PLASMIN: A POTENT PRO-INFLAMMATORY FACTOR

By

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Umeå 2008
To my family

Science is a wonderful thing if one does not have to earn one's living at it.

Albert Einstein
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<tr>
<td>α₂-AP</td>
<td>α₂-antiplasmin</td>
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<td>AIA</td>
<td>antigen-induced arthritis</td>
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<td>C5aR</td>
<td>C5a receptor</td>
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<td>CFU</td>
<td>colony-forming unit</td>
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<td>CIA</td>
<td>collagen type II-induced arthritis</td>
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<td>CII</td>
<td>collagen type II</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<td>IL</td>
<td>interleukin</td>
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<td>kDa</td>
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<td>LIA</td>
<td>local injection-induced arthritis</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>mBSA</td>
<td>methylated bovine serum albumin</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>MMPs</td>
<td>matrix metalloproteinases</td>
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<td>PA</td>
<td>plasminogen activator</td>
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<td>PAI-1</td>
<td>PA inhibitor type 1</td>
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<td>PN-1</td>
<td>protease nexin-1</td>
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<td>RA</td>
<td>rheumatoid arthritis</td>
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<td>S. aureus</td>
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<td>TLR</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>uPA</td>
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<td>uPAR</td>
<td>uPA receptor</td>
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ABSTRACT
Plasmin: A Potent Pro-inflammatory Factor
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Plasmin, the central molecule of the plasminogen activator system, is a broad-spectrum serine protease. Plasmin is important for the degradation of fibrin and other components of the extracellular matrix (ECM) during a number of physiological and pathological processes. The aim of this thesis was to elucidate the functional roles of plasmin during pathological inflammation and infection in autoimmune and non-autoimmune diseases. For this purpose, mouse models of rheumatoid arthritis (RA), bacterial arthritis, infection, and sepsis have been used.

Previous studies from our laboratory have shown that plasminogen-deficient mice are resistant to the development of collagen type II-induced arthritis (CIA). In contrast, others have shown that plasmin plays a protective role in antigen-induced arthritis (AIA). To investigate the contrasting roles of plasminogen deficiency in models of CIA and AIA, a new animal model of arthritis called local injection-induced arthritis (LIA) was developed. In this model, we replaced methylated bovine serum albumin, which is normally used as an immunogen in the AIA model, with collagen type II (CII) to induce arthritis. When wild-type and plasminogen-deficient mice were injected intra-arterially with CII or 0.9% NaCl following CIA induction, plasminogen-deficient mice developed typical CIA, but the disease was less severe than in wild-type mice and was restricted to the injected joints. When the AIA model was used, plasminogen-deficient mice developed a much more severe arthritis than the wild-type mice. These results indicate that both the antigen and joint trauma caused by the local injection are critical to explaining the contrasting roles of plasminogen deficiency in CIA and AIA. This indicates that CIA and AIA have distinct pathogenic mechanisms and plasmin plays contrasting roles in different types of arthritis models.

To study the functional roles of plasmin in the host inflammatory response during infectious arthritis, a Staphylococcus aureus-induced bacterial arthritis model was established. When wild-type mice were injected intra-articularly with $1 \times 10^6$ colony-forming units (CFU) of *S. aureus* per joint, all the bacteria were completely eliminated from the injected joints in 28 days. However, in the plasminogen-deficient mice, the *S. aureus* counts were 27-fold higher at day 28 than at day 0. When human plasminogen was given to the plasminogen-deficient mice daily for 7 days, the bacterial clearance was greatly improved and the necrotic tissue in the joint cavity was also completely eliminated. Supplementation of plasminogen-deficient mice with plasminogen also restored the expression level of interleukin-6 (IL-6) in the arthritic joints. In summary, plasmin has protective roles during *S. aureus*-induced arthritis by enhancing cytokine expression, removing necrotic tissue, and mediating bacterial killing and inflammatory cell activation.

The functional roles of plasmin during infection and sepsis were also studied in mice. Infection was induced by injecting $1 \times 10^7$ CFU of *S. aureus* intravenously and the sepsis model was induced by injecting $1.6 \times 10^8$ CFU of *S. aureus*. In the infection model, the wild-type mice had a 25-day survival rate of 86.7%, as compared to 50% in the plasminogen-deficient group. However, when sepsis was induced, the average survival for plasminogen-deficient mice was 3 days longer than for wild-type mice. Twenty-four hours after the induction of sepsis, the serum levels of IL-6 and IL-10 as well as the bacterial counts in all organs investigated were significantly higher in wild-type mice than in plasminogen-deficient mice. In wild-type mice, blockade of IL-6 by intravenous injection of anti-IL-6 antibodies significantly prolonged the onset of mortality and improved the survival rate during sepsis. These data indicate that plasmin plays different roles during infection and sepsis. Furthermore, plasmin appears to be involved in the regulation of inflammatory cytokine expression during sepsis.

Taken together, our data indicate that plasmin plays multifunctional pro-inflammatory roles in different autoimmune and non-autoimmune diseases. The pro-inflammatory roles of plasmin include activation of inflammatory cells, regulation of cytokine expression, and enhancement of the bacterial killing ability of the host.

Key words: plasmin, inflammation, rheumatoid arthritis, bacterial arthritis, infection, sepsis, cytokine, signal transduction.
PUBLICATION LIST

This thesis is based on the following articles, which are referred to in the text by Roman numerals (I–III):


INTRODUCTION

The extracellular matrix (ECM) is the extracellular part of animal tissue that supports the cells, in addition to performing various other important functions. The ECM includes the interstitial matrix and the basement membrane. Degradation of ECM proteins by proteolysis can lead to rapid and irreversible responses to changes in the cellular microenvironment. Such activities are involved in the host inflammatory responses to self- and non-self challenges. The plasminogen activator (PA) system has been suggested to play a key role in many physiological and pathological processes that involve proteolytic degradation of the ECM (Mignatti and Rifkin, 1993).

The PA system is a versatile enzymatic cascade involved in the control of fibrin degradation, matrix remodeling, and cell invasion. The key component of this system is the broad-spectrum serine protease plasmin. Plasmin is formed from plasminogen by either of the two plasminogen activators (PAs), tissue-type PA (tPA) or urokinase-type PA (uPA), which are subject to time- and space-dependent regulation. The PA system is also regulated by several specific inhibitors, including PA inhibitor type 1 (PAI-1) and PA inhibitor type 2 (PAI-2). These inhibitors are directed against PAs, whereas $\alpha_2$-antiplasmin ($\alpha_2$-AP) is directed against plasmin (Saksela and Rifkin, 1988).

THE AIMS OF THIS THESIS were to study the roles of plasmin during inflammation and infection in autoimmune and non-autoimmune disease models. The studies were done using mainly wild-type and plasminogen-deficient mice. Initially, by comparing the phenotypes of plasminogen-deficient mice in two autoimmune arthritis models, collagen-induced arthritis (CIA) and antigen-induced arthritis (AIA), the distinct roles of plasmin in autoimmune arthritis models with different disease pathogenesis were investigated. Furthermore, a bacterial arthritis model in mice was established to investigate the functions of plasmin in non-autoimmune diseases. The functional roles of plasmin in inflammation were further studied in an infection model and a sepsis model. Together, these studies show that plasmin has novel pro-inflammatory roles during inflammation and infection, including roles in activating inflammatory cells, stimulating cytokine expression, enhancing ECM remodeling, and enhancing the bacterial killing ability of the host.

1. THE PLASMINOGEN ACTIVATOR (PA) SYSTEM

The PA system is a versatile, temporally controlled enzymatic system. The central molecule of the PA system, plasmin, is formed from proteolytic activation of the precursor protein plasminogen by tPA, uPA, or kallikrein. The activation of plasminogen is controlled by the levels of production of tPA and uPA, as well as by PAI-1 and PAI-2 (Saksela and Rifkin, 1988; Vassalli et al., 1991). Plasmin activity is controlled by the protease inhibitors $\alpha_2$-AP and $\alpha_2$-macroglobulin. Plasmin is a highly potent serine protease that degrades a large group of ECM substrates including fibrin, gelatin, fibronectin, and proteoglycans (Alexander and Werb, 1989). It also activates precursors of matrix metalloproteinases (MMPs) that, once activated, degrade components of the ECM that are barriers to cellular migration. Certain cells also have a specific cell-surface receptor for uPA that can direct proteolytic activity to the cell
surface (Stoppelli et al., 1985; Vassalli et al., 1985). A simplified diagram of the PA system and its regulation is given in Figure 1.

![Schematic representation of the PA system and its regulation.](image)

**Figure 1.** Schematic representation of the PA system and its regulation. The synthesis of tPA and uPA by specific cells is regulated by hormones, growth factors, and cytokines. In the extracellular space, the activities of PAs and plasmin are controlled by the specific inhibitors PAI-1, PAI-2, and \(\alpha_2\)-AP, respectively. Binding of PAs and plasmin to cellular binding sites (R) can result in localized proteolytic activity on the cell surface.

### 1.1. Plasminogen/plasmin

Plasminogen is a 791-amino acid single-chain glycoprotein with a molecular weight of approximately 92 kilodaltons (kDa). Plasminogen is mainly synthesized and secreted by the liver (Raum et al., 1980). The concentration of plasminogen in plasma and body fluids is approximately 200 µg/ml and it has a half-life of 2.2 days (Ogston, 1980). Plasminogen exists in two different molecular forms, Glu-plasminogen and Lys-plasminogen. Native uncleaved plasminogen has an amino-terminal glutamic acid residue, and this is termed Glu-plasminogen. After cleavage of the Lys\(^{76}\)-Lys\(^{77}\) bond by the autocatalytic action of plasmin, Lys-plasminogen is formed, which is 76 amino acids shorter and has an amino-terminal lysine residue (Wallen and Wiman, 1970; Wallen and Wiman, 1972). Enzymatically inactive plasminogen is converted to plasmin after cleavage of the Arg\(^{561}\)-Val\(^{562}\) bond by uPA or tPA, yielding the two-chain, disulfide-linked plasmin molecule (Robbins et al., 1967; Sottrup-Jensen et al., 1975). The structural features of the plasmin molecule include an A-chain (N-terminal) and a B-chain (C-terminal). The A-chain part has a pre-activation peptide (from 1–77) and is followed by five tandem structures called kringle domains. Kringle domains
participate in binding to fibrin and to the cell surface (Ponting et al., 1992). The B-chain (C-terminal) contains the catalytic protease domain with the characteristic His-Asp-Ser triad of serine proteases (Parry et al., 2000).

