Stony Brook University

The dual role of the Plasminogen Activation System in the dissemination of *Borrelia burgdorferi* and the inflammatory migration of immune cells

A Dissertation Presented

By

Woldeab Berhane Haile

To

The Graduate School

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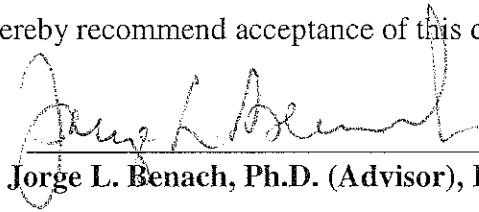
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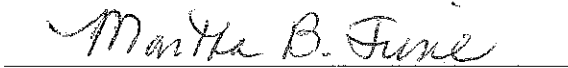We, the dissertation committee for the above candidate for the

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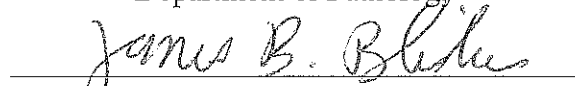
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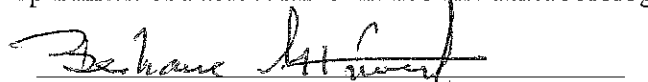
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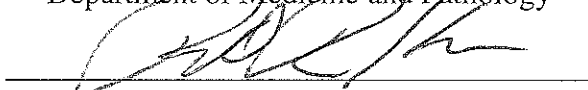
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Abstract of the Dissertation

The dual role of the Plasminogen Activation System in the dissemination of *Borrelia burgdorferi* and the inflammatory migration of mammalian host

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Lyme disease is the most common vector borne disease in the United States. It is caused by a spirochete, *Borrelia burgdorferi*. One of the aspects of Borrelia infection that has been the focus of investigation is its dissemination from its site of entry to the secondary infectious sites to become systemic.

It has previously been shown that *B. burgdorferi* interacts with the mammalian plasminogen activation system (PAS). In this dissertation, I further investigated the interaction of *B. burgdorferi* with the PAS. I showed that Borrelia induces urokinase plasminogen activator (uPA) and its physiological inhibitors, plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2). Induction of uPA resulted in increased penetration of *B. burgdorferi* across extracellular matrix (ECM) components. Using transmigration assays, I showed that the direct coincubation of *B. burgdorferi* with monocytic cells, Mono Mac 6 (MM6) cells, and peripheral blood monocytes, enhances bacterial invasion across a barrier coated with fibronectin, mediated by uPA. Moreover, PAI-2 does not have a significant effect on the uPA-potentiated transmigration of *B. burgdorferi*,

showing that bacterial invasion is not hampered by the inhibitor. Using invasion and adhesion assays of two different monocytic cell lines, MM6 and THP-1, I also showed that the interaction of *B. burgdorferi* with the PAS profoundly affects the invasive ability of monocytic cells due to the induction of PAI-2 by decreasing the adhesive and migratory capacity of the cells.

Kinetic studies of the inductions of these proteins revealed that uPA and PAI-2 inductions are temporally modulated to benefit the pathogen in a twofold way. First, the early induction of uPA ensures sufficient time for uPA to bind to the bacteria before it is permanently inhibited by PAI-2. Second, although PAI-2 is induced after uPA, it is induced early enough to inhibit monocyte invasion.

Finally, using uPAR deficient mice, it was shown that dissemination of bacteria and the inflammatory infiltration of immune cells were not different in this phenotype. Overall this dissertation showed that the PAS plays a two fold role in the pathogenesis of *B. burgdorferi* infection, both by enhancing bacterial dissemination and by diminishing host-cell inflammatory migration.

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Acknowledgments

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Chapter 1 Introduction:

Biology of *Borrelia burgdorferi*:

Borrelia burgdorferi, a tick transmitted pathogen, is the causative agent of Lyme

disease (Benach *et al.*, 1983; Burgdorfer *et al.*, 1982). Similar to all spirochetes, Borrelia

has a long protoplasmic cylinder encased by a cytoplasmic membrane, which in turn is

surrounded by a periplasm containing about 7-11 flagella anchored at each tip. The

periplasm is also surrounded by a loosely attached outer membrane (Barbour and Hayes,

1986). The bacterium is cylindrical in morphology, 0.2-0.3 μm in width and 20-30 μm in

length (Hovind-Hougen *et al.*, 1986). *B. burgdorferi* has a genome of approximately 1.5

megabases; including 950 kilobases of linear chromosome as well as 9 circular and 12

linear plasmids (Casjens *et al.*, 2000; Fraser, 1997).

