-catalytically inactivated plasmin.

The exact mechanism linking the cleavage and dissociation of the receptor complex and activation of the intracellular signaling remains elusive consistent with previous data (Laumonnier et al. 2006). Plasmin cleaves annexin A2 at a single position at the lysine 27 releasing an annexin A2 amino-terminal peptide (residues 1-27, A2NP) and C-terminal peptide (A2IC) (**Figure 30**). In order to test the possibility that one of the proteins generated by cleavage is acting as a ligand initiating signaling, the following experiments were performed. Firstly, we analyzed whether the N-terminal peptide of annexin A2, the A2NP, can stimulate monocytes. As shown in **Figure 31**, increased phosphorylation of JAK1 was detectable after stimulation with either A2NP or plasmin. Thus, A2NP might be

involved in the plasmin-mediated monocytes activation. To analyze whether the effect of A2NP is specific, we used a scrambled peptide, which contains the same amino acids as A2NP, as control. As shown in **Figure 32**, stimulation of monocytes with the A2NP peptide (100 μM), but not with the scrambled peptide, increased the phosphorylation of JAK1/STAT3 similar to that observed in the cells stimulated with 0.43 CTA U/ml plasmin.

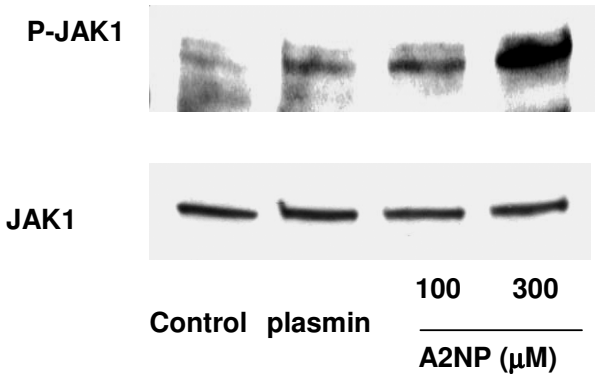


Figure 31. A2NP triggers concentration-dependent activation of JAK1. Monocytes ($2x10^6$/assay) were stimulated with plasmin (0.43 CTA U/ml) or A2NP (100 μM, 300 μM) in serum-free RPMI 1640 for 15 min. Whole cell lysates were separated and analyzed by Western immunoblotting with antibodies against phospho-JAK1 (P-JAK1). Expression levels of JAK1 - loading controls. Results are representative of at least 3 independent

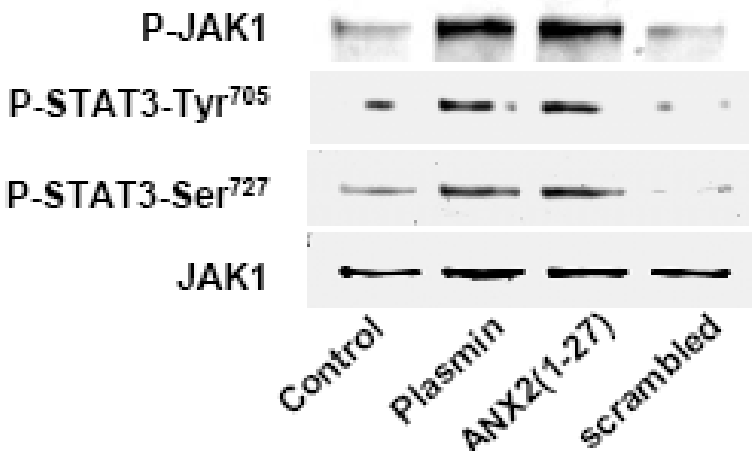


Figure 32. A2NP-induced activation of JAK1/STAT3 is similar to that of plasmin. Monocytes ($3x10^6$/assay) were stimulated with plasmin (0.43 CTA U/ml), A2NP and scrambled (each 100 μM) in serum-free RPMI 1640 for 15 min. Whole cell lysates were separated and analyzed by Western immunoblots with antibodies against phospho-JAK1

(P-JAK1), P-STAT3-Tyr^{705} and P-STAT3-Ser^{727}**.** Expression levels of JAK1 - loading controls. Results are representative of at least 3 independent experiments.

3.2.2. A2NP-induced expression of MCP-1 is similar to that of plasmin

As the A2NP may act as a new activating ligand, which mediates the plasmin-induced signaling in monocytes, we have analyzed whether A2NP similar to plasmin triggers expression of chemotactic MCP-1. MCP-1 protein levels were assayed in the media from monocytes stimulated with 0.43 CTA U/ml plasmin, different concentrations of A2NP and srambled peptide for 24 h by ELISA (**Figure 33**). A2NP triggered concentration-dependent release of MCP-1 from monocytes. A2NP (100 μM). The induced release of MCP-1 (2.6 ng/10^6 cells) was a bit higher than MCP-1 released by 0.43 CTA U/ml plasmin (1.9 ng/10^6 cells).

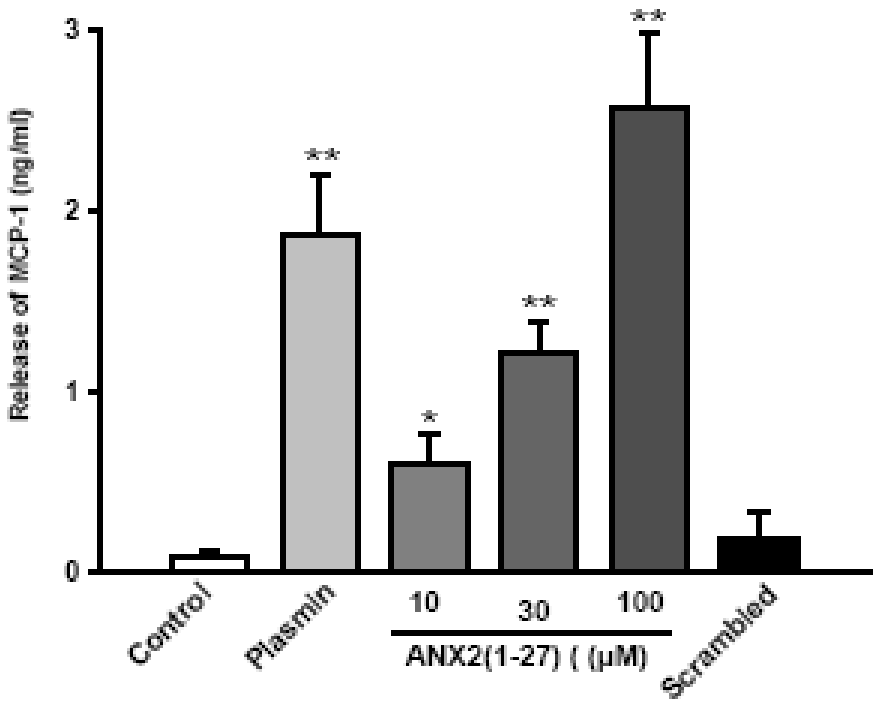


Figure 33. Release of MCP-1 from monocytes stimulated with plasmin, A2NP or scrambled peptide. Monocytes were stimulated with plasmin (0.43 CTA U/ml), A2NP (1-27) (10 μM, 30 μM and 100 μM), or scrambled peptide (100 μM) for 24 h. The supernatants were recovered and expression of MCP-1 was measured by ELISA. Data are presented as the mean ± SEM of 3-6 independent experiments. ** P<0.01 vs control.