The major function of plasmin is the dissolution of fibrin clots through the degradation of fibrin into soluble fragments (Collen and Lijnen, 1991). Plasmin also has substrate specificities for several other components of the ECM, including laminin, fibronectin, proteoglycans and gelatin, indicating that plasmin also plays an important role in ECM remodeling (Saksela and Rifkin, 1988; Mignatti and Rifkin, 1993). Plasmin can also indirectly degrade additional components of the ECM by activating some pro-MMPs to active MMPs (HE et al., 1989; Carmeliet et al., 1997). Thus, it has been suggested that plasmin may be an important upstream regulator of extracellular proteolysis, and may thus be involved in many tissue degradation-related pathological and physiological processes such as cell migration, ovulation, tissue remodeling, and inflammation (Saxne et al., 1993; Schaff and Eisenberg, 1997; Plow et al., 1999; Collen, 2001). In addition, plasmin has the ability to activate latent forms of certain growth factors (Rifkin et al., 1990; Andreasen et al., 1997). In vitro, plasmin cleaves components of the complement system, thereby releasing chemotactic complement fragments (Lachmann et al., 1982; Schaff and Eisenberg, 1997).

Changes in plasminogen levels have been associated with different physiological or disease conditions. For instance, elevated levels of plasminogen have been found in the earlier stages of pregnancy (Bonnar et al., 1969). Reduced levels of plasminogen have been reported in several clinical conditions such as during sepsis, leukemia, and liver disease (Biland et al., 1978; Sutor, 1979; Gallimore et al., 1980; Smith-Erichsen et al., 1982). The decrease in plasminogen in these conditions is associated with poor prognosis.

1.2. Plasminogen activators

uPA and tPA are the two major physiological PAs that have been identified. Both PAs are immunologically distinct molecules encoded by different genes, although both can activate plasminogen (Dano et al., 1985). Even though the enzymes are highly similar in their basic structures, the homology between tPA and uPA at the amino acid level is only about 40% (Saksela and Rifkin, 1988). The synthesis of PAs is modulated by a variety of effector molecules such as peptide hormones, steroid hormones, and growth factors. Expression of both PAs has been detected in a number of different tissues (Saksela and Rifkin, 1988). Traditionally, it was suggested that there are different biological functions for the two PAs, tPA being primarily involved in vascular fibrinolysis and uPA mediating tissue remodeling and invasion processes (Mignatti and Rifkin, 1993). However, functional studies in PA-deficient mice have suggested that tPA and uPA may have partially overlapping physiological functions (Carmeliet et al., 1994; Khokha et al., 1995; Carmeliet and Collen, 1996). The PAs are also involved in the processes of mammary cell growth and involution, as well as in the innate immune system by enhancing inflammatory cell migration and activation (Politis, 1996; Plow et al., 1999).
1.2.1. Urokinase-type plasminogen activator (uPA)

uPA is a single-chain, 411-amino acid glycoprotein with a molecular weight of 53 kDa (Wun et al., 1982; Nielsen et al., 1982). Single-chain uPA is an inactive pro-enzyme that is converted to an active disulfide-linked, two-chain molecule by proteolytic cleavage of the Lys$^{158}$-Ile$^{159}$ bond by enzymes such as plasmin, kallikrein, factor XIIa, and cathepsin B (Ichinose et al., 1986; Andreasen et al., 1997). Active uPA is a two-chain form held together by a single disulfide bond. uPA has its own specific cell-surface receptor, termed uPA receptor (uPAR), which directs the activity of bound uPA to the cell surface (Solberg et al., 1994; Romer et al., 1994). It has also been shown that uPAR is involved in the internalization of uPA/inhibitor complexes and also in cell migration and cell signaling events (Blasi, 1996; Andreasen et al., 1997). uPA and its receptor can be synthesized by numerous different cell types, including activated T lymphocytes, dendritic cells, and macrophages (Gyetko et al., 1999; Tchougounova and Pejler, 2001). Increased levels of expression of uPA and/or uPAR are found in cancer cells from tumor tissues of breast, colon, ovary, stomach, cervix, bladder, kidney, and brain (Andreasen et al., 1997; Han et al., 2005).

1.2.2. Tissue-type plasminogen activator (tPA)

tPA is secreted as a single-chain glycoprotein with a molecular weight of about 70 kDa (Rijken and Collen, 1981; Pohl et al., 1984). tPA can be cleaved at the Arg$^{275}$-Ile$^{276}$ bond by plasmin to form a two-chain molecular form held together by a single disulfide bond. The carboxyl-terminal (light-chain) part of tPA contains the active site, while the non-catalytic amino-terminal (heavy-chain) part of tPA contains structural domains. Unlike uPA, both the two-chain and single-chain forms of tPA are active. This property makes tPA unique among serine proteases (Rijken et al., 1982; Tachias and Madison, 1996). The finger and kringle domains of tPA are important in binding to fibrin (Collen, 1999). This binding not only enhances plasminogen activation, but also serves to localize plasmin to its substrate fibrin. This provides the targeted local proteolysis which is an important characteristic of vascular fibrinolysis (Collen and Lijnen, 1991). tPA is mainly produced by endothelial cells (Levin, 1983; Levin and del Zoppo, 1994; Levin et al., 1997), but also by keratinocytes, melanocytes, and neurons (Chen et al., 1993; Bizik et al., 1996; Teesalu et al., 2002). Although cellular binding sites for tPA have been described on different cell types (Vassalli et al., 1991), a unique cell-surface receptor that binds tPA exclusively has yet not been identified (Hajjar et al., 1987; Verrall and Seeds, 1989).

1.3. Inhibitors of the PA system

The PA system is delicately regulated by specific inhibitors that are directed against PAs and active plasmin. The major inhibitors of the PA system are PAI-1, PAI-2, protease nexin I (PN-I), and $\alpha_2$-AP. All of these inhibitors are members of the serine protease inhibitor (serpin) superfamily (Irving et al., 2000; Gettins, 2002). The serpin family members are structurally related proteins with similar tertiary structure and
function. The serpins act through a mechanism called “suicide inhibition” that mimics the cleavage of the substrate but traps the enzyme in an inactive, stable serpin-protease complex (Bode et al., 1994). Based on the strong similarities in structure and functional mechanisms, it has been postulated that the serpins have evolved from a common ancestor. It has also been well documented that the inhibitors of the PA system are involved in all major proteolytic cascades in the body such as fibrinolysis, coagulation, inflammation, apoptosis, angiogenesis, and complement activation (Richardson et al., 2006).

1.3.1. Plasminogen activator inhibitor type 1 (PAI-1)

PAI-1 is a 45-kDa, 379-amino acid single-chain glycoprotein serpin. It is present in human plasma at a concentration of 20 ng/ml, and it is synthesized by many tissues and cells (Kristensen et al., 1990). PAI-1 efficiently inhibits single-chain tPA, two-chain tPA and two-chain uPA, and is therefore an important regulator of plasminogen activation (Berrettini et al., 1989). In the blood and ECM, PAI-1 is mainly found in complex with the adhesion protein vitronectin (Declerck et al., 1988). This binding stabilizes and maintains PAI-1 in its active conformation, but does not interfere with the inhibition of PAs (Seiffert et al., 1990). PAI-1 inhibits target proteolysis by rapidly forming covalently bound 1:1 complexes (Lawrence et al., 1989; Fa et al., 1995; Wilczynska et al., 1997). A primary function of PAI-1 in vivo is to balance the proteolytic activity of the PAs during fibrinolysis (Schleef and Loskutoff, 1988). Besides its role in regulating homeostasis, PAI-1 also has roles in the regulation of cell adhesion, cell migration, and in PA- or plasmin-dependent tumor invasion (Kjoller et al., 1997; Waltz et al., 1997). Studies in transgenic mice have also revealed a functional role for PAI-1 in wound healing, atherosclerosis, metabolic disturbances such as obesity and insulin resistance, tumor angiogenesis, chronic stress, bone remodeling, asthma, rheumatoid arthritis (RA), fibrosis, glomerulonephritis, and sepsis (Lijnen, 2005).

1.3.2. Plasminogen activator inhibitor type 2 (PAI-2)

PAI-2 is a single-chain, 425-amino acid serpin that is produced by a few cell types including monocytes/macrophages, keratinocytes, and epithelial cells. Its plasma concentration is below detectable levels (Kruithof et al., 1986; Jensen, 1997). PAI-2 exists in two isoforms, a 60-kDa extracellular glycosylated form and a 47-kDa intracellular non-glycosylated form (Genton et al., 1987). The biological function of extracellular PAI-2 is to inhibit both uPA and two-chain tPA, although PAI-2 is a poor inhibitor of single-chain tPA. The inhibition efficiency of PAI-2 is 20- to 100-fold less than that of PAI-1 (Kruithof et al., 1986). The extracellular form of PAI-2 is also considered to be a regulator of uPA activity in blood vessels and in the ECM during the processes of pregnancy, cancer formation, and inflammation (Montemurro et al., 1999; Kucharewicz et al., 2003). The role of intracellular PAI-2 still remains to be elucidated, although several studies have suggested that it may play a role in protecting cells from apoptosis (Dickinson et al., 1995; Kruithof et al., 1995). Recent studies have indicated that PAI-2 can be spontaneously polymerized, and the CD-loop
of PAI-2 functions as a redox-sensitive molecular switch that converts PAI-2 between a stable active monomeric conformation and a polymeric conformation, suggesting that the redox status of the cell may be a regulator of PAI-2 polymerization (Wilczynska et al., 2003).