Borrelia burgdorferi has minimal biosynthetic proteins and virulence factors, and

thus, it does most likely depend on its host for most of its nutritional requirements. A

characteristic feature of *Borrelia burgdorferi* is that it has a large number of predicted

and known lipoproteins. Some of these lipoproteins are differentially expressed rendering

the pathogen able to live in different environments in its enzootic cycle. A known group

of lipoproteins is the outer surface proteins (Osps A-F) (Lahdenne *et al.*, 1997; Li *et al.*,

1997; Schwan *et al.*, 1995; Lam *et al.*, 1994; Marconi *et al.*, 1994; Marconi *et al.*, 1993;

Bergstrom *et al.*, 1989). OspA and OspB are known to be expressed in the tick midgut

but subsequently downregulated during tick feeding. This is followed by the upregulation

of OspC as the organism traverses to the tick salivary gland and into the host (Schwan

and Piesman, 2000; Lahdenne *et al.*, 1997; Montgomery *et al.*, 1996; Schwan *et al.*,

1995). Similarly, other bacterial proteins are differentially expressed during early

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infections, including fibronectin binding protein (Probert and Johnson, 1998) and decorin binding proteins A and B (DbpA and DbpB) (Feng *et al.*, 1998).

Life cycle and animal host:

Lyme disease is the most common vector borne disease in the United States (Orloski *et al.*, 2000). It is transmitted by a tick vector that belongs to the *Ixodes ricinus* complex (Burgdorfer and Keirans, 1983; Steere and Malawista, 1979; Steere *et al.*, 1978; Wallis *et al.*, 1978). These ticks have larval, nymphal, and adult stages and require a blood meal at each stage. In northeastern and midwestern United States, *B. burgdorferi* goes through an efficient cycle between the larval and nymphal stages of *Ixodes scupularis* and white-footed mouse (Spielman, 1994). The life cycle of the spirochete depends on horizontal transmission: from infected nymphs to the mice in early summer, and in late summer from the infected mice to larvae, which then molt to become infected nymphs that begin the cycle again in the following year (Matuschka and Spielman, 1986). White-footed mice are tolerant to *B. burgdorferi* infection which allows the mice to stay spirochetemic throughout the summer without inflammatory response to the infection (Levine *et al.*, 1985). After a larval tick feeds on an infected mouse, the spirochete remains in the midgut of the tick until the following year, when as a nymph it attaches itself to another host. The organisms then migrate to the tick's salivary gland and are injected with its saliva as it feeds (Benach *et al.*, 1987; Ribeiro *et al.*, 1987). Because of the two drastically different environments, the bacteria have to adapt to the changing environment by differentially expressing genes in response to their environment (Tokarz *et al.*, 2004; Montgomery *et al.*, 1996).

***Borrelia burgdorferi* pathogenesis and dissemination:**

Following the inoculation of *Borrelia burgdorferi* into the host, the organisms disseminate through the skin. Days to weeks later Borrelia infect distant organs such as the heart, joint, and central nervous system (Garcia-Monco and Benach, 1995; Steere *et al.*, 1977). The pathogenesis of Lyme disease is caused by the infection of different host tissues with the spirochetes amplified by the inflammatory response of the host's immune system (Szczepanski and Benach, 1991; Garcia-Monco and Benach, 1989). As an extracellular pathogen, a critical requirement for the systemic infection of *Borrelia burgdorferi* is the ability to degrade host tissue barriers such as the extracellular matrix (ECM) and basement membranes (BM). *Borrelia burgdorferi* lacks proteases that can degrade ECM and BM proteins and therefore depends on its host for proteolytic enzymes. A known enzyme that is recruited by many pathogenic bacteria for dissemination purposes is the broad-spectrum serine protease, plasminogen (PLG) (Lahteenmaki *et al.*, 2005; Coleman *et al.*, 1999; Boyle and Lottenberg, 1997; Lottenberg *et al.*, 1994). The employment of PLG and its activators by different bacteria is discussed in detail later on in this document.

Clinical manifestations:

Generally, the course of Borrleia infection can be divided into stage 1 or early infection followed within days or weeks by stage 2 or disseminated infection, succeeded by stage 3 or persistent infection, which usually begins months to years after the onset of the disease, sometimes following long periods of latent infection.