A2NP is one of the fragments generated from cleavage of annexin A2 heterotetramer receptor by plasmin in monocytes. These data imply that A2NP which is released during

monocyte stimulation with plasmin may act as a new ligand and may activate monocytes through some known or unknown functional receptor.

3.2.3. Effect of inhibition of protease-activated receptor 1 (PAR1) on plasmin-induced cytokine release in human monocytes

Thus, the A2NP peptide, a new proteolytically-generated signaling molecule, might activate a transmembrane receptor. Plasmin induced-signaling triggers activation of JAK1/STAT3 pathway in monocytes. The JAK/STAT pathway is activated through different types of receptors, mainly the type I cytokine receptor and the G-protein coupled receptors (GPCR), such as PAR1.

Protease-activated receptors are expressed on monocytes (Colognato et al. 2003). Human monocytes express mainly PAR1, a thrombin receptor, and less PAR3. Stimulation of monocytes or macrophages with thrombin or PAR1-AP, triggers expression of MCP-1 (Colognato et al. 2003). Recently, the thrombin receptor PAR1 was implicated in the plasmin-induced expression of the Cyr61 gene in fibroblasts (Pendurthi et al. 2002). In a different study, integrin-bound plasmin enhanced cell migration by a PAR1-dependent mechanism (Majumdar et al. 2004).

The effect of PAR1 on plasmin-induced signaling on monocytes was examined in this study. Monocytes were preincubated either in the presence or absence of increasing concentration of SCH79797 (PAR1 inhibitor) (Strande et al. 2006) for 30 min, then stimulated with plasmin, LPS or thrombin for 24 h. MCP-1 release was measured by ELISA (**Figure 34**). Treatment with plasmin induced MCP-1 release and preincubation with SCH79797 had no inhibitory effect.

To ensure the specific inhibition of PAR1 by SCH79797, two controls were included: LPS, which does not require PAR1 for cell activation (Naldini et al. 2004) and thrombin, which activates cells via PAR1 (Nieman and Schmaier 2007). Thus, treatment of monocytes with LPS served as a negative control and treatment of monocytes with thrombin served as a positive control. Preincubation with SCH79797 had no effect on the LPS-induced MCP-1 release. By contrast, the thrombin-induced MCP-1 release was inhibited by pre-incubation with SCH79797 (**Figure 34**).

Thus, plasmin does not utilize the thrombin receptor PAR1 to activate human monocytes. Plasmin might signal through a type I cytokine receptor or others.

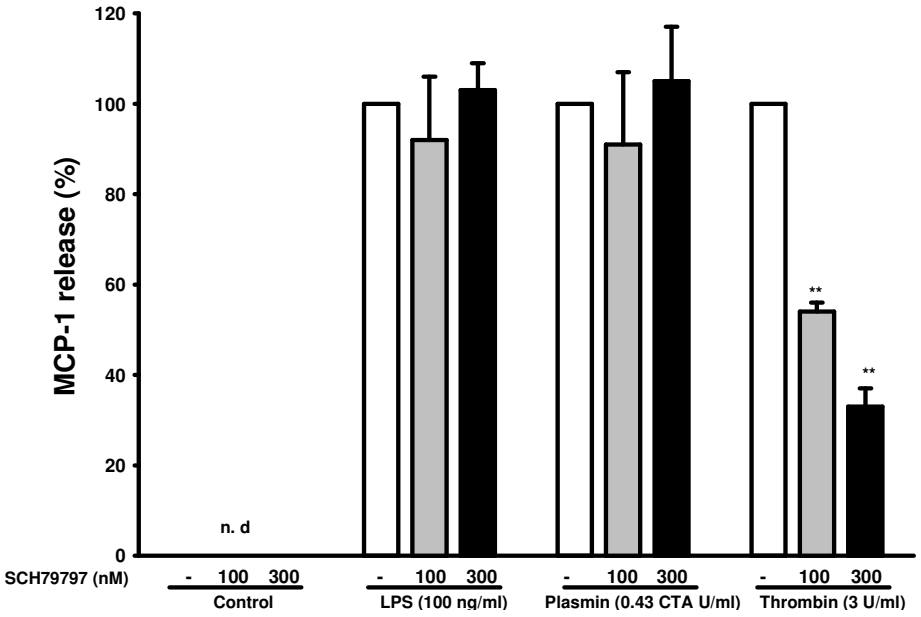


Figure 34. PAR1 is not required for the plasmin-induced MCP-1 release in monocytes. Monocytes preincubated with SCH79797 (100 nM and 300 nM, a PAR1 inhibitor) for 30 min were stimulated with LPS (100 ng/ml), plasmin (0.43 CTA U/ml), and thrombin (6 U/ml). MCP-1 was analyzed by ELISA after 24 h of stimulation. The amount of MCP-1 released in the absence of the inhibitor was set to 100%. The amount of MCP-1 released by LPS was 1.823 ng/10^6 cells, plasmin 0.501 ng/10^6 cells and thrombin 1.225 ng/10^6 cells. The data represent the mean ± SEM of 3 independent experiments.

3.2.4. Expression of members of gp130 family receptors in human monocytes

Plasmin induced-signaling triggers activation of the JAK1/STAT3 pathway in monocytes, which may be due to activation of the type I cytokine receptors of gp130 family.