1.3.3. Protease nexin-1 (PN-1)

PN-1 is a secreted 392-amino acid glycoprotein with a molecular weight of about 45 kDa, and is expressed in several tissues and cell types (Baker et al., 1980; Vassalli et al., 1993). PN-1 is a broad and rapid inhibitor of a number of serine and cysteine proteases including tPA, uPA, plasmin, trypsin, and thrombin (Silverman et al., 2001). PN-1 inhibits certain regulatory serine proteases by forming a covalent complex with the catalytic-site serine residue; the complex then bound to the cell surface and is internalized and degraded (Farrell and Cunningham, 1987). Studies have shown that PN-1 is expressed exclusively in granulosa cells in ovarian follicles of mice and rats (Hagglund et al., 1996; Hasan et al., 2002). PN-1 deficient male mice have dysfunctional semen, which makes these mice less fertile than their wild-type counterparts (Murer et al., 2001).

1.3.4. α₂-antiplasmin (α₂-AP)

α₂-AP is the major physiological inhibitor of plasmin and is synthesized in the liver. It is a single-chain, 452-amino acid glycoprotein with a molecular weight of 70 kDa and a plasma concentration of 70 μg/ml (Collen and Wiman, 1979). The concentration may fall to below 30% of the normal level in severe cases of liver disease or intravascular coagulation (Collen, 1980). Free plasmin is rapidly inhibited by α₂-AP in order to limit the activity of plasmin. This inhibition occurs both at the reactive center and the lysine binding sites of plasmin (Longstaff and Gaffney, 1991). The binding of plasmin to fibrin involves the same lysine binding sites as those involved in the binding to α₂-AP (Collen and Wiman, 1979; Sasaki et al., 1986). This means that fibrin-bound plasmin is protected from inhibition until the fibrin has been dissolved. In the circulation, this mechanism is thought to ensure that plasmin activity is restricted to fibrin (Longstaff and Gaffney, 1991). Lack of α₂-AP increases platelet microaggregation, resulting in thrombus formation (Takei et al., 2002).

2. STUDIES ON GENE-DEFICIENT MICE

The PA system is involved in various tissue remodeling processes. The possibility of creating strains of mice that lack individual proteins through gene targeting or “knockout” technology has provided useful research tools for investigation of the functions of these proteins in vivo. Mice with deficiencies in most of the different components of the PA system, including tPA, uPA, uPAR, PAI-1, PAI-2, and plasminogen have been produced. These strains provide useful model systems to study the role of the PA system in vivo (Carmeliet et al., 1993; Carmeliet et al., 1994;
2.1. Plasminogen-deficient mice

In the mouse, the plasminogen gene is located on chromosome 17 (Degen et al., 1990). Plasminogen-deficient mice were generated by two research groups using two separate strategies to inactivate the plasminogen gene (Ploplis et al., 1995; Bugge et al., 1995). One group eliminated exons 15 to 17 (Ploplis et al., 1995) and the other group deleted proximal promoter sequences as well as exons 1 and 2 (Bugge et al., 1995). Surprisingly, the plasminogen-deficient mice survived into adulthood. However, the mice had impaired thrombolysis, extensive fibrin deposition, retarded growth, spontaneous ulceration of the gastrointestinal tract, rectal prolapse, poor lactation with reduced mammary epithelial content, and also reduced ovulation efficiency, fertility, and life-span (Bugge et al., 1995; Ploplis et al., 1995; Ny et al., 1999; Green et al., 2006). Furthermore, plasminogen-deficient mice with C57BL/6J background have higher frequency and severity of palpebral and bulbar conjunctivitis compared to those with 129/black Swiss background (Drew et al., 1998; Drew et al., 2000). Supplementation of these mice with murine plasminogen has been shown to normalize the thrombolytic potential and resolve endogenous fibrin deposits significantly, indicating that plasminogen is critical in dissolution of fibrin in vivo. Interestingly, plasminogen-deficient mice develop chronic otitis media with various degrees of inflammatory changes (Eriksson et al., 2006). The acoustic startle reflex is also markedly reduced in these mice (Hoover-Plow et al., 2001).

Induced phenotypes in plasminogen-deficient mice have been found from studies of animal models for inflammation, infection, vascular remodeling, wound healing, neurodegeneration, RA, and also cancer growth and metastasis. For example, plasminogen-deficient mice have been found to show impaired wound healing of the corneal and skin epithelium (Romer et al., 1996; Kao et al., 1998), elevated deposition of fibrin, and exacerbated joint inflammation in an RA model (Busso et al., 1998). Most of these studies indicate reduced recruitment of inflammatory cells and disturbed tissue remodeling processes in plasminogen-deficient mice. A strong correlation between plasminogen and fibrin(ogen) has been suggested to contribute to these processes (Ploplis et al., 1995).

Inflammation and infection have been of interest in studies on the functional roles of plasminogen. Phenotypes of plasminogen-deficient mice in this context include compromised recruitment of macrophages and lymphocytes (Ploplis et al., 1998), reduced spirochete load in Borrelia fever (Gebbia et al., 1999), resistance to Yersinia pestis infection (Goguen et al., 2000), severe functional and histological exacerbation of glomerular injury after glomerulonephritis (Kitching et al., 1997), in addition to enhanced collagen-fibrin deposition and diminished macrophage recruitment after challenge with bleomycin (Swaisgood et al., 2000).

Although a strong correlation between plasminogen and fibrin(ogen) in many pathological and physiological processes has been proposed, the results from mice that are doubly deficient in plasminogen and fibrinogen have indicated that the
substrate specificity of plasmin in vivo is more diverse (Bugge et al., 1996; Kao et al., 1998). For example, necrosis has been found in both plasminogen-deficient and plasminogen/fibrinogen doubly deficient mice in many pathological models, suggesting that plasmin may have a critical role through a fibrin-independent pathway. In a chronic liver injury model, plasminogen deficiency leads to impaired lobular reorganization and matrix accumulation after chronic liver injury. Furthermore, it was found that the combined genetic loss of plasminogen and fibrinogen did not correct the abnormal phenotype (Pohl et al., 2001).

Plasminogen deficiency may also be beneficial for the body. For example, reduced transplant arteriosclerosis, in particular less infiltration of macrophages and less migration of smooth muscle cells, has been found in plasminogen-deficient mice (Ploplis et al., 1998; Moons et al., 1998; Bugge et al., 1998).

2.2. Human plasminogen abnormalities

The human plasminogen gene is located on chromosome 6 at position 6q26 (Murray et al., 1987). The size of the gene is about 52.5 kb of DNA, and it consists of 19 exons separated by 18 introns (Petersen et al., 1990). The first report of a patient with abnormal plasminogen was in 1978. The report described a 31-year-old man suffering from a history of intracranial, mesenteric venous thrombosis and pulmonary embolism. Although the plasminogen antigen concentration was normal, the patient had only 37% of normal functional plasminogen activity (Aoki et al., 1978).

To date, two types of plasminogen deficiency have been identified (Ichinose et al., 1991). Type-I plasminogen deficiency is also called hypoplasminogenemia, which is characterized by a complete deficiency of both the immunoreactive plasminogen antigen level and the functional activity. Type-II plasminogen deficiency or dysplasminogenemia is a disorder in which the patients have reduced plasminogen activity but a normal antigen level (Ichinose et al., 1991). Type-II plasminogen deficiency is associated with ligneous conjunctivitis, periodontitis, and vaginitis (Schuster et al., 1997; Kurtulus et al., 2007; Lotan et al., 2007). Many of the patients who carry dysfunctional plasminogen variants (of either type-I or type-II) suffer from recurrent thrombosis. However, most of the cases reported hitherto have been type-II plasminogen deficiency, so called Tochigi disease. Such mutations include Ala$^{601}$→Thr, Val$^{355}$→Phe and Ser$^{572}$→Pro substitutions (Aoki et al., 1978; Ichinose et al., 1991; Azuma et al., 1993).

One disease particularly associated with plasminogen deficiency is ligneous conjunctivitis. Ligneous conjunctivitis is a rare and unusual form of chronic pseudo-membranous conjunctivitis, which usually starts in early infancy (Schuster and Seregard, 2003). The disease also includes pseudo-membranous lesions of other mucous membranes such as the mouth, trachea, or female genital tract. Replacement therapy with Lys-plasminogen leads to rapid regression of the pseudo-membranes and normalization of respiratory tract secretions and wound healing (Schott et al., 1998).
2.3. **tPA-deficient mice**

tPA-deficient mice do not show any overt physiological phenotypes, although they have a reduced capacity for clot lysis and an increased thrombotic tendency after injection of endotoxin (Carmeliet et al., 1994). tPA appears to have a protective role in RA, as tPA-deficient mice have been found to have a more severe arthritis during AIA and CIA (Yang et al., 2001; Cook et al., 2002). Further studies have indicated that tPA plays a role in neuronal processes including long-term potentiation (learning) and neuronal degeneration (Tsirka et al., 1995; Huang et al., 1996). However, neural damage is reduced in tPA-deficient mice after spinal cord injury (Abe et al., 2003). In addition, tPA also appears to be important in protecting against inflammatory renal injury (Kitching et al., 1997).

2.4. **uPA-deficient mice**

uPA-deficient mice are generally healthy, although susceptible to pro-inflammatory thrombotic agents. This susceptibility appears to be caused by an impaired fibrinolytic function of macrophages rather than reduced vascular fibrinolysis, as in the case of tPA-deficient mice (Carmeliet et al., 1994). uPA-deficient mice have more severe AIA and impaired liver regeneration, and they are more susceptible to bacterial infection than wild-type mice (Roselli et al., 1998; Busso et al., 1998; Gyetko et al., 2002). However, uPA deficiency may be beneficial under certain conditions. For example, metastasis of transgenic mammary cancer is reduced in uPA-deficient mice (Almholt et al., 2005).

2.5. **tPA/uPA doubly deficient mice**

tPA/uPA doubly deficient mice have similar phenotypes to that of plasminogen-deficient mice. They have shorter life-span, retarded growth, impaired thrombolytic capacity, chronic ulceration, rectal prolapse, and fibrin deposition in several organs including the lung, liver, and kidney (Carmeliet et al., 1994; Kitching et al., 1997). However, skin wound healing is less impaired in tPA/uPA doubly deficient mice than in plasminogen-deficient mice (Lund et al., 2006). In our studies with a *Staphylococcus aureus*-induced sepsis model, we observed that the tPA/uPA doubly deficient mice have a significantly higher survival rate and delayed onset of septic death as compared to wild-type mice (Paper III).