Early infection (localized infection):

After it is injected by the tick, *B. burgdorferi* spreads locally in the skin in most patients and results in erythema migrans, which is sometimes accompanied by flu like symptoms (Steere *et al.*, 1986; Steere *et al.*, 1983b). At this stage, both arms of the immune response are weak or non existent. Mononuclear cell response is minimal (Sigal *et al.*, 1986) and specific antibody to the spirochetes is often lacking (Shrestha *et al.*, 1985).

Early infection (disseminated infection):

Within days or weeks after inoculation, the Lyme disease spirochetes may spread to different sites of the patient via the blood and lymphatic systems. Disseminated infection is accompanied by characteristic symptoms of the skin, nervous system or musculoskeletal sites (Steere *et al.*, 1983b). During this stage, there appears to be an elevated responsiveness of mononuclear cells to *B. burgdorferi* antigens (Sigal *et al.*, 1986). Specific IgM response peaks between the third and sixth week (Steere *et al.*, 1983a). Subsequent to the IgM response, specific IgG antibody develops primarily of the IgG1 and IgG3 subclasses (Hechemy *et al.*, 1988). There is an infiltration of lymphocytes within different tissues.

Late infection (persistent infection):

At this stage of infection, there are longer episodes of arthritis and an onset of chronic arthritis with characterstics of continual joint inflammation (Steere *et al.*, 1987; Lawson and Steere, 1985; Steere *et al.*, 1979b). There is a heavy infiltration of mononuclear cells in the inflamed joint. In severe cases, chronic Lyme arthritis may lead to the erosion of the cartilage and bone (Steere *et al.*, 1987; Herzer *et al.*, 1986; Lawson and Steere, 1985;

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Steere *et al.*, 1979a) and occasionally to permanent joint disability (Steere *et al.*, 1987).

Some patients may also develop symptoms of acute neuroborreliosis during or shortly

after the disseminated infection (Garcia-Monco and Benach, 1995; Steere, 1989).

The plasminogen activation system and its components:

The plasminogen activation system is a complex system that is involved in many

physiological and pathological conditions. It comprises of serine proteases, membrane

receptors and serine protease inhibitors (Figure 1.1).

Plasminogen:

Plasminogen (PLG) is a circulating zymogen that can be activated into plasmin by

proteolytic cleavage. PLG is secreted in the liver as a glycoprotein and is abundantly

present in most extracellular fluids, with a concentration of about 2 μM in the plasma

(Lijnen and Collen, 1982). Plasminogen has 709 amino acid residues with a molecular

weight of 92 kDa (Wiman, 1977; Wiman, 1973). It is found in two isoforms: glu-

plasminogen named as such for the glutamic acid residue found at the N-terminus of the

molecule and lys-plasminogen, that is formed by a proteolytic cleavage of the glu-

plaminogen at positions Arg^{67} - Met^{68}, Lys^{76} – Lys^{77} and Lys^{77} – Val^{78} by plasmin

(Collen and de Maeyer, 1975; Wallen and Wiman, 1972). Conversion of glu-plasminogen

to lys-plasminogen enhances the rate of PLG activation to plasmin by plasminogen

activators.

Plasminogen is activated by the cleavage of the molecule at position Arg^{560} – Val^{561}

(Robbins *et al.*, 1967). This yields a heterodimer with A and B chains joined through two

disulfide bridges (Ranson and Andronicos, 2003). The N-terminal, A chain, contains five

triple looped structures known as kringles that are responsible for the binding of PLG to

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lysine and lysine analogs such as ε-amino caproic acid (Markus *et al.*, 1978). It also can

bind lysine rich components at the surfaces of cells and extracellular matrix (ECM). The

C-terminal B chain contains the enzymatic domain that contains the catalytic triad, His^{602},

Asp^{645} and Ser^{740}, of the active site.

Plasmin is a broad-spectrum protease with a wide range of substrates. Formerly

believed to be mainly involved in the degradation of fibrin, plasmin can degrade multiple

protein components of the ECM such as fibronectin, vitronectin, and laminin and also

activates pro-enzyme forms of the matrix metalloproteinases (MMPs) to the active form

(Ranson and Andronicos, 2003; Andreasen *et al.*, 1997). Due to its wide substrate

specificity, plasmin is subject to a tight regulation in the plasma by a specific serine

protease inhibitor known as α-2 antiplasmin (Travis and Salvesen, 1983). During

fibrinolysis and inflammation, plasminogen is bound to fibrin or lysine-rich cell surfaces

which allow the generation of plasmin in a focused way. This binding also allows

plasmin to be resistant to α-2 antiplasmin inhibition (Plow *et al.*, 1986), and this is

believed to be through the competitive binding on the kringle domains.