The type I cytokine receptors include the gp130 subunit as a common co-receptor, and OSMRβ, LIFR as most well-known receptors. Recently a new subunit, IL31Rα was found expressed in monocytic cell line THP1 and was proposed as a functional alternative to gp130 (Diveu et al. 2003; Diveu et al. 2004)

Expression and function of IL-31Rα and most other gp130 family members in human peripheral monocytes has not been investigated, yet. RT-PCR and quantitative real-time PCR demonstrated that human monocytes express high levels of gp130, lower levels of IL-31R mRNA and no LIF-R or OSM-R (**Figure 35A,B**). Western blotting analysis

confirmed expression of gp130 and IL-31Rα proteins (**Figure 36A**). Flow cytometry and immunofluorescence microscopy showed that both receptors are present at the surface of monocytes (**Figure 36B,C**). Primary monocytes were identified by detection of the surface marker CD14 by FACs (**Figure 36B**).

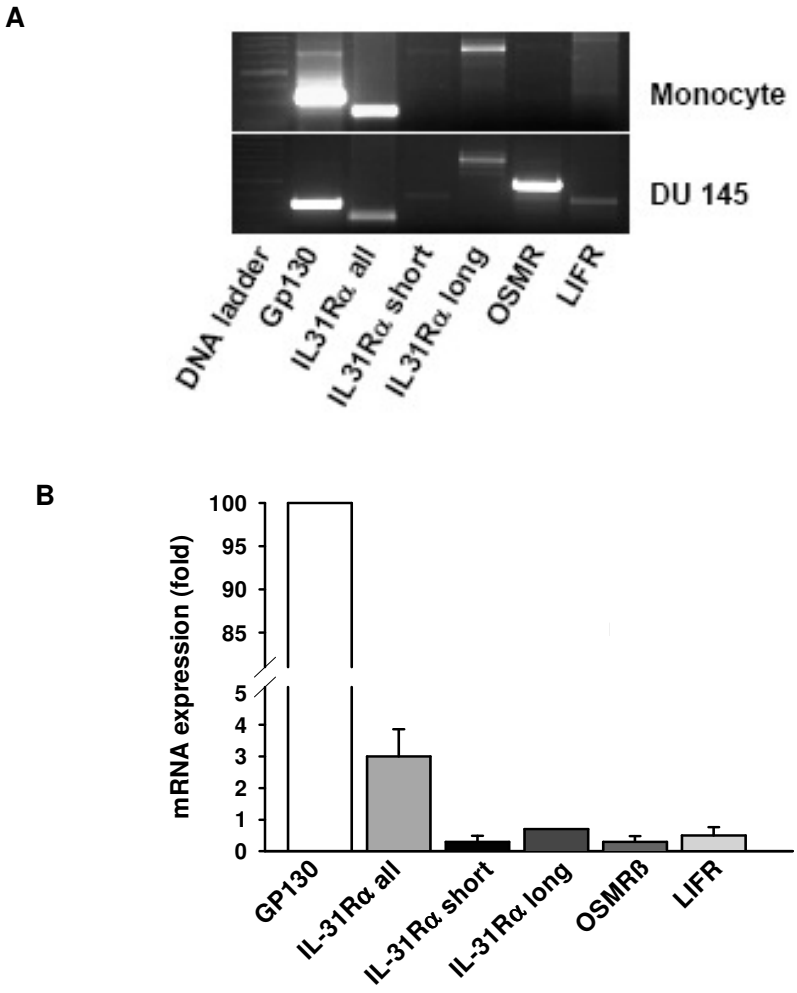


Figure 35. mRNA expression profile of IL-31Rα and other gp130-family members in human peripheral monocytes. A, Gp130, IL-31Rα, OSM-R or LIF-R mRNA levels were analyzed by RT-PCR in monocytes and the DU145 cell line used as positive control for IL-31Rα. cDNAs were synthesized from 2 µg of total RNA by random hexamer primers using MMLV reverse transcriptase. Reverse transcription products were subsequently amplified by 35 cycle of PCR. The picture shows one representative experiment out of two. B, Quantitative real-time PCR analysis using two-step PCR, Applied Biosystems 7300 system and the $2^{-\Delta\Delta ct}$ method. GAPDH served as control. The amount of gp130 mRNA was set to 100%. In each case, results are means ± SEM of 3 independent experiments.