2.6. **PAI-1-deficient mice**

PAI-1-deficient mice are generally healthy, although the disruption of the gene appears to induce a mild hyperfibrinolytic state and a greater resistance to venous thrombosis, but not to impair hemostasis (Carmeliet et al., 1993). The induced phenotypes in PAI-1-deficient mice include accelerated neointima formation and
wound closure, as well as reduced lung inflammation, angiogenesis, and arteriosclerosis. Taken together, these studies confirm that PAI-1 plays an important role in fibrinolysis (Carmeliet and Collen, 1996; Bajou et al., 2001).

2.7. PAI-2-deficient mice

PAI-2-deficient mice are indistinguishable from their wild-type littermates in terms of development, survival, and fertility. No overt phenotypic differences were found between PAI-2-deficient and wild-type mice during bacterial infection or endotoxin infusion, or in epidermal wound healing processes (Dougherty et al., 1999). A recent study has, however, shown that the development of adipose tissue was impaired in PAI-2-deficient mice that had been put on a high-fat diet (Lijnen et al., 2007). Until recently, studies in PAI-1 and PAI-2 doubly deficient mice have not revealed any overlap in function between these two serpins (Almholt et al., 2003).

3. INFLAMMATION

Inflammation is a complex local biological response in the vascular tissues that serves to destroy, dilute, or wall-off both the initial cause of harmful stimuli and the consequences of such stimuli. Inflammation can be triggered by tissue injury or pathogen invasion including hypoxia, trauma, and infection. Without inflammation, wounds and infections would never heal, resulting in progressive destruction of the tissue with high morbidity and mortality. However, persistent inflammation would lead to a chronic disease in itself such as hay fever or atherosclerosis. Furthermore, when the body’s own immune system triggers an inflammatory response against its own substances, this causes autoimmune diseases such as RA (Haynes, 2007) and systemic lupus erythematosus (Abou-Rayya and Abou-Rayya, 2006). It is for this reason that inflammation is normally tightly regulated by the body. In addition, overwhelming inflammation may lead to severe conditions such as systemic inflammation responses syndrome during sepsis (Gruber et al., 1999).

The process of inflammation can be separated into two phases: acute and chronic. Both forms are amplified as well as propagated as a result of the recruitment of humoral and cellular components of the immune system (Kay, 1987; McCartney-Francis et al., 2003). Acute inflammation is the body’s initial response to harmful stimuli. During acute inflammation, a cascade of biochemical events propagates and matures the inflammatory response. This involves delivery of blood plasma and cellular components to the extravascular tissue spaces and the formation of tissue edema. This leads to destruction of the infectious agents and clearance of necrotic debris, and also the release of cytokines and subsequent initiation of the healing process. During the chronic phase of inflammation, another cascade of pathophysiological events occurs—including a progressive shift in the type of cells, an increase in cell proliferation and migration, tissue destruction, and simultaneous healing of the tissue (Jackson et al., 1997). However, when tissues are chronically exposed to inflammatory mediators, it may also lead to cell mutagenesis and oncogene activation (Maisonneuve and Lowenfels, 2002).
The migration, accumulation, and subsequent activation of leukocytes are central events in the pathogenesis of virtually all forms of inflammation. Of the inflammatory leukocytes, neutrophils are the most important type of effector cells. Neutrophils also form the first line of defense. Normally, the bone marrow of a healthy adult produces more than $10^{11}$ neutrophils per day and this number increases to more than $10^{12}$ per day during acute inflammation (Scheel-Toellner et al., 2004b; Peng, 2006). After release from the bone marrow to the circulation, neutrophils are in a non-activated state before marginating and entering tissue pools, where they survive for 1–2 days (Scheel-Toellner et al., 2004a). In general, cytokines generated by neutrophils, monocytes, and macrophages are thought to mediate the development of inflammation.

Several plasma-derived inflammatory mediators act in parallel to propagate and mature the inflammatory response. These mediators are derived from plasma or cells, and are triggered by the inflammatory stimuli (Villoslada and Genain, 2004; Streetz et al., 2001). Once activated, these mediators amplify the inflammatory response and modify its evolution (Kollias et al., 1999). Interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α) are among the most important mediators of inflammatory reactions (Murch, 1998; Catania et al., 1999).

Inflammation is terminated when the injurious stimulus has been removed and the mediators have either been dissipated or inhibited. Inflammation plays a central role in the pathogenesis of autoimmune diseases and non-autoimmune diseases (such as RA), infection, and wound healing. Further understanding and better control of this complex inflammatory system represent both a formidable challenge and a great opportunity for medical research.

3.1. The roles of the plasminogen activator system in inflammation

Traditionally, the PA system has been regarded as a potent protease system in the degradation of fibrin and other ECM proteins. Thus, the PA system is involved in the inflammatory processes that facilitate the migration of inflammatory cells and the remodeling of inflammatory tissue. For instance, uPA and tPA are believed to have important roles in inflammatory cell infiltration, fibrin deposition, and joint destruction in RA patients, and also in RA animal models such as AIA and CIA. Clinical studies have demonstrated that tPA expression is significantly higher in inflamed pulp tissue (Huang et al., 2005; Huang et al., 2006). Lack of uPA or plasminogen was found to be associated with impairment of the influx of macrophages into the injured area, which results in delayed or abolished wound healing (Heymans et al., 1999; Creemers et al., 2000).

In recent years, research on the PA system in inflammation has shown that the active protease plasmin may not only initiate fibrinolysis and tissue destruction, but may also be involved in various kinds of inflammatory processes. For example, active plasmin triggers the aggregation of neutrophils, the degranulation of platelets, and the release of arachidonate from endothelial cells (Montrucchio et al., 1996). Plasmin also
induces expression of cytokines and tissue factor (TF) in human monocytes (Syrovets et al., 1997; Weide et al., 1996; Syrovets et al., 2001). In vitro studies have shown that through its lysine-binding sites in the heavy chain, plasmin can bind to a variety of cell types—including neutrophils and monocytes. Such cell-surface binding leads to full-scale pro-inflammatory activation of the cells. The activation effects of plasmin are specific, require the active catalytic center, and can be antagonized by lysine analogs (Syrovets et al., 2001). However, the exact functional roles of plasmin in inflammation in vivo remain essentially unknown.

In this thesis, I have attempted to elucidate the functional roles of plasmin and other components of the PA system during different inflammatory diseases such as RA, bacterial arthritis, infection, and sepsis. The studies have been based on the use of gene-deficient mice lacking different components of the PA system. The results indicate that plasmin has a multifunctional role during inflammation and infection by activation of inflammatory cells, enhancement of cytokine expression, improvement of bacterial killing, removal of necrotic tissue, and degradation of fibrin and other ECM components. Together, these data suggest that plasmin is not only a broad-spectrum ECM protease, but also a central player in the host inflammatory response.

3.2. Novel roles of the plasminogen activator system in signal transduction during inflammation

Plasmin(ogen) can bind to various cell types through its lysine binding sites. Traditionally, the pericellular effects of plasmin have been regarded mainly in terms of membrane-associated fibrinolytic or proteolytic activity (Carmeliet et al., 1998). However, plasmin can trigger profound functional changes in a number of cells, suggesting receptor-mediated signaling (Syrovets and Simmet, 2004). Recent studies have shown that another serine protease, thrombin, can activate a number of cells via proteolytic cleavage of the so-called protease-activated receptors and therefore induce signal transduction intracellularly (Vergnolle, 2000; Vergnolle et al., 2001; Resendiz et al., 2007). Considering the similarities between thrombin and plasmin regarding their protease activity and cellular binding properties, it is very intriguing to speculate that plasmin may exert its intracellular effects by a similar mechanism.

Although the cell activation-specific plasminogen receptors and the interaction mechanism with plasminogen remain to be identified, the intracellular signal transduction effects have been observed in various in vivo studies. In platelets, plasmin triggers the release of Ca$^{2+}$ from intracellular stores (Nakamura et al., 1995). In bovine endothelial cells, plasmin stimulates the arachidonic acid cascade, which leads to activation of prostacyclin biosynthesis. Lipid mediator studies have indicated that plasmin is an activator of the 5-lipoxygenase pathway in monocytes (Simmet and Weide, 1991). Stimulation of macrophages with plasmin leads to nuclear translocation of transcriptionally active STAT3 (Li et al., 2007). Moreover, recent studies have shown that plasmin activates multiple signaling pathways, in particular the JAK1/STAT, p38 MAPK, ERK1/2, and the NF-κB pathways (Syrovets et al., 2001; Kawao et al., 2007). Notably, the MAPK pathway is one of the major signaling pathways that control the expression of different pro-inflammatory genes (Burysek et al., 2002). Overall, these studies reveal novel aspects of plasmin function besides its
known role in fibrinolysis. More studies will be necessary to determine the physiological relevance and the underlying mechanism of plasmin-mediated cell activation. Such studies are important for our understanding of the body’s overall mechanism of inflammation activation and may eventually lead to the identification of novel therapeutic targets for intervention in different inflammatory processes.

4. RHEUMATOID ARTHRITIS (RA)

RA is a chronic systemic autoimmune disease affecting around 1–2% of the human population. The disease starts with an immunological attack against cartilage in the joints, resulting in chronic inflammation of synovial membranes (synovitis), increased thickness of the synovial lining (hyperplasia), tissue and bone destruction, and eventually deformity of the affected joints (Paleolog, 2002; Youssef et al., 1998). RA progresses, with relapses, in three stages. The first stage is the swelling of the synovial lining, which causes pain, warmth, stiffness, redness, and swelling around the joint. The second stage is the rapid growth of cells or pannus, which causes the synovium to thicken. In the third stage, the inflamed cells release enzymes that may digest bone and cartilage, often causing the involved joint to lose its shape and alignment, which in turn causes loss of movement (Lee and Weinblatt, 2001). Although the exact mechanism of tissue destruction in RA remains unclear, several studies have suggested that the PA and the MMP systems are involved (Saxne et al., 1993; Busso et al., 1997).