Plasminogen activators:

Urokinase plasminogen activator:

Urokinase plasminogen activator (uPA) is a glycoprotein with 411 amino acids and an

approximate molecular weight of 50-55 kDa (Gunzler *et al.*, 1982a; Gunzler *et al.*,

1982b). It is synthesized by several cell types including monocytes, hepatocytes and

lymphocytes (Carpen *et al.*, 1986) and circulates in the plasma at a low concentration of

about 2 pM (Andreasen *et al.*, 1997). The protein structure of uPA consists of three

distinct domains: (1) a growth-factor like domain, which bears a resemblance to the

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receptor-binding regions in epidermal growth factor and transforming growth factor α (Derynck *et al.*, 1984; Gunzler *et al.*, 1982b), and is responsible for the binding of uPA receptor (uPAR); (2) one kringle structure whose function is not known yet; and (3) the catalytic domain in the carboxy-terminus, similar to other serine proteinases (Strassburger *et al.*, 1983; Gunzler *et al.*, 1982b), and contains the active site of the enzyme with the catalytic triad His^{204}, Asp^{255} and Ser^{356} (Figure 1.2).

uPA is synthesized and secreted as a single chain molecule (Scu-PA) with a catalytic activity several hundred fold lower than its active form (Andreasen *et al.*, 1997). Cleavage of the Scu-PA at Lys^{158}-Ile^{159} peptide bond gives a catalytically active two chain uPA (tcu-PA), with its two subunits, A and B, joined via a disulfide bond (Andreasen *et al.*, 1997). The A chain contains the growth factor domain that has a short stretch of amino acids, known as the amino terminal fragment (ATF), responsible for binding to uPAR (Appella and Blasi, 1987; Appella *et al.*, 1987). The B chain consists of the C-terminus with the catalytic domain (Verde *et al.*, 1984; Strassburger *et al.*, 1983). Although the exact enzyme that cleaves and activates uPA *in vivo* is not yet identified, several proteases, including plasmin, have been shown to activate uPA *in vitro* (Andreasen *et al.*, 1997; Dano *et al.*, 1985). An alternate hydrolysis of the Lys^{135}-Lys^{136} peptide bond with plasmin, following previous cleavage of the Lys^{158}-Ile^{159} bond, can result in a low molecular weight (33 kDa) form of tcu-PA that is enzymatically active. Low molecular weight uPA lacks most of the A chain and therefore cannot bind its membrane receptor (Steffens *et al.*, 1982).

uPA has a restricted range of enzymatic specificity. Although PLG is the main substrate for uPA (Mignatti and Rifkin, 1993; Saksela and Rifkin, 1988; Dano *et al.*,

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1985), other substrates, such as fibronectin and uPAR, are also cleaved by uPA (Hoyer-Hansen *et al.*, 1992; Gold *et al.*, 1989; Quigley *et al.*, 1987). The activity of uPA drastically increases upon its binding to its receptor, uPAR. Moreover, binding of uPA to uPAR focuses proteolysis to the surface of the cell.

Tissue type plasminogen activator (tPA):

tPA is mainly synthesized and secreted by endothelial cells as a single chain zymogen of molecular size of about 70 kDa (Rijken, 1995; Fisher *et al.*, 1985; Pennica *et al.*, 1983). tPA is found in the plasma at a concentration of 5-10 ng/ml (Lijnen and Collen, 1995; Rijken, 1995; van Hinsbergh *et al.*, 1991). Activation of tPA is brought about by a proteolytic cleavage of the single chain molecule into two chain tPA that contains the A and B chains held together via a disulphide bridge. The N-terminal A chain domain is followed by the epidermal growth factor like domain (EGF), two kringle domains and the C-terminal domain which contains the active site of the enzyme consisting of the catalytic triad, His^{322}, Asp^{371} and Ser^{478}. The presence of one more kringle domain in tPA compared to uPA is critical for the binding of tPA to fibrin, making tPA the main protease in fibrinolysis. Binding of tPA to fibrin drastically increases its enzymatic activity leading to the activation of PLG and the subsequent degradation of fibrin.