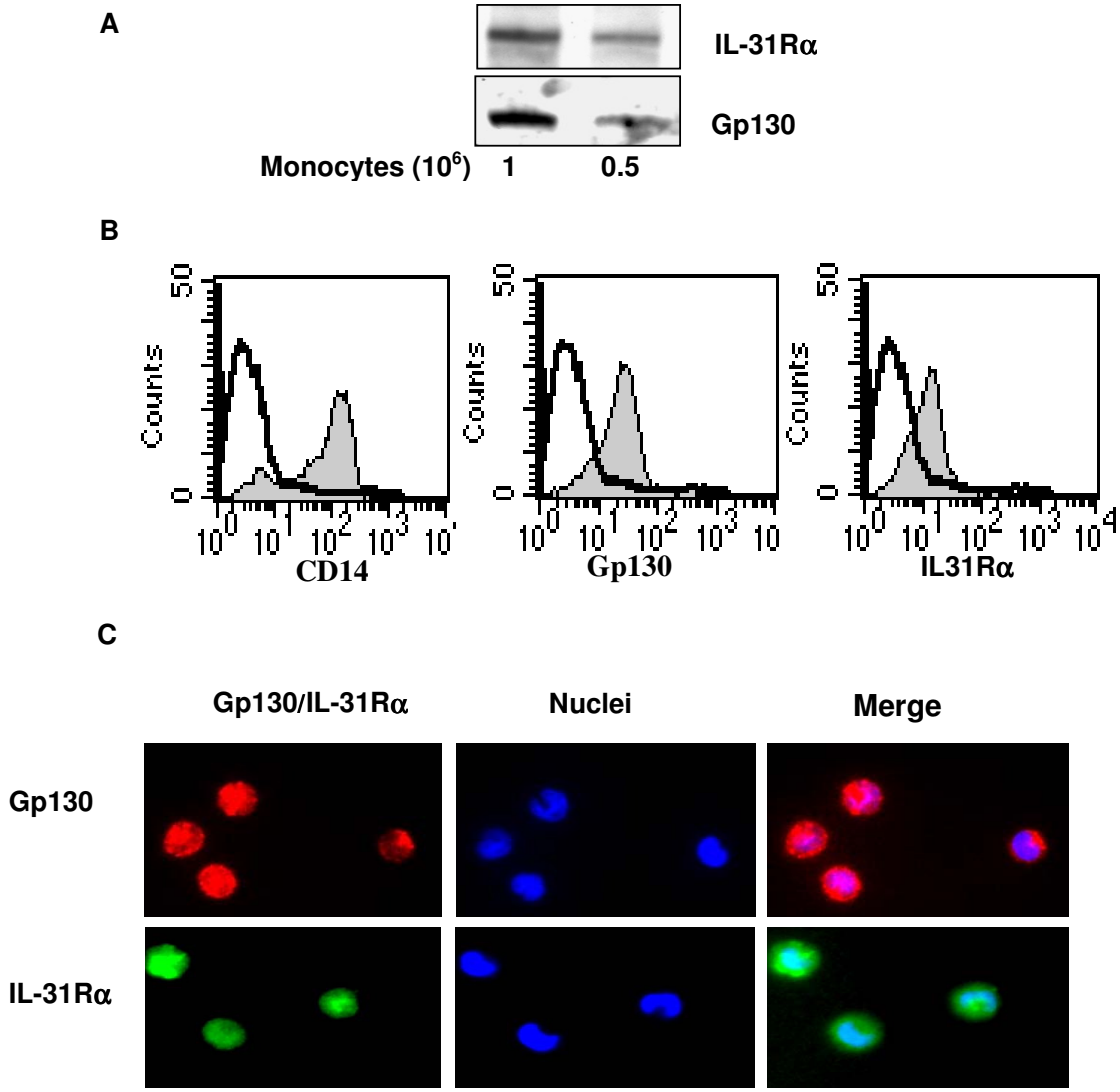


Figure 36. Expression of gp130 and IL-31Rα proteins in human peripheral monocytes. A, Immunoblot. Different amounts of monocytes were analyzed by immunoblotting using antibodies against gp130 and IL-31Rα. B, Flow cytometry. Cells were stained with either control mouse IgG (empty peaks), gp130 or IL-31Rα antibodies (grey peaks). Primary antibodies were visualized by PE-conjugated anti-mouse $F(ab)_2$ and cells were analyzed by FACS. Monocytes-specific staining with CD14 was used as a positive control. C, Immunofluorescence microscopy. Monocytes were fixed and stained with rabbit antibodies against gp130 followed by anti-rabbit Cy5-labeled IgG $F(ab)_2$ or mouse anti-IL-31Rα antibody followed by anti-mouse FITC-labeled IgG $F(ab)_2$. Cells were analyzed by fluorescence microscopy. Results are representative of three independent experiments.

3.2.5. Down-regulation of IL31Rα but not gp130 impairs the plasmin-dependent STAT3 activation

In order to define the type of receptor involved in the signaling induced by plasmin in monocytes. An antisense ODN approach was used to down-regulate the expression of gp130 or IL31Rα and the downstream signaling was investigated.

The treatment of primary monocytes for 12 hours with an antisense ODN targeting gp130, but not a sense control, decreased expression of gp130 by 50%. Similarly, antisense ODN against IL31Rα led to a decrease in the expression of IL-31Rα (**Figure 37A**). To estimate the functionality of this down-regulation of gp130 and IL31Rα expression, human monocytes were stimulated with plasmin (0.43 CTA U/ml) and the phosphorylation status of STAT3 protein at the position of the serine 727 was assessed by immunoblotting.

The cells pretreated with the antisense ODN targeting IL31Rα, but not the cells treated with the sense ODN, plasmin-induced phosphorylation of STAT3 was impaired. Inversely, cells treated with either sense or antisense ODN against gp130 did not show any decrease in the STAT3 phosphorylation upon plasmin stimulation (**Figure 37B**).

Thus, the STAT3 phosphorylation after monocyte tretatment with plasmin is dependent on IL31Rα, but not on gp130.