4.1. Animal models of rheumatoid arthritis

Animal models of RA are used extensively in research and pharmaceutical industry to investigate the pathogenesis of RA and to test potential anti-arthritis agents. Several types of animal models for RA have been established including CIA and AIA. These models provide important insights into the pathogenetic mechanisms of human RA (Bendele, 2001).

Collagen-induced arthritis (CIA): CIA is the most commonly used and well-characterized model for studies of human RA (Holmdahl et al., 2002). Immunization of genetically susceptible strains of rodents and primates with both heterologous and autologous collagen type II (CII) leads to the development of a severe polyarticular arthritis. This type of arthritis is characterized by marked cartilage destruction associated with immune complex deposition on articular surfaces, bone resorption and periosteal proliferation, and moderate to marked synovitis and peri-articular inflammation mediated by an autoimmune response (Brand et al., 2007). The susceptibility of CIA is strongly associated with major histocompatibility complex (MHC) II molecules. In particular, H-2q- and H-2r-bearing haplotypes are the most susceptible in mice (Holmdahl et al., 1988; Gustafsson et al., 1990; Kjellen et al., 1998).

Antigen-induced arthritis (AIA): Unlike CIA, AIA is a monoarticular arthritis model. Virtually any animal species can be used in AIA studies. The animal is pre-
immunized (subcutaneous or intradermal injections) with an antigen usually consisting of a cationic substance such as methylated bovine serum albumin (mBSA), which will induce an immunological reaction against the antigen. The antigen is then injected into one or both knee joints—where it binds to negatively charged cartilage and can be retained in the joint. The immunization and subsequent antigen binding results in an acute inflammatory reaction that is characterized by exudation of neutrophils and fibrin. Consequently, it proceeds to a chronic arthritis with synovial hyperplasia, infiltration of mononuclear cells, and then cartilage and bone destruction. Retention of the antigen in the joints is considered to be important for the chronicity of the inflammation. The histopathological changes of AIA are similar to those that occur in RA (Petrow et al., 1996b; Pohlers et al., 2004). AIA is a T cell-dependent experimental arthritis and the susceptibility is not associated with MHC II molecules (Brauer et al., 1994; Petrow et al., 1996a).

4.2. Cytokines in rheumatoid arthritis

Cytokines play a very important role in the destructive process of RA. During the inflammation stage of RA, macrophages actively produce the pro-inflammatory cytokines IL-1 and TNF-α as well as other cytokines such as IL-6, IL-12, IL-15, and IL-18 (Feldmann et al., 1996; Carteron, 2000; Arend, 2001). Thus, analysis of cytokine expression and regulation may yield effective therapeutic targets in inflammatory diseases. For instance, TNF-α is one of the major pro-inflammatory mediators in RA. TNF-α stimulates the production of a number of other cytokines such as IL-1 and IL-6, which results in enhanced inflammation of the joint (Chabaud and Miossec, 2001; Jain et al., 2006). Thus, TNF-α blockade may reduce inflammation either by directly diminishing the activity of TNF-α or by indirectly diminishing the level of IL-1 (Feldmann et al., 2001). In fact, administration of anti-TNF-α antibody to RA patients has shown marked clinical benefit (Maini and Feldmann, 2002; Feldmann and Maini, 2002).

4.3. Roles of the plasminogen activator system in rheumatoid arthritis

The PA system has been suggested to have important roles in inflammatory cell infiltration, fibrin deposition, and joint destruction associated with RA (Busso et al., 1997; Busso and Hamilton, 2002). Most cells that are present in the inflamed joint express uPA, together with variable amounts uPAR and PA inhibitors. Elevated levels of uPA, uPAR, PAI-1, and PAI-2 in RA synovial tissue and fluids have been correlated with the clinical severity of the disease (Weinberg et al., 1991; Brommer et al., 1992b; Ronday et al., 1996; Busso et al., 1997). Secreted pro-uPA might induce plasmin-independent effects such as mitogenic, migratory, and adhesiveness responses (Pepper, 2001). Alternatively, active uPA could generate plasmin and thereby further degrade fibrin deposition and activate and mobilize latent forms of growth factors that can influence the growth and differentiation of cellular constituents in arthritic joints (Busso and So, 1997). Furthermore, the activated plasmin may also act indirectly through the activation of latent MMPs (Ronday et al.,
1997). In contrast, there is reduced tPA expression in synovial tissues and fluids in RA. This reduction in tPA probably accounts for the increase in fibrin deposition in the arthritic joints and thereby helps to create a local pro-inflammatory environment (Brommer et al., 1992a; Belcher et al., 1996; Stringer, 2000).

Studies on animal models of RA have also shown the involvement of the PA system in development of the disease. For example, data from the CIA model indicate that uPA-deficient mice develop a milder form of CIA than wild-type mice, with minimal levels of inflammation and joint destruction (Li et al., 2005). In another study, it has been found that uPA mRNA levels increase during development of CIA (Busso and Hamilton, 2002). Surprisingly, plasminogen-deficient mice are completely resistant to CIA (Li et al., 2005). Studies of AIA showed that uPA-deficient or plasminogen-deficient mice had significantly more severe arthritis (Busso et al., 1998). Interestingly, tPA-deficient mice develop more severe disease than wild-type mice, with sustained inflammation and fibrin accumulation within the joints in both CIA and AIA models (Yang et al., 2001; Cook et al., 2002). Thus, it appears that tPA and uPA can either be deleterious or beneficial, depending on the animal model used. These findings highlight the complex nature of RA and the relative importance of the PA system in the pathogenesis of AIA and CIA.

In order to explain the contrasting phenotypes of plasminogen-deficient mice in CIA and AIA, we developed a new animal model of arthritis called local injection-induced arthritis (LIA) (Paper I in this thesis). In this model, we followed the induction procedure used in AIA, the only difference being that we replaced mBSA with CII. After LIA induction, plasminogen-deficient mice developed arthritis in joints that had been injected with CII, or even just saline. The arthritis was, however, milder than that in the wild-type littermates. This study clearly indicates that both the antigen and the joint trauma caused by the local injection are critical in explaining the contrasting roles of plasminogen deficiency in CIA and AIA. The results further suggest that CIA and AIA have distinct pathogenic mechanisms. Together, these studies have helped to clarify the overall role of the PA system in the pathogenesis of arthritis in vivo and should facilitate the development of new therapeutic strategies for RA.

5. INFECTION

Infectious diseases result from invasion of pathogens such as bacteria, viruses, and some eukaryotic organisms. Pathogens often colonize the host when the host is in direct contact with its environment. These pathogens invade by using the host’s resources to multiply and they can cause chronic wounds, loss of an infected limb, and even death of the host. Bacterial infection accounts for almost 70% of the overall causes of infection. Some pathogenic bacteria contain virulence factors that mediate interactions with the host. Such interactions include adherence and further invasion of the epithelial surfaces, and also elicitation of particular responses from the host cells. These interactions eventually promote the replication and spread of the pathogen. In the case of viruses, they rely on subverting the machinery of the host cell by using receptor-mediated endocytosis to gain entry, for example, and then they replicate and express their genomes (Aderem, 2003).
5.1. The host defense against bacteria

Despite the various mechanisms that pathogens have developed to invade the host, there are only limited patterns by which the machinery of the host defense reacts against the infection. The body’s innate and acquired immunity together constitute the host defense to protect the host from the deleterious effects of pathogens. The innate immunity relies on the body’s ability to recognize conserved features of pathogens. During the initial hours and days of exposure of the host to a new pathogen, the innate immunity is the first line of defense against invading pathogens. At later stages of infection, however, the active acquired immunity becomes involved to further activate inflammatory cells through the humoral and cellular pathways (mediated by antibodies and T cells, respectively) (Davenport et al., 2003).

In the host defense, the phagocytic cells use a combination of degrading enzymes, anti-microbial peptides, and reactive oxygen species to kill the invading microorganisms (Hauschildt and Kleine, 1995). In addition, phagocytic cells release signaling molecules to trigger an inflammatory response and begin to marshal the forces of the adaptive immune system (Chertov et al., 2000). To counteract this, bacteria have developed different strategies to escape from phagocytes such as inhibiting chemotaxis and phagocytosis, and also killing or colonizing the phagocytes (Chensue, 2001; Supuran et al., 2002). Bacteria have also developed different strategies directed against the adaptive immune system such as molecular mimicry, suppression of antibodies, hiding inside cells, or release of antigen into the bloodstream (Chertov et al., 2000).

The innate immune system provides general protection against infectious diseases caused by pathogens. However, the adaptive immune response must be induced or turned on by exposure of the host to a pathogen during infection. Unlike the innate immune system, the adaptive immune system is not immediately ready to come into play until after the host has been appropriately exposed to pathogens.

In the past, it has generally been agreed that regardless of the various types of microbial pathogens that cause an infection, the host response is similar and involves initiation of the systemic inflammatory response with activation of the pro-inflammatory cytokines and mediators. However, in recent years there has been increasing evidence to show that the host responses to Gram-positive and Gram-negative bacterial pathogens are rather different (Bone, 1994; Feezor et al., 2003). For instance, in infection by Gram-negative bacteria, endotoxin—such as the lipopolysaccharide (LPS) moiety of the outer membrane—is a prime activator of both immune and non-immune cells. Exploration of the interaction between LPS and host cells has led to the identification of key molecules, including the LPS binding protein and the CD14 receptor (a pattern recognition molecule in the innate immune response against microorganisms). Gram-positive bacteria, on the other hand, can provoke severe inflammation by at least two distinct mechanisms. Firstly, Gram-positive bacteria such as staphylococci or streptococci have been shown to release exotoxins that act as superantigens. Secondly, cell wall components of Gram-positive bacteria have been found to activate monocytes and macrophages to release pro-inflammatory mediators (Calandra, 2001).
Among all the sophisticated host defense mechanisms conducted by the innate immune system, the complement system and Toll-like receptors (TLRs) are especially important in providing critical danger signals to adaptive immune system. The complement system and TLRs are the most ancient, conserved components of the immune system. The complement system is the major humoral component of the innate immune system. It is a biochemical cascade in the blood, consisting of a number of small proteins for clearing pathogens from the body. TLRs are a class of single membrane-spanning non-catalytic receptors that recognize structurally conserved molecules derived from microbes. Binding of such molecules to TLRs results in activation of immune cell responses. Local or systemic infections are likely to activate both the complement system and TLRs, and the inflammatory cells at the same time, which suggests that complement receptor signaling pathways may intersect with TLR signaling pathways (Marth and Kelsall, 1997; Hawlisich and Kohl, 2006).