Urokinase plasminogen activator receptor (uPAR):

The urokinase plasminogen activator receptor (uPAR) is a glycosylphosphatidylinositol (GPI) anchored membrane protein that binds uPA on the surface of cells. uPAR, also called CD87, is related to the members of the Ly6 superfamily (Ploug and Ellis, 1994). The mature uPAR protein consists of three domains of about 90 residues each designated as D1, D2 and D3 (Behrendt *et al.*, 1991). The first

amino terminal domain, D1, of the molecule is involved in the binding to uPA. Despite

evidence showing the involvement of D1 in uPA binding, isolated D1 molecule does not

bind uPA with the same affinity as intact uPAR (Behrendt et al., 1996), indicating that

the other domains might be important for optimum binding. The interaction of uPA with

uPAR exhibits some species specificity in that human uPA does not bind to murine

uPAR, and vice versa (Solberg et al., 1992; Estreicher et al., 1989). However, human

uPA does interact with bovine (Reuning and Bang, 1992) and hamster (Fowler et al.,

1998) cells with high affinity.

uPAR can bind pro-uPA and uPA with about the same affinity without affecting the

enzymatic activity of uPA (Behrendt et al., 1995; Nykjaer et al., 1994). uPA binds uPAR

in an autocrine (Stoppelli et al., 1986) or paracrine fashion leading to the saturation of the

receptor and the regulation of uPA activity. uPAR plays a regulatory role in the activity

of uPA in two ways: (1) uPAR positively regulates the function of uPA by enhancing its

activity (Stephens et al., 1989) (2) Binding of uPA inhibitors, plasminogen activator

inhibitors 1 and 2, to uPAR bound uPA leads to the internalization and subsequent

degradation of uPA and the inhibitors (Cubellis et al., 1990; Estreicher et al., 1990;

Jensen et al., 1990). Since uPAR is a GPI anchored molecule, internalization of the

uPA/PAI complex by uPAR and recycling of uPAR is aided by transmembrane proteins,

α 2-macroglobulin receptor/low density lipoprotein-related protein (α 2MR/LRP)

(Nykjaer et al., 1997).

uPAR also exists as a soluble form in the plasma in normal and pathological

conditions (Garcia-Monco et al., 2002; Mizukami et al., 1995; Ronne et al., 1995). It is

formed by an alternative splicing of the mRNA. It can be cleaved between D1 and D2

domains resulting in a chemoattractant peptide with the D2 and D3 domains.

The uPA/uPAR and cell invasion:

The uPA/uPAR complex is involved in cell invasion and migration in physiological

and pathological conditions. The migration of cells involves the degradation of biological

barriers such as the protein components of the ECM and the basement membranes of

endothelial cells by plasmin. In addition to proteolysis, uPA/uPAR complex facilitates

cellular invasion by mediating cell adhesion and subsequent migration. Furthermore, uPA

and uPAR serve as chemoattractants enhancing the directional migration of cells.

uPA/uPAR system and pericellular proteolysis:

The binding of uPA to uPAR facilitates cell migration via surface proteolysis in two

ways. First, binding to uPAR focuses proteolysis to the leading edge of the migrating

cells where uPAR is concentrated (Estreicher *et al.*, 1990). This is also beneficial because

it limits plasmin-mediated collateral tissue damage. Secondly, the binding of uPA to

uPAR markedly increases the enzymatic activity of uPA by several orders of magnitude.

This guarantees a quick and effective proteolysis of the ECM proteins.

The uPA/uPAR system and cell adhesion:

uPAR is a high-affinity receptor for vitronectin suggesting that uPAR plays a role in

cell adhesion (Waltz and Chapman, 1994; Wei *et al.*, 1994). uPAR binds to both the

cleaved form of vitronectin present in plasma and the full-length form (Hoyer-Hansen *et

al.*, 1997). The vitronectin-binding site on uPAR is distinct from the uPA binding site.

Competition studies with synthetic peptides suggest that the region between residues 364

and 380 close to the carboxy terminus of vitronectin may be involved in uPAR binding

(Waltz et al., 1997). Vitronectin-uPAR binding is enhanced by the simultaneous binding of uPA, but is inhibited by active, but not latent or cleaved, plasminogen activator inhibitors (Deng et al., 1996; Kanse et al., 1996). The simultaneous recognition of vitronectin by uPAR and the $\alpha_5\beta_3$ integrin co-localizes these two receptors to adhesion structures and directs the proteolytic activity of the plasminogen system to the matrix (Wei et al., 1996; Yebra et al., 1996). On the other hand, when PAI-1 binds to vitronectin, it interferes with vitronectin recognition by integrin $\alpha_5\beta_3$, thereby stimulating release of cells from the matrix and supporting cell migration (Seiffert and Loskutoff, 1996; Stefansson and Lawrence, 1996). Competition studies with purified uPAR domains suggest that more than one domain is involved in the vitronectin binding reaction and perhaps that the intact uPAR is required for efficient binding (Hoyer-Hansen et al., 1997). Vitronectin has been shown to concentrate the uPA/uPAR-complex to cell surfaces and ECM sites (Chavakis et al., 1998), which leads to the accumulation of plasminogen activator activity required for cell migration and tissue remodeling.