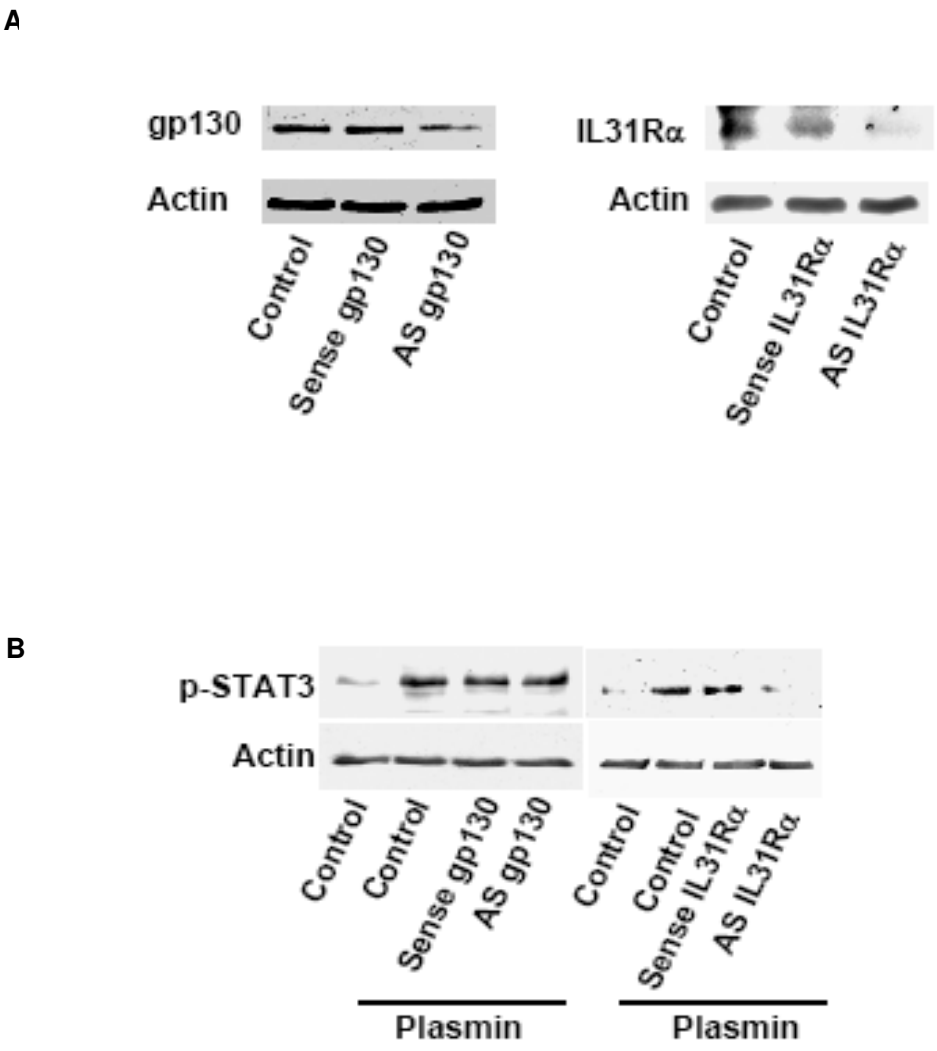


Figure 37. Down-regulation of IL31Rα, but not gp130, impairs the plasmin-dependent STAT3 activation. A, Specific antisense ODNs decrease the expression of gp130 and IL31Rα. B, Decreased expression of IL31Rα, but not of gp130, impairs the plasmin-induced activation of STAT3. Monocytes were treated for 12 h or 48 h with 10 mM of the corresponding ODNs (sense or antisense) directed against either gp130 or IL31Rα, respectively. After 12 h or 48 h recovery in RPMI 1640 without lysine, the cells were lysed, or stimulated with plasmin (0.43 CTA U/ml) for 30 min and then lysed, the proteins were separated and analyzed by immunoblotting with antibodies against gp130, IL-31Rα and phospho-STAT3- (Ser^{727}), actin served as loading control. Results are representative of at least 3 independent experiments. The IL-31Rα antibody was kindly provided by Dr. Gascan (Angers, France).

3.2.6. Plasmin-mediated activation of human monocytes. Summary II

Plasmin might induce monocyte activation through the cleavage of annexin A2 and the subsequent release of the annexin A2 N-terminal peptide.

The N-terminal annexin A2 fragment might interact with the type I cytokine receptor IL31Rα to induce activation of human monocytes (**Figure 38**).

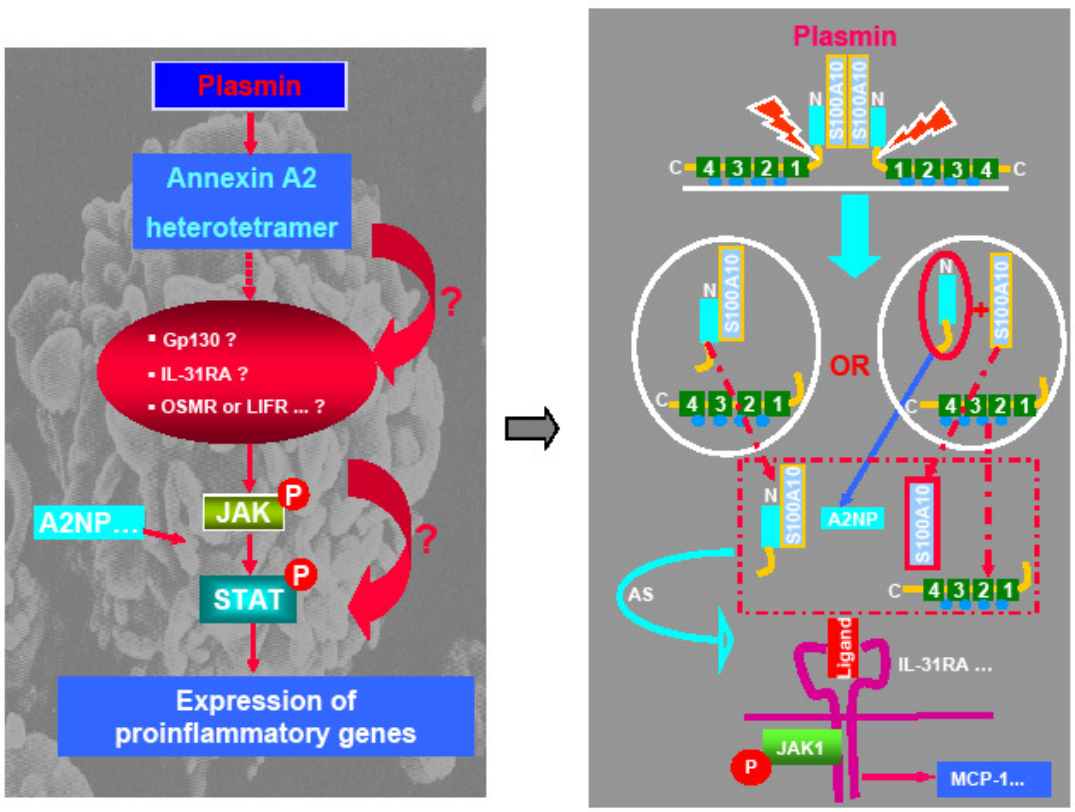


Figure 38. Schematic presentation of the plasmin-mediated monocyte activation.

4. Discussion

4.1. Plasmin a proinflammatory activator of human monocytes-derived macrophages

Macrophages possess crucial effector functions in a variety of chronic inflammatory diseases including atherosclerosis (Hansson 2005) and rheumatoid arthritis (Syrovets and Simmet 2004), where due to contact activation generation of plasmin occurs.

Here we show for the first time that the serine protease plasmin upregulates expression of proinflammatory cytokines such as TNF-α and IL-6 in human macrophages. Plasmin induces activation of multiple signaling pathways including JAK/STAT, p38 MAPK, ERK1/2 and the NF-κB signaling pathway. At the molecular level, similar to monocytes (Laumonnier et al. 2006), the annexin A2 heterotetramer mediates the plasmin signaling in macrophages. Cleavage of annexin A2 by plasmin is a critical event for subsequent cell activation.

Plasmin induces gene expression in macrophages via JAK1 activation, as shown by the use of the JAK inhibitor AG490. The activation kinetics show an early, within 5 min, phosphorylation of JAK1, p38 and STAT3 on Tyr^{705} followed by a second activation phase starting 15 min after plasmin stimulation and involving Akt/NF-κB signaling leading to Akt-mediated activation of IKK, phosphorylation of IκBα and activation of NF-κB.

The JAK family members, encompassing JAK1-3 and TYK2, are generally considered as early activated kinases in the type I and AII interferon receptor signaling (Platanias 2005). Such an early JAK also occurs in plasmin-stimulated macrophages and may drive the crucial downstream phosphorylation events. The classical downstream targets of JAK1 are the STAT proteins, which become phosphorylated at their tyrosine residues. In addition, the phosphorylation of STAT3 on Ser^{727}, which is induced by ERK1/2 and p38 MAPK, is pivotal for full transcriptional activity of STAT3 (Burysek et al. 2002; Xuan et al. 2005). We observed a double phosphorylation of STAT3 in response to plasmin stimulus. Moreover, JAK1 can also target IκBα degradation through phosphorylation of Akt. Indeed, JAK proteins activate the phosphoinositide 3-kinase (PI3K) (Platanias 2005), a kinase upstream of Akt. Akt, in turn, may activate NF-κB signaling through the stimulation of the IκB kinase complex (Ozes et al. 1999). The plasmin-induced Akt activation coincidized with IκB phosphorylation and subsequent translocation of NF-κB confirming activation of the IκB kinase complex via Akt.