As discussed above, the inflammatory cells (neutrophils and macrophages) play a central role in the host defense mediated by both the innate and adaptive immune systems against all kinds of infections, ranging from viruses and bacteria, single-celled fungi and protozoa, to large parasitic worms (Smith, 1994). The inflammatory cells actively seek, engulf, and destroy pathogens directly or through a variety of cell-surface receptors such as TLRs and complement receptors. If a pathogen is too large, a group of macrophages and neutrophils will gather around the invader and secrete inflammatory mediators to constrain the infection and enhance the local inflammation. Activated macrophages also recruit additional phagocytic cells to sites of infection. Inflammatory cells also secrete a variety of signaling molecules to mediate and amplify the inflammatory response. In the B cell-mediated adaptive immune response against infectious pathogens, newly generated antibodies bind to the antigens on the pathogens through the Fab fragment. These antibodies also bind to the surface receptors on the inflammatory cells (mainly macrophages) through Fc fragment, thus linking the inflammatory cells and pathogens and leading to death of the latter (Mellman et al., 1984).

5.2. Septic arthritis

Septic arthritis, also called infectious arthritis or bacterial arthritis, is a clinical arthritic disease in the joint space caused by a bacterial infection or, more rarely, by a fungal infection. Bacteria are carried to the joint either by the bloodstream from an infectious focus elsewhere or through a skin lesion that penetrates the joint, or by extension from adjacent tissue (Berendt and Byren, 2004). Septic arthritis may affect any joints but it is most frequently found in the knee, hip, shoulder, wrist, elbow, and finger joints. Septic arthritis occurs most often in people who have had a recent traumatic injury to a joint, and/or in people who currently have a blood infection. Microorganisms can spread from an original site of infection into the blood and they can then be transported into the joint space. Therapy is usually with intravenous antibiotics, analgesia, and washout/aspiration of the joint to dryness. However, in recent years, bacterial resistance to antibiotics has increasingly become a huge medical challenge in the treatment of septic arthritis (Tarkowski et al., 2001).
Staphylococci are Gram-positive spherical bacteria that occur in microscopic clusters resembling grapes, about 1 µm in diameter. There are about 30 species of staphylococci, and most of them are completely harmless. Only *S. aureus* and *S. epidermidis* are significant in their interactions with humans. *S. aureus* is always considered to be a potential pathogen. It is the most common cause of nosocomial pneumonia, septic arthritis, and operative wound infections (Slaughter *et al*., 1995).

*S. aureus* is the most common pathogen in septic arthritis. In fact, around 75% of all clinical septic arthritis cases are caused by an *S. aureus* infection. *S. aureus*-mediated septic arthritis results in synovial inflammation, cartilage and bone destruction, and eventually joint deformity. Various animal species including mammals, birds, and reptiles have been found to develop spontaneous *S. aureus*-mediated arthritis and are therefore potential models for induction of the disease. The mouse *S. aureus*-induced septic arthritis model has been regarded to be the optimal because of the striking resemblances between the immune and inflammatory systems of mice and humans. Furthermore, the availability of genetically and immunologically well-characterized mouse strains has enabled in-depth analyses of host variables during infection. Transgenic and gene-deficient mice are of obvious interest. However, it is important to bear in mind that certain staphylococcal factors might be restricted in their host interactions (Tarkowski *et al*., 2001).

### 5.3. Roles of the plasminogen activator system during infection and bacterial arthritis

During infection, the PA system has been suggested to be involved at several stages and by various mechanisms, both with regard to bacterial invasion and the host defense against infection (Lahteenmaki *et al*., 2001). As discussed above, the PA system participates in the host defense against infection through its important roles in the host inflammatory response. However, the PA system also participates in the bacterial invasion processes, by interaction with bacteria and bacterial components. A vast number of pathogens express plasmin(ogen) receptors to immobilize plasminogen (Broder *et al*., 1991; Berge and Sjobring, 1993). Such binding enhances pericellular plasminogen activation by host PAs, and consequently turns the bacterium into a proteolytic organism by use of a host-derived system (van Gorp *et al*., 1999; Lahteenmaki *et al*., 2001). Bacteria also influence the secretion of PAs and their inhibitors from mammalian cells (Brandtzaeg *et al*., 1990; Fuchs *et al*., 1996). For example, production of uPA has been found to be enhanced in cells infected by various bacteria (Fuchs *et al*., 1996). Furthermore, the periodontal pathogen *Porphyromonas gingivalis* and the plague bacterium *Yersinia pestis* inactivate the plasmin inhibitors α2-AP and α2-macroglobulin (Grenier, 1996; Kukkonen *et al*., 2001). The bacterially-encoded PAs streptokinase and staphylokinase are not enzymes themselves, but they form 1:1 complexes with plasminogen and plasmin, and acquire a remarkable ability to activate plasminogen (Parry *et al*., 2000). Overall, such interactions between bacteria and the PA system promote damage of the ECM and further facilitate bacterial spread and organ invasion during infections.

To date, most studies on the role of the PA system in bacterial arthritis have focused on the interaction between the invading bacteria and the PA system. Research
methodology includes a variety of microbiological approaches such as mutating one or more bacterial surface proteins, or using different bacterial strains. Although it is known that the PA system can play a double role during bacterial infection by both facilitating the bacterial invasion and enhancing the host defense, there have been essentially no publications that have attempted to clarify the enigma of the critical functional roles of plasmin. During my thesis work, I have tried to investigate this question by making use of a local *S. aureus*-induced septic arthritis model in wild-type mice and in mice lacking components of the PA system. Our data indicate that the PA system plays a key role in septic arthritis by activating the host defense rather than by facilitating bacterial invasion (Paper II in this thesis). This suggests a novel therapeutical strategy whereby plasminogen could be used as a drug candidate to enhance the host defense in order to fight infectious arthritis and other infectious diseases. As we face the increasing problem of bacterial antibiotic resistance in the global healthcare system, this novel anti-infection strategy has a unique advantage over the traditional antibiotic treatments, in that it will not induce the side effect of bacterial resistance.

6. **SEPSIS**

Sepsis, also known as systemic inflammatory response syndrome, is a serious medical condition caused by the overwhelming inflammatory response of the whole body to an infection. Pathways and mediators involved in sepsis include innate and adaptive immune systems, pro-inflammatory and anti-inflammatory cytokines and molecules, pro-coagulation and anticoagulation pathways, and also hypoxia, apoptosis, and immunosuppression molecules (Russell, 2006). Symptoms of sepsis are often related to the overactive anti-infectious processes such as vigorous inflammation, fever, and leukocytosis.

The vigorous immunological response is one of the major aspects of sepsis and can lead to widespread inflammation, resulting in dysfunction of the circulatory system and eventually multiple organ failure and death. The peptidoglycan of Gram-positive bacteria and the LPS of Gram-negative bacteria bind to TLR-2 and TLR-4 and initiate a brisk inflammatory response (Chowdhury *et al*., 2006). Such binding activates intracellular signal transduction pathways that lead to the activation of cytosolic NF-κB and increase the transcription of the cytokine pool such as TNF-α, IL-1β, and IL-10 (Faure *et al*., 2000; Higgins *et al*., 2003). Sepsis also enhances the activity of inducible nitric oxide synthase and synthesis of the potent vasodilator nitric oxide. Upregulated adhesion receptors activate endothelial cells and enhance the binding of neutrophils, monocytes, macrophages, and platelets. These effector cells release mediators such as proteases, oxidants, prostaglandins, and leukotrienes, which in turn injure endothelial cells, leading to increased permeability, further vasodilation, and alteration of the procoagulant-anticoagulant balance (Faure *et al*., 2000).

Alteration of the procoagulant-anticoagulant balance is an important aspect of sepsis. Sepsis initiates coagulation by activating endothelium to increase the expression of TF. Activation of the coagulation cascade leads to the formation of thrombin-α, which converts fibrinogen to fibrin, leading to the formation of microvascular thrombi. Such microvascular thrombi can amplify injury by microvascular obstruction, which causes enhanced procoagulation such as distal ischemia and tissue hypoxia. In addition,
during sepsis, the invading bacteria and cytokines also increase the synthesis of PAI-1 and reduce the levels of protein C, protein S, antithrombin III, and TF pathway inhibitor, which together contribute to impaired anticoagulation in the body (Faust et al., 2001).

Treatment strategies for severe sepsis are directed at its infectious etiology and at the rapidly cascading events that lead to the lethal deterioration in physiological function (Hotchkiss and Karl, 2003). Early diagnosis is essential because the efficacy of early, goal-directed therapy directs the refinement of the antibiotic regimen, control of the source of sepsis, and evaluation of resolution of the signs of the systemic inflammatory response syndrome. Thus, emphasis has been placed on discovering the causes of infection and eradicating the nidus of infection by appropriate antibiotic therapy, surgical drainage, and other anti-infective strategies. Notably, despite the extensive use of antibiotics, antibiotic treatment does not seem to show a satisfying outcome. Consequently, studies focused on dissecting the complex underlying mechanisms of sepsis are greatly needed. Only efforts such as these would lead to novel and effective therapeutic strategies (Lolis and Bucala, 2003; Rice and Bernard, 2005).

6.1. Cytokines and sepsis

The binding of microbial products to pattern recognition receptors on innate immune cells activates several intracellular signaling pathways. This results in the activation of transcription factors, the expression of immune response genes, and the release of a broad range of effector molecules. Cytokines are an important class of mediators derived mainly from mononuclear phagocytic cells, and are involved in virtually every facet of immunity and inflammation.