The β_2 subfamily of integrins plays a critical role in cell adhesion and migration. Association of uPAR with the β_2 integrin CD11b/CD18 (also known as Mac-1 and complement receptor-3 [CR3]) has been found in studies of co-capping and co-immunoprecipitation with neutrophils and monocytes (Bohuslav et al., 1995; Xue et al., 1994). Incubating monocytes with antibodies to CR3 impaired uPAR-dependent adhesion to vitronectin, and engagement of uPAR by uPA or vitronectin inhibited CR3 dependent fibrinogen degradation (Simon et al., 1996). Down-regulation of uPAR with antisense oligonucleotides in monocytes reduced the adhesiveness of CR3 (Sitrin et al., 1996). The interaction of β_2 integrins with the T cell antigen-receptor complex has been shown to

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increase uPAR mRNA and protein and to promote migration of T cells through an ECM *in vitro*. In an *in vivo* study, the β_2 integrin-dependent recruitment of leukocytes to the inflamed peritoneum of uPAR deficient mice was significantly reduced relative to that in wild-type animals (May *et al.*, 1998). This finding indicates that β_2 integrin-mediated leukocyte-endothelial cell interactions and recruitment to inflamed areas both require the presence of uPAR. The association between uPAR and β_2 integrins clearly initiates a variety of direct and indirect regulatory mechanisms for both adhesive and proteolytic processes (Preissner *et al.*, 1997). In some cells that do not express β_2 integrin, uPAR interacts with β_1 and β_3 integrins in a similar way (Xue *et al.*, 1997; Gladson *et al.*, 1995).

In addition to pericellular proteolysis and cell adhesion, uPA and soluble uPAR serve as chemoattractants (Resnati *et al.*, 1996). This results in a directional migration of inflammatory cells. The invasive ability conferred by the uPA/uPAR system is used in normal physiological conditions such as wound healing, angiogenesis, and tissue remodeling as well as in pathological situations such as cancer metastasis and inflammation.

The uPA/uPAR system and cell growth:

Besides cell adhesion and proteolysis, the uPA/uPAR system has been shown to play a role in cell proliferation (Koopman *et al.*, 1998; Kirchheimer *et al.*, 1989). Occupancy of uPAR with uPA leads to increased proliferation of cells which could be antagonized with the presence of PAI-2 (Yu *et al.*, 2002; Hibino *et al.*, 1999). Also, the addition of specific antibodies to receptor-bound uPA reduces proliferation showing the autocrine or paracrine binding of uPA to membrane uPAR plays a role.

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Because of the broad enzyme specificity of plasmin and the multiple physiological and pathological functions of the PAS, the system is under a tight regulation. Plasmin is regulated by its physiological inhibitor, α-2 antiplasmin. It is also regulated at the level of the activators uPA and tPA through proteinase inhibitors that belong to the serpin superfamily, namely plasminogen activator inhibitor 1 and 2 (PAI-1 and PAI-2).

Serine protease inhibitors

Serine proteinase inhibitors (serpins), also called suicide inhibitors for their mechanism of inhibition, block their target proteases by forming a covalent bond. The inhibitor acts as a pseudo-substrate where the enzyme attacks and gets trapped. Inhibitory and non-inhibitory serpins constitute the majority of plasma proteinase inhibitors (Travis *et al.*, 1990). They are single chain proteins with extensive secondary structures that include α-helices and β-sheets (Silverman *et al.*, 2001; Whisstock *et al.*, 1998; Stein and Carrell, 1995; Potempa *et al.*, 1994; Gettins *et al.*, 1992; Travis *et al.*, 1990). The most important structural feature of the serpins is the presence of a reactive center loop (RCL) which is exposed to the surface of the protein and plays an important role in the inhibitory mechanism.

The primary sequence of the RCL domain is the most variable among serpins and it determines the specificity of the inhibitor (Travis *et al.*, 1990). Serpins have a single reactive site in their RCL and the amino acids in this group are designated as ....P_3-P_2-P_1-P_1-P_2-P_3....COOH, where the reactive site is the bond between P_1 and P_1 (Gettins *et al.*, 1992). This bond provides a reactive pseudo-substrate for the target proteinase forming a covalent bond between the active site of the proteanse and the P_1-P_1 group of the inhibitor trapping the enzyme in an inactive form (Andreasen *et al.*, 1997). Following the covalent

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bond formation, the RCL inserts itself into the β-sheet of the serpin rendering it more stable (Silverman *et al.*, 2001). It is the stability of the complex that leads to the non-reversibility of the reaction.