JAK1 stimulates the MEK/ERK1/2 pathway through the recruitment of SHP-2/Grb2/SOS and activation of the Ras/Raf/MEK pathway (Heinrich et al. 2003). Stimulation of macrophages with plasmin triggers ERK1/2 activation. The plasmin-induced ERK1/2 phosphorylation is inhibited by the JAK inhibitor AG490 demonstrating dependence of ERK1/2 activation on JAK1, but not on p38 MAPK. Moreover, activation of p38 upon plasmin treatment depends on another JAK kinase, TYK2. Plasmin, similar to interferon α and β, activates TYK2, which might phosphorylates p38 MAPK through activation of Rac1 (Uddin et al. 2000).

The signaling pathways induced by plasmin in macrophages are converging at the nuclear translocation of the STAT3 and NF-κB transcription factor p65 triggering expression of TNF-α, IL-6 and MIP-3α. At the transcriptional level, TNF-α, IL-6 and MIP-3α genes are regulated by recruitment of NF-κB to their promoters. The promoter region of TNF-α contains 2 binding regions for NF-κB (Kuprash et al. 1999). In addition to NF-κB, the plasmin-induced expression of TNF-α is apparently regulated by p38. Hence, the TNF-α release was significantly inhibited when p38 activation was blocked by SB203580. In human macrophages the p38 MAPK is not only involved in the translational control of TNF-α due to mRNA stabilization by regulation of the polyadenylation status and the translation rate, but it also contributes to the transcriptional regulation through NF-κ (Campbell et al. 2004).

Apart from a single binding site for C/EBPβ, the IL-6 promoter likewise contains functional canonical binding sites for NF-κB. Both transcriptional factors can synergistically activate transcription of IL-6 (Matsusaka et al. 1993). Similar to plasmin-stimulated macrophages, during myoblast differentiation a cross-talk takes place between the p38 MAPK and NF-κB in terms of IL-6 expression; here, the p38 MAPK participates in the regulation of IL-6 in dual ways, by increasing the IκBα degradation and by potentiating the transactivation of p65 through the coactivator CBP/p300 of the basal transcriptional machinery (Baeza-Raja and Munoz-Canoves 2004).

Similar to TNF-α and IL-6, MIP-3α is a chemokine whose transcription is controlled by the NF-κB transcription factor. The human MIP-3α promoter contains a functional NF-κB site between -82 and -91 that is responsible for the MIP-3α expression (Sugita et al. 2002). Inhibition of MIP-3α release by the p38 inhibitor SB203580 indicates that the p38 MAPK is involved in the plasmin-induced expression of MIP-3α. The p38 MAPK is also indispensable for the expression of MIP-3α in human gingival fibroblasts (HGF)

stimulated with IL-1β, TNF-α and E. coli LPS (Nakanishi et al. 2005). Reibman et al. reported that ERK and p38 MAPK are involved in the MIP-3α production by airway epithelial cells stimulated with IL-1β or TNF-α (Reibman et al. 2003).

The plasmin-induced activation of macrophages is expected to be of particular importance for cardiovascular disease, where the severity of atherosclerotic lesions positively correlates with the expression of the plasminogen activator uPA (Syrovets and Simmet 2004; Nicholl et al. 2006). The release of cytokines like TNF-α, IL-6 and MIP-3α is a key feature in chronic inflammatory disease. All cytokines may act as autocrine or paracrine molecules on the cells involved in the atherosclerotic process. TNF-α regulates the expression of receptors involved in lipid uptake such as the oxidized-LDL receptor LOX1 in macrophages and the scavenger receptor (ScR) in smooth muscle cells (Li et al. 1995; Tedgui and Mallat 2006), thereby promoting foam cell formation. Consistently, ApoE/TNF-α double knockout mice show a significant decrease in relative lesion size (Branen et al. 2004).

Likewise, IL-6 is associated with atherosclerosis; it is expressed in atherosclerotic plaques (Tedgui and Mallat 2006) and enhanced plasma levels have been observed in patients with progressive disease (Tzoulaki et al. 2005). In mice, IL-6 participates in the macrophage lipid uptake by increasing the expression of CD36 (Keidar et al. 2001). Consistently, also IL-6 was shown to enhance fatty lesion development in mice. Further, endothelial dysfunction can be triggered by the cytokine expression, because eNOS expression can be decreased either by TNF-α, which represses the transcription and destabilizes the mRNA, or by IL-6, which induces a STAT3-mediated transcriptional repression (Saura et al. 2006).

MIP-3α is a chemokine for the cells bearing CCR6, the receptor for MIP-3α, which is not shared by any other known chemokine. MIP-3α is expected to play a critical role in trafficking and homing of many CCR6 positive cells, including memory T cells and immature dendritic cells, to inflammatory sites, such as atopic dermatitis (Nakayama et al. 2001), hepatitis (Shimizu et al. 2001), arthritis (Ruth et al. 2003) and periodontal disease (Hosokawa et al. 2002). MIP-3α released by plasmin might be important for infiltration of CCR6 positive leukocytes in inflamed tissues and it might be involved in the pathogenesis of chronic inflammation.

MIP-3α was observed in human inflamed pulp sections, and was mostly expressed by macrophages that had accumulated in the area adjacent to carious lesions. CCR6

expression was also observed in inflammatory infiltrating lymphocytes. It was suggested that MIP-3α may play a major role in the advancement of pulpal inflammation via the recruitment of CCR6-expressing lymphocytes (Nakanishi et al. 2005). Plasmin activated macrophages release MIP-3α that will, in turn, recruit CCR6 positive cells to macrophages. This might have a modulating effect on the immune response. Interaction of the CCR6-positive T cells with plasmin-activated macrophages should be addressed in future studies.

Finally, the expression of chemokines, such as monocyte chemoattractant protein-1, and cytokines, such as IL-6, generated by plasmin-activated macrophages, might promote leukocyte activation and recruitment into areas of inflammation (Haddy et al. 2003; Ito and Ikeda 2003; Ruth et al. 2003; Tedgui and Mallat 2006) and augment the immune response.