Cytokines can be functionally classified into pro-inflammatory (TNF-α, IL-1, IL-6) and anti-inflammatory (IL-4, IL-10) molecules. During an inflammatory response, pro-inflammatory cytokines are induced first, followed by production of the anti-inflammatory cytokines. In experimental and human clinical studies, pro-inflammatory cytokine production has been strongly associated with the pathogenesis of sepsis. Extremely high levels of IL-6 or TNF-α in the sera of septic patients or mice are associated with increased mortality (Rink and Kirchner, 1996; Latifi et al., 2004; Remick et al., 2005). In addition, infusion of TNF-α into animals induces symptoms characteristic of sepsis (Michie et al., 1988; Riedemann et al., 2003a), while passive immunization with anti–TNF-α antibodies is protective (Michie et al., 1988). Suppressing the IL-6 or TNF-α levels by antibody treatment can somewhat improve the survival of patients and survival in murine models (Riedemann et al., 2003b). However, none of these treatments appear to be especially effective. This may be because the agents were not sufficiently potent, or because the target mediators were either too distal in the inflammatory cascade or not of central importance in effecting systemic toxicity.

Besides stimulating the synthesis of pro-inflammatory cytokines, the septic response also stimulates the production of anti-inflammatory mediators such as IL-10. As an anti-inflammatory cytokine, IL-10 levels in serum are elevated in cecal ligation and
puncture-induced sepsis model. Subcutaneous treatment with recombinant human IL-10 delays the onset of sepsis and improves long-term survival in both wild-type and IL-10-deficient mice (Latifi et al., 2002). However, inhibition of IL-10 by injecting neutralizing antibodies actually increases mortality if injected at the time of induction of sepsis. In contrast, inhibition of IL-10 improves the survival if the antibodies are administered 12 hours after induction of sepsis (Song et al., 1999). These data suggest that IL-10 can be either protective or harmful, depending on the stage of development of sepsis, and they further demonstrate the complex nature of sepsis.

6.2. Complement and sepsis

In the innate immune defense, the complement system plays a key role in recognizing invading microbial agents. However, this system also acts as a link between innate immunity and the activation, the regulation, and the effector arms of the adaptive immunity (Fearon, 1998; Carroll, 2000). The complement system consists of a complex group of serum proteins, glycoproteins, and soluble or membrane-bound receptors (Muller-Eberhard and Schreiber, 1980). Complement can be activated through three pathways: the classical, the alternative, or the lectin pathway. The three pathways converge at the point of activation of C3.

There is strong evidence that the complement system is actively involved in the very early stage of sepsis in both humans and animals. Clinical studies have shown that the levels of C3a, C4a, and C5a in sera are all significantly higher in non-survivors than in survivors, and that they closely reflect the severity of sepsis (Nakae et al., 1994; Stove et al., 1996; Messias-Reason et al., 2002). In a murine cecal ligation and puncture-induced sepsis model, the excessive generation of C5a during sepsis appears to desensitize neutrophils, leading to high mortality (Ward, 2004). In line with these observations, blockade of C5a production with antibodies during the onset of sepsis has been shown to greatly improve survival in rodents (Czermak et al., 1999). Since the effect of C5a on neutrophils is mediated by the C5a receptor (C5aR), similar findings were made when the C5aR was blocked either by antibodies or by a small molecular inhibitor (Huber-Lang et al., 2002a; Riedemann et al., 2002). Furthermore, blockade of C5aR results in reduced IL-6 plasma levels (Riedemann et al., 2002). In turn, blockade of IL-6 was found to inhibit the increase in C5aR in the organs and resulted in improved survival during cecal ligation and puncture-induced sepsis (Riedemann et al., 2003b).

Based on the above studies, in recent years a hypothesis has been proposed for the roles of the complement system in sepsis (Figure 2). Briefly, overwhelming bacterial infection leads to excessive generation of C5a at the onset of sepsis. The excessive amounts of C5a induce an overwhelming pro-inflammatory response, which causes a significant pro-coagulation effect and a significant impairment of the innate immune functions of blood neutrophils (Huber-Lang et al., 2002b; Guo et al., 2003). Overexpression of cytokines such as IL-6 also contributes to enhanced C5aR expression in various cell types in solid organs, and also contributes to the sensitivity to C5a effects. IL-6 and C5a can cause massive production of reactive oxygen species in phagocytic cells, increase in vascular permeability, damage to various organs, and eventually leading to multiple organ failure. Based on this hypothesis, a novel
A therapeutic strategy has been developed whereby the interaction of C5a and C5aR can be interfered during sepsis (Riedemann et al., 2002). Currently, research on complement inhibitors for the treatment of sepsis is a very active field in the pharmaceutical industry.

![Diagram](image.png)

**Figure 2.** The key role of C5a/C5aR activation in the development of sepsis and multi-organ failure. The diagram depicts the pro-inflammatory and immunosuppressive effects of C5a in the context of sepsis. Modified from (Riedemann et al., 2002).

### 6.3. Coagulation and sepsis

As discussed above, sepsis is not just an inflammatory state exclusively. During sepsis, exotoxins and endotoxins initiate both the inflammatory and coagulation cascades. These cascades act in concert and augment each other, with a role in the morbidity and mortality associated with the disease (Levi et al., 2006). In addition, the body’s systemic response—predominantly the release of cytokines such as TNF-α and IL-1—stimulate both inflammation and coagulation, which alter homeostasis to favor thrombus formation. The enhanced expression of TF stimulated by pro-inflammatory cytokines further downregulates thrombomodulin expression on endothelial surfaces, and thereby enhances coagulation (Taylor, Jr. et al., 1996).

Formed TF can initiate the coagulation cascade, resulting in the formation of thrombin and fibrin clots. Thrombin stimulates the formation of fibrin clots in the microcirculation, in an attempt to confine the infection to the local site. These diffuse intravascular microthrombi create regional areas of hypoperfusion and lead to local tissue hypoxia, coagulation necrosis, organ dysfunction, and eventually multiple organ failure (Zeerleder et al., 2005). In addition to their coagulation effects, thrombin, TF-VIIa complex, and activated factor X also act as potent inflammatory mediators: by stimulating neutrophil migration and releasing pro-inflammatory cytokines, they...
fuel additional thrombin and fibrin clot formation (Faust et al., 2001; Hotchkiss and Karl, 2003).

Based on the above findings, new therapeutic strategies have been directed at interference with the coagulation pathway. In fact, treating sepsis patients with antithrombin, acute-phase protein, or TF pathway inhibitor has led to significant attenuation of disseminated intravascular coagulation and inflammation, resulting in improved survival (Faust et al., 2001).

6.4. Roles of the plasminogen activator system in sepsis

Under normal circumstances, the anticoagulation system acts in concert with the coagulation system in order to preserve homeostasis in the body. However, under conditions of sepsis, a bimodal response of overall fibrinolytic activity occurs: there is a marked increase in PAs and overall fibrinolytic activity for a few hours, followed by prolonged suppression of fibrinolytic activity which is paralleled by a sustained increase in PAI-1 (Lorente et al., 1993). Accordingly, different conclusions about the roles of the PA system in sepsis have been drawn based on different experimental conditions such as the animal models used, the time points of sepsis when the fibrinolysis parameters were measured, and which patient groups were selected.

In some reports, the authors have claimed that, although some plasmin-induced fibrinolysis still occurs, the high levels of PAI-1 and reduced levels of both plasminogen and plasmin indicate that the fibrinolytic response is insufficient to maintain homeostasis (Watanabe et al., 2001; Wada et al., 2003). Another study showed that the levels of uPA in the sera of patients with bacterial meningitis were dramatically elevated and associated with an increase rate of mortality (Winkler et al., 2002b; Abraham et al., 2003). uPA-deficient mice were found to be protected from acute inflammatory processes in LPS-induced acute lung injury disease (Abraham et al., 2003), whereas tPA-deficient mice were found to have impaired host defense against *Escherichia coli*-induced peritonitis and reduced survival (Renckens et al., 2006). However, elevated production of tPA and higher concentrations of tPA-PAI complex in the plasma of septic patients have been suggested to be correlated with severity of multiple organ failure (Hoshino et al., 2001; Winkler et al., 2002a). It is more generally accepted that PAI-1 levels are consistently elevated in sepsis and highly predictive of an unfavorable clinical outcome in humans (Lorente et al., 1993; Zeerleder et al., 2005).

In our study of *S. aureus*-induced sepsis in mice, we found that mice lacking genes of different components of the PA system have improved levels of survival, but to different extents (Paper III in this thesis). Wild-type mice have the earliest onset of death from sepsis and the lowest survival rate, whereas uPA- or tPA-deficient mice and plasminogen-heterozygous mice have delayed onset of death from sepsis and a higher survival rate than wild-type mice. Strikingly, tPA/uPA doubly deficient and plasminogen-deficient mice have the most delayed onset of septic death and the highest survival rate compared to all the other groups. These results can hardly be explained by the “traditional” role of plasmin as a protease that degrades fibrin and other ECM components. Our results therefore suggest that the PA system may have...
novel functional roles in sepsis. The lack of active plasmin in either plasminogen-deficient or tPA/uPA doubly deficient mice most likely results in an impaired inflammatory response during the early stage of \textit{S. aureus}-induced sepsis. This may diminish the overwhelming level of inflammatory response that is seen in wild-type mice, and consequently improve the survival rate and delay the onset of septic death (Paper III).

7. SUMMARY OF THE PRESENT STUDIES

This chapter summarizes the three research papers (Papers I–III) that make up this thesis. The figures and tables cited correspond to those used in the original papers.

7.1. Contrasting roles of plasminogen deficiency in different rheumatoid arthritis models (Paper I)

Both AIA and CIA are commonly used murine models of RA. AIA is induced by immunization with mBSA in Complete Freund’s Adjuvant with heat-killed \textit{Bordetella pertussis} at day 0 and day 7, followed by intra-articular injection of mBSA into the knee joint at day 21. The disease is chronic, antigen-specific, and T cell-dependent. CIA is induced by immunization with CII in the presence of Complete Freund’s Adjuvant, which contains heat-killed mycobacteria. This results in activation of natural killer lymphocytes and in multiplication of T and B lymphocytes that recognize the animal’s own collagen. These cells produce antibodies and cytokines, which cause inflammation of joint tissues. AIA and CIA differ in several respects including antigen challenge, disease induction, and clinical features. Whereas AIA is a monoarthritis model restricted to locally injected knee joints, CIA is a polyarthritis model in which the arthritis can develop in all the peripheral joints. The severity of the disease in CIA can be followed by a macro-scoring system, whereas the severity of AIA is evaluated by histology.