There are several members of the serpin superfamily. It includes PAI-1, PAI-2 and α-2 antiplasmin which are members of the PAS and therefore are considered in this dissertation.

Plasminogen activator inhibitor 1 (PAI-1):

PAI-1 was first described in conditioned media of human endothelial cells (Loskutoff and Edgington, 1977) and later detected in a wide variety of cell types (Kruithof, 1988). PAI-1 level in plasma is variable but it is approximately 20 ng/ml (Kruithof *et al.*, 1987). It is a single chain glycoprotein with 379 amino acids of approximately 52 kDa (Andreasen *et al.*, 1986a; Erickson *et al.*, 1984) with the active site located at Arg[346]-Met[347] (Sanzo *et al.*, 1987; Andreasen *et al.*, 1986b; Ny *et al.*, 1986). PAI-1 is highly sensitive to oxidation (Baker *et al.*, 1990), relatively unstable and rapidly converts into a latent conformation at 37°C (Reilly *et al.*, 1990; Levin and Santell, 1987; Mimuro *et al.*, 1987). This latent form can be reactivated by denaturation. Interaction of active PAI-1 with the matrix prote*in vitro*nectin stabilizes PAI-1 in its active form and prevents the formation of the latent PAI-1. PAI-1 inhibits tPA and uPA with similar affinity. PAI-1 also binds soluble and membrane bound uPA.

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Plasminogen activator inhibitor 2 (PAI-2):

PAI-2 is found in the placenta (Kawano *et al.*, 1970) and is also synthesized by mononuclear cells in response to certain inflammatory agents such as phorbol esters (Kruithof *et al.*, 1995). It resides intracellularly as a low molecular weight form of about 47 kDa and in the pericellular space as a secreted, high molecular weight glycosylated form of about 60 kDa (Kruithof *et al.*, 1986). PAI-2 is a serpin with a higher affinity for uPA than for tPA as fibrin bound tPA is resistant to PAI-2 inhibition (Leung *et al.*, 1987). Its mode of inhibition is that of a typical serpin. It forms a stable and irreversible complex in a one to one ratio with uPA.

PAI-2 gene structure and biochemistry:

PAI-2 is synthesized by a variety of cell types including monocytic cells (Ritchie and Booth, 1998; Haj *et al.*, 1995; Schwartz and Bradshaw, 1992; Wohlwend *et al.*, 1987a) and fibroblasts (Pytel *et al.*, 1990). It is 415 amino acids long in both the glycosylated and unglycosylated forms and is encoded by a single mRNA of approximately 2.0 kb in length (Kruithof *et al.*, 1995). The transcription initiation site for PAI-2 is found between 22 to 25 base pairs (bp) downstream of the consensus TATAAAA sequence (Samia *et al.*, 1990; Ye *et al.*, 1989; Kruithof and Cousin, 1988).

The polypeptide chain of PAI-2 has three potential N-glycosylation sites (Asn[75], Asn[115] and Asn[339]) which appear to be fully occupied in the glycosylated form (Ye *et al.*, 1989). There are two common variants of PAI-2 which differ at three amino acid positions: Asn[120], Asn[404] and Ser[413] for type A compared to Asp[120], Lys[404] and Cys[413] for type B (Ye *et al.*, 1989; Ye *et al.*, 1987). PAI-2 is mainly found intracellularly in almost all cell types studied (Belin *et al.*, 1989; Medcalf *et al.*, 1988b; Medcalf *et al.*, 1988a;

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Genton *et al.*, 1987; Wohlwend *et al.*, 1987a). Cellular localization of PAI-2 may be modulated, as extracellular levels of PAI-2 was reported to be very low under normal conditions but may increase significantly under certain pathological conditions (Quax *et al.*, 1990; Ye *et al.*, 1988). The differential translocation of the two forms of PAI-2 could be explained by the presence of two internal hydrophobic signal regions. Increasing the hydrophobicity of these regions enhances the extracellular translocation of PAI-2 (Belin, 1993; von Heijne *et al.*, 1991).