This work clearly demonstrates that the serine protease plasmin triggers proinflammatory cytokine expression in human macrophages via the annexin A2 heterotetramer and several downstream signaling pathways. These findings significantly expand the relevance of plasmin generated in inflamed tissues beyond its roles in proteolysis and matrix metalloproteinase activation.

4.2. Mechanism of plasmin-induced monocyte activation

A number of studies have highlighted the role of plasmin in inflammation. Plasmin is able to induce a pro-inflammatory state in particular in cells of the innate immune system such as in monocytes or macrophages. This induction promotes the release of leukotrienes (Weide et al. 1996) and pro-inflammatory cytokines and chemokines, such as TNF-α and CCL2/MCP-1, by monocytes (Syrovets et al. 2001; Burysek et al. 2002) and, as it was identified in the present study, TNF-α, IL-6 and MIP-3α in macrophages (Li et al. 2007). The signaling plasmin receptor on the monocytes is not identified, yet.

At the molecular level, the annexin A2 heterotetramer, consisting of two molecules annexin A2 and 2 molecules S100A10, has been identified as a binding site important for the plasmin-induced signaling (Laumonnier et al. 2006). The annexin A2 heterotetramer contains no transmembrane domain and is known to bind to the cell membrane surface via the Ca^{2+}/phospholipid layer (Waisman 1995). Thus, annexin A2 can't serve as a link between plasmin and downstream signaling pathways. It is, however, known that after addition of plasmin to monocytes, plasmin cleaves annexin A2 at the N-terminus. A newly

generated annexin A2 lacking the N-terminus (A2Nt) does not contain the binding site to S100A10 (Kube et al. 1992). Therefore, dissociation of the annexin A2 heterotetramer occurs (Laumonnier et al. 2006). The 27 amino acids long N-terminal part of annexin A2 may be still attached to S100A10. S100A10, the N-terminal part of the annexin A2 or its C-terminal part, A2Ct) might finally serve as a ligand to a signaling receptor.

Annexin A1, a member of the annexin family proteins, has been found to augment anti-CD3/CD28-mediated CD25 and CD69 expression and cell proliferation of activated T cells, inducing MAP kinase and NF-κB activation (D'Acquisto et al. 2007). A N-terminal peptide of annexin A1, is sufficient to engage the formylated peptide receptors (FPRs) and elicit chemotaxis in human peripheral blood granulocytes and monocytes (Ernst et al. 2004). The annexin A2 heterotetramer can cause rapid phosphorylation of several MAP kinases, as well as translocation of p65 NF-κB to the nucleus and expression of TNF-α, IL-1β, and IL-6 as well as several members of the chemokine family in human monocytes-derived macrophages (Swisher et al. 2007).

The C-terminal and core domains of annexin A2 are also important for the functions of annexin A2. The C-terminal part of annexin A2 binds to and modulate the actin cytoskeleton (Filipenko and Waisman 2001). The core domain comprises the intracellular binding sites for Ca^{2+}, phospholipids and F-actin (Filipenko and Waisman 2001; Gerke and Moss 2002). Binding of Ca^{2+} regulates association of annexin A2 with membranes and cytoskeleton (Gerke and Moss 2002; Rescher and Gerke 2004). The annexin A2-S100A10 complex, i.e. the annexin A2 heterotetramer, which regulates exocytosis in endothelial cells, forms a ternary complex with tryptophanyl-tRNA synthetase (TrpRS). The interaction of TrpRS with AA2t regulates the TrpRS function by retaining it in the cytosol. Dissociation of the annexin A2 heterotetramer from TrpRS allows TrpRS export to the cell exterior. Once outside the cells, plasmin or another protease cleaves the TrpRS to generate angiostatic fragments. These fragments can inhibit the Akt signaling pathway through the interaction with VE-cadherin (Kapoor et al. 2008).

The activation of different signaling pathways such as the MAPK pathway, the STAT1/3 pathway and the NF-κB pathway by plasmin seems to start with the phosphorylation of the tyrosine kinases of JAK family, JAK1 and TYK2. The cleavage of annexin A2 by plasmin generates a C-terminal part of annexin A2, which may interact with some proteins to influence vital monocyte functions In this study, it was demonstrated that a peptide derived from the unique N-terminal domain of annexin A2 (A2NP) induces JAK1/STAT3 phosphorylation and MCP-1 release in human monocytes. Thus, A2NP

alone or in a complex with some functional proteins is capable of activating an unknown receptor to induce downstream signaling. This activation induces then JAK1/STAT3 phophorylation.

So far, it is unknown A2NP activates JAK1/STAT3 in human monocytes. Different types of receptors have been reported currently identified to activate the JAK/STAT pathways. An alternative pathway involving the JAK/STAT is the activation of G-protein coupled receptors (GPCR), such as the factor VA2I, the thrombin receptor PAR1 or the angiotensin receptor (Banes et al. 2005). The best candidate among the GPCR as plasmin coreceptor would be PAR1, which was proposed to act as a plasmin receptor in fibroblast and endothelial cells (Pendurthi et al. 2002; Tarui et al. 2002). However, the plasmin-induced signaling in monocytes does not rely on PAR1 activation, because it persisted in the presence of the PAR1 inhibitor SCH79797, whereas the thrombin-induced release of MCP-1 is mediated by PAR1 (Colognato et al. 2003), was greatly impaired. In addition, GPCR often activate JAK2 (Ferrand et al. 2005), a kinase which was not activated in plasmin-stimulated monocytes (Burysek et al. 2002) or macrophages.

The JAK/STAT pathways are also activated in response to the type I cytokine receptors, including interleukin receptors, colony stimulation factor receptors and growth hormone receptor, prolactin receptor, oncostatin M receptor and leukemia inhibitory factor receptor (Kisseleva et al. 2002). In monocytes, plasmin induced phosphorylation of STAT3 is similar to that induced by cytokines IL-6 and IL-31. The IL-6 family cytokines signal either through gp130 or IL-31Rα as the common receptors (Bando et al. 2006) or the interferon α/β receptors (Mitani et al. 2001). Activation of interferon receptors with cognate ligand induces the activation of JAK1 through the IFNRα subunit and Tyk2 by IFNRβ subunit, the later is not occurring in monocytes (Burysek et al. 2002) suggesting no involvement of the IFN signaling pathway.