In a previous study using the AIA model, it was shown that plasminogen-deficient mice develop more severe arthritis than wild-type control mice (Busso et al., 1998). In contrast, our studies using the CIA model have shown that plasminogen-deficient mice are completely resistant to CIA. It is possible that the apparent discrepancy of the plasminogen-deficient phenotype during CIA and AIA might be explained by differences in the experimental set-up or by the mouse strains used. However, it is more likely that the apparent discrepancy may reflect the different pathogenic mechanisms of these two arthritis models.

The aim of this study was to gain a deeper understanding of the molecular mechanism by which plasmin affects the pathogenesis of RA in the two different RA models. To do this, we developed a new experimental animal model for RA, which we have called LIA. In this model, the administration was performed as for AIA, but mBSA used in AIA as the antigen was replaced by CII. The difference between LIA and CIA is the administration: a local trauma is induced in the knee joint in LIA, whereas there is no trauma in CIA. The local trauma will also initiate a wound healing-like process.
in which the innate immune response against trauma is involved. By using these
different models, the different functional roles of plasminogen in AIA and CIA can be
interpreted and the different mechanisms between the models can be elucidated more
precisely.

In the CIA model, we found that after immunization with CII alone, wild-type mice
developed arthritis in most of the paws and in the knee joints, whereas plasminogen-
deficient mice were totally resistant to the disease. Local knee injections of CII or
saline slightly enhanced the severity of the knee arthritis in wild-type mice over a 60-
day experimental period. Unexpectedly, the plasminogen-deficient mice developed
arthritis in joints that had been injected with CII or saline. However, the arthritis was
milder than that in wild-type littermates. Sustained tissue necrosis was found only in
the plasminogen-deficient mice after the local injection. These results demonstrate
that the local trauma is critical to the disease profile of arthritis. Taken together, these
data show that both the antigen and the joint trauma caused by the local injection can
explain the difference in response between the CIA and AIA models. This further
indicates that CIA and AIA have distinct pathogenic mechanisms and suggests that
plasmin may be an absolute requirement in arthritis models that are critically
dependent on complement activation.

7.2. Protective effects of plasmin(ogen) in a mouse model of
Staphylococcus aureus-induced arthritis (Paper II)

*Staphylococcus aureus* is the microorganism most frequently associated with bacterial
arthritis, which results in synovial inflammation, cartilage and bone destruction, and
eventually joint deformity. The PA system is a general proteolytic system that has
been suggested to play an important role in the development of different types of
arthritis. Plasminogen can be activated to form the broad-spectrum protease plasmin
by either of the two physiological PAs, tPA or uPA. There is also increasing evidence
that plasmin induces pro-inflammatory responses that are independent of its
proteolytic properties.

The PA system has been shown to have dual but contrasting roles during bacterial
arthritis, enhancing both bacterial invasion and host defense. Several pathogenic
microorganisms have been shown to bind plasminogen. The subsequent activation of
plasminogen to plasmin may contribute to the virulence of these microorganisms.
However, during bacterial arthritis, plasmin may also be important for the host
defense by killing bacteria or degrading ECM components during tissue repair.

To study the functional roles of plasmin(ogen) in the host inflammatory response to
infection, bacterial arthritis was induced in plasminogen-deficient mice and wild-type
littermates by local injection of $1 \times 10^6$ colony-forming units (CFU) of *S. aureus* into
the knee joints. Following induction of bacterial arthritis, both histological and
immunohistochemical comparisons between wild-type mice and plasminogen-
deficient littermates were made.

Our data showed that in wild-type mice, the *S. aureus* counts had declined within 2
days, and by day 28, the bacteria had been completely eliminated. *S. aureus* was still
detectable in all the injected joints of plasminogen-deficient mice, however, and the bacterial counts were 27-fold higher at day 28 than at day 0. The extent of macrophage and neutrophil recruitment to the infected joints was comparable for wild-type and plasminogen-deficient mice at days 1, 7, and 14. The activation of these inflammatory cells appeared to be impaired in plasminogen-deficient mice. However, treatment of plasminogen-deficient mice with antibiotic (cloxacillin) resulted in successful killing of the bacteria, but the necrotic tissue remained in the infected joints. When human plasminogen was given intravenously to the plasminogen-deficient mice daily for 7 days, bacterial clearance was greatly improved compared to their untreated counterparts, and the amount of necrotic tissue in the joint cavity was dramatically reduced. The expression of IL-6 and IL-10 was higher in wild-type mice than in plasminogen-deficient mice during bacterial arthritis.

In summary, plasmin protects against *S. aureus*-induced arthritis by enhancing cytokine expression, removing necrotic tissue, and mediating bacterial killing. These results indicate that plasmin plays an essential role in the host defense against bacterial arthritis and they suggest a novel strategy in the treatment of infectious joint diseases.

### 7.3. Beneficial and detrimental effects of plasmin(ogen) during infection and sepsis (Paper III)

Sepsis is a serious medical condition caused by the whole body’s overwhelming response to an infection. Sepsis can lead to widespread inflammation and blood clotting. Sepsis caused by *S. aureus* is a life-threatening condition that may lead to multiple organ failure and eventually death. It is well known that disorders of coagulation and fibrinolysis play a major role in the development of organ dysfunction during sepsis. The key component of the PA system, plasmin, is responsible for fibrinolysis in the vascular system. Besides fibrinolysis, the PA system plays an important role in several aspects of the innate immune system such as activating the STAT3 signaling pathway and stimulating the release of cytokines and other inflammatory mediators by different cell types. Thus, it is of interest to study the roles of the PA system under septic conditions as in the *S. aureus*-induced sepsis model.

The aim of this study was to investigate the functional roles of plasmin during *S. aureus*-induced sepsis and infection by the use of plasminogen-deficient mice. To do this, we used two models. We defined injection of $1 \times 10^7$ CFU of *S. aureus* as the infection model and the injection of $1.6 \times 10^8$ CFU of *S. aureus* as the sepsis model. Twenty-five days after induction of the infection, wild-type mice had significantly higher survival rates than plasminogen-deficient mice. However, when mice were given sepsis-inducing doses of bacteria, the mean onset time of death in plasminogen-deficient mice was delayed by 58 h compared to wild-type mice. For the infection model, 24 h after bacterial inoculation there was no significant difference (between plasminogen-deficient and wild-type mice) in bacterial counts in all the organs studied except brain. In contrast, 24 h after induction of sepsis, the bacterial counts in all organs studied were significantly higher in wild-type mice than in plasminogen-deficient mice. Furthermore, levels of inflammatory cytokines such as serum TNF-α,
IL-6, and IL-10 were significantly higher in wild-type mice than in plasminogen-deficient mice. Levels of both phosphorylated and total STAT3 protein were dramatically lower in the spleen neutrophils of plasminogen-deficient mice than in those of wild-type mice. In wild-type mice, blockade of IL-6 by intravenous injection of anti-IL-6 antibodies significantly prolonged the onset of mortality and improved the survival during sepsis. We also investigated the effects of different components of PAs during sepsis. The survival curves in sepsis showed that wild-type and tPA/uPA doubly heterozygous mice have the lowest survival rate and that tPA/uPA doubly deficient and plasminogen-deficient mice have the highest survival rate, whereas plasminogen-heterozygous, uPA-deficient, and tPA-deficient mice have intermediate rates of survival.

In conclusion, in the infection model plasmin is of benefit in the antibacterial immune responses of the host. However, in the sepsis model, plasmin appears to promote the production of inflammatory cytokines and tissue destruction, cripples neutrophil function, and impairs the capacity to kill bacteria. These data thus indicate that functional plasmin plays a key role in the mortality of normal mice during sepsis.
CONCLUSIONS

Plasminogen-deficient mice are resistant to CIA, develop a less severe disease in LIA, and a more severe disease in AIA as compared to wild-type controls.

The apparent discrepancy in arthritis phenotypes in plasminogen-deficient mice during CIA and AIA reflects the different pathogenic mechanisms of these two arthritis models.

Plasminogen plays essential roles in activating inflammatory cells, killing bacteria, reducing necrosis formation, and enhancing cytokine expression during bacterial arthritis.

Inflammatory cell migration is not impaired in plasminogen-deficient mice during bacterial infection.

Plasminogen-deficient and tPA/uPA doubly deficient mice have significantly delayed onset of death and improved survival as compared to wild-type mice, indicating that active plasmin has critical and deleterious roles during *S. aureus*-induced sepsis.
ACKNOWLEDGEMENTS

I would like to thank all the people who work at the Department of Medical Biochemistry and Biophysics, for making the place so very special.

First, my deepest gratitude goes to my supervisor Tor Ny, for everything you have done for me both in science and in life in general, and for your continuous support and never-ending encouragement and enthusiasm.

Special thanks to Jinan Li, for being a good friend and collaborator; you have given me so much help and encouragement. We share data, ideas and an office, although sometimes we cannot agree. Your optimistic and enterprising spirit will lead you to great success.

To my collaborator, Elin Hagström, thank you for your kind help.

Kui Liu and Malgorzata Wilczynska, thank you for all your suggestions about experiments and life. Patrik Wahlberg, thank you for all your kind help.

Nina Ahlskog and Rima Sulniute, thank you for all your suggestions about experiments. I am really happy to discuss things with you.

Thanks to all my Chinese friends, Yunsheng Zhao and Yafang Mei, you have given me so much help. Shouye Wang, Bo Xiong, Yue Shen, Chao Zou, Chun Du, and Wenjing Zheng, best wishes to all of you.

Peter Hägglöf, Sergei Lobov, Deepak Adhikahi, Pradeep Reddy, Krishna Jagarlamudi, Thord Johansson and Assar Bäckman, all members of the group.

Ingrid Råberg, thank you very much for helping me with all the paperwork. I cannot find a better way to express my thankfulness.

Thanks to all my friends at BISAM Lena Gustafsson, Marianne Borgström, Renita Högström and Sara Forsmark for helping me to take care of my mice and rats.

This study was supported by research grants from the Swedish Research Council and the Medical Faculty of Umeå University.

Special thanks to Jie Zhao, my dear wife and family for their support, patience, patience, and more patience over the years.
REFERENCES