Biological functions of PAI-2:

Although the physiological roles of PAI-2 have not been clearly elucidated, PAI-2 appears to play a role in certain physiological and pathological conditions such as pregnancy, cancer and inflammation. The plasma levels of PAI-2 are not detectable under normal conditions. However, in pregnancy, PAI-2 levels spike up to 250 μg/ml, suggesting that PAI-2 plays a role in this condition (Halligan *et al.*, 1994; Stegnar *et al.*, 1993; Koh *et al.*, 1992; Wright *et al.*, 1988; Kruithof *et al.*, 1987; Lecander and Astedt, 1987). PAI-2 levels are modulated in certain cancers in which the elevation of PAI-2 correlates with low metastasis and good prognosis (Rosenberg, 2003). Induction of PAI-2 levels with pro-inflammatory agents such as phorbol esters and down-regulation by anti-inflammatory agents suggest a role for PAI-2 in inflammation (Dear *et al.*, 1997; Kruithof *et al.*, 1995).

Since PAI-2 is an effective inhibitor of uPA, it serves to enforce a regulatory mechanism on uPA/uPAR mediated biological processes. PAI-2 can bind both soluble and receptor bound uPA. Binding of PAI-2 to receptor-bound or soluble uPA culminates

in uPAR mediated internalization and degradation of the complex (Al-Ejeh *et al.*, 2004). This effectively removes uPA and blocks uPA mediated activation of plasminogen.

PAI-2 also inhibits uPA/uPAR mediated adhesion and cell migration in two ways: first, it facilitates clearance of uPA and hence its occupancy of surface uPAR and second, by directly binding to vitronectin (Radtke *et al.*, 1990), it poses a physical barrier for the binding of uPAR to vitronectin.

As recent evidence shows, PAI-2 is also involved in the cell proliferation and differentiation processes (Yu *et al.*, 2002). Although the exact mechanisms and effect of PAI-2 are not completely worked out yet, and there is some conflicting evidence as to its role, it is clear that PAI-2 affects cell growth.

The interaction of the PAS and microorganisms:

An important requirement for the dissemination of pathogens within the host is the ability to cross tissue barriers, particularly ECM and basement membranes as well as the fibrin deposit that is used to confine the dissemination of pathogens. Some pathogens recruit plasmin for the degradation of these barriers (Sun, 2006; Lahteenmaki *et al.*, 2005; Coleman and Benach, 1999; Boyle and Lottenberg, 1997; Lottenberg *et al.*, 1994). Some bacteria can bind PLG on their cell surfaces and subsequently activate it to plasmin using endogenous plasminogen activator (Sun *et al.*, 2004; Christner and Boyle, 1996; Berge and Sjobring, 1993; Lottenberg *et al.*, 1992; Sodeinde *et al.*, 1992; Kuusela and Saksela, 1990; Reddy and Markus, 1972). Others such as *B. burgdorferi*, however, depend on host-derived plasminogen activators to activate cell surface-bound PLG (Coleman *et al.*, 1995; Klempner *et al.*, 1995; Fuchs *et al.*, 1994).

Bacteria that have endogenous PLG activators:

Functionally, bacterial PAS fall into two groups: 1) non-proteolytic PLG activators, such as streptokinase (SK) and staphylokinase (SAK); and 2) proteolytic PLG activators such as Pla. Streptokinase and staphylokinase are extracellular proteins secreted by several members of groups A and C streptococci and staphylococci bacteria, respectively. SK has a molecular weight of 47 kDa (Radek and Castellino, 1989), while SAK is a smaller molecule with molecular weight of 18 kDa (Rabijns et al., 1997). Although these proteins do not share sequence homology, they have a similar function in that they form equimolar active complexes with PLG (Parry et al., 1998; Wang et al., 1998; Rabijns et al., 1997). This binding results in the exposure of the active site of plasmin without a proteolytic cleavage of PLG (Parry et al., 2000). It was shown that binding of PLG to lysine residues on SK enhances formation of the SK/PLG catalytic complex (Panizzi et al., 2006). Soluble SAK-plasmin complex is inhibited by α-2 antiplasmin while the cell bound one is not. In contrast, free or cell-bound SK-PLG complex is resistant to inhibition by α-2 antiplasmin (Sakai et al., 1989; Cederholm-Williams et al., 1979).

In contrast to the SK and SAK, surface protease Pla activates PLG by proteolytically cleaving it at the same site as tPA and uPA. Pla is an outer membrane protein encoded by a virulence plasmid in *Yersinia pestis* (Sodeinde et al., 1992). Pla derives its name from the fact that it can activate the mammalian plasma proenzyme plasminogen into plasmin (Beesley et al., 1967). Pla is required for the migration of *Y. pestis* from the subcutaneous infection site into the circulation (Sodeinde et al., 1992). Bacterium-induced formation of plasmin potentiates