The family of gp130-type receptors induce also a JAK/STAT activation (Guschin et al. 1995). This receptor family includes gp130, IL-31Rα, LIFR and OSMRβ. IL-31Rα protein was found to be expressed predominantly on human monocytes activated by IFNγ stimulation (Dillon et al. 2004) and detectable at low to moderate levels on CD14-positive cells (Ghilardi et al. 2002). Monocytes and dendritic cells were also found to express consistent amounts of IL-31Rα transcripts (Diveu et al. 2003). The result of this study is consistent with these findings. The monocytes contain primarily gp130, low levels of IL-31Rα and no LIFR and OSMRβ. The gp130 and IL-31Rα receptors are also expressed at

the surface of monocytes. Thus, either IL-31Rα and/or gp130 receptor may act to mediate plasmin-induced signaling.

Plasmin and the N-terminal peptide of annexin A2 activate JAK1/STAT3 in human monocytes. We show that plasmin generates the N-terminal peptide of annexin A2 and hypothesized that this peptide alone or in a complex with another functional protein could serve as a new ligand for IL-31Rα or gp130, receptors expressed by monocytes. Downregulation of IL-31Rα and gp130 expression in monocytes demonstrated that plasmin-induced phosphorylation of JAK1 and STAT3 were decreased after down-regulation of IL-31Rα but not gp130. It means that plasmin mediated JAK/STAT phosphorylation to induce many inflammatory functional cytokines release, which occur via IL-31Rα receptor complexes in human monocytes. IL-31Rα expressed on blood cells is a signaling receptor chain possibly involved in the development and function of monocytes and macrophages (Dreuw et al. 2004).

IL-31 is a specific ligand for IL-31Rα, till now no other ligand was found to activate IL-31Rα. IL-31 signals via a heterodimeric receptor composed of IL-31Rα and OSMRβ to activate STAT1, STAT3, STAT5 but not STAT6 in transfected BaF3 cells (Dillon et al. 2004). IL-31 also recruits JAK1, JAK2 and activates Pi3-K/Akt cascade (Diveu et al. 2004; Dreuw et al. 2004). IL-31-binding to a IL-31Rα/OSMRβ heterodimer, but to IL-31Rα/gp130 heterodimer, initiated signaling by particularly strong recruitment of the STAT3, ERK, JNK and Akt pathways and achieving arrest of proliferation by down-regulation of the expression and activity of cell cycle-controlling proteins in the human alveolar epithelial cell line A549 (Chattopadhyay et al. 2007). Also in colorectal cancer derived intestinal epithelial cell (IEC) lines, IL-31 alters expression of cell cycle proteins (Sonkoly et al. 2006). Also IEC express the functional IL-31Rα receptor complex, binding of IL-31 to it leads to phosphorylation of STAT3/1, Akt and ERK MAP kinases (Dambacher et al. 2007).

The demand for OSMRβ or gp130 as coreceptors for IL-31Rα was confirmed in studies, where shimeric receptor constructs were analyzed. All constructs contained the same extracellular part (IL-5R) and could be activated by the addition of IL-5. The signaling in terms of ERK1/2 activation in the cells trasfected with such chimera receptors was observed only when the receptor containing the intracellular portion of IL-31Rα was coexpressed with either OSMRβ or gp130-containing chimeras. The authors concluded that IL-31Rα is not functional in form of a homodimer, but needs a coreceptor, either OSMRβ or gp130 (Diveu et al. 2003; Dreuw et al. 2004). We could show that OSMRβ is

not expressed in monocytes and gp130 is not involved in the plasmin-induced monocyte activation. Hence IL-31Rα needs a coreceptor to induce a downstream signaling, this would indicate that monocyte might express another, yet unknown, receptor, which is able to heterodimerize with IL-31Rα to form a functional complex.

Taken together, these data suggest that plasmin activates monocytes through generation of N-terminal peptide of annexin A2. N-terminal peptide of annexin A2 may act as a ligand for IL-31Rα forming a complex with some, yet unknown, receptor.

5. Summary

Macrophages are key regulators of the inflammatory responses. Contact activation in inflamed tissues induces generation of the serine protease plasmin, which is a known activator of human monocytes. Plasmin activates monocytes via the annexin A2 heterotetramer, composed of annexin A2 and S100A10.

This study demonstrates that macrophages express higher amounts of the annexin A2 heterotetramer compared to monocytes as analyzed by Western immounblotting and flow cytometry. Activation of macrophages by plasmin leads to cleavage of the annexin A2 subunit of the receptor, followed by activation of JAK1 signaling, which results in STAT3 activation, Akt-dependent NF-κB activation, and phosphorylation of MAPK p38 and ERK1/2. Moreover, the plasmin-induced activation of STAT3 and p65/NF-κB transcription factors and their translocation to the nucleus and binding to the corresponding response DNA sequences, trigger expression and release of the proinflammatory cytokines TNF-α and IL-6.

Pharmacological inhibitors of JAK, p38 and NF-κB revealed that these signaling pathways are indispensable for the plasmin-mediated expression of the TNF-α and IL-6 genes. By contrast, the ERK1/2 activation is essential only for the expression of IL-6 gene. The observed MIP-3α release is dependent on p38 activation. Furthermore, at the molecular level activation of macrophages depends on the proteolytic activity of plasmin as shown by the use of plasmin that had been catalytically inactivated by the specific chloromethyl ketone VPLCK.

Plasmin activated macrophages via a receptor composed of annexin A2 and S100A10, so-called annexin A2 heterotetramer. Consistently, down-regulation of the expression of either annexin A2 or S100A10 by antisense oligonucleotides abolished the plasmin-induced expression of proinflammatory cytokines.

Plasmin initiates signaling in monocytes by proteolytic cleavage of the annexin A2 subunit of the receptor, generation of the N-terminal peptide of annexin A2 and the dissociation of the annexin A2 heterotetramer. The N-terminal peptide of annexin A2 is able to mimic the plasmin-induced activation of the tyrosine kinase JAK1 and the transcriptional factor STAT3 as well as the expression of CCL2/MCP-1. Activation of this pathway may require a new member of the type I cytokine receptor, IL31Rα. Consistently, down-regulation of

the expression of IL31Rα, but not of gp130, impairs the plasmin-induced activation of human monocytes.

In conclusion, these data show that the serine protease plasmin is a potent proinflammatory activator of human macrophages and monocytes acting via the annexin A2 heterotetramer and multiple downstream signaling pathways. Thus, plasmin generated at the sites of inflammation will profoundly affect immune response.

6. References

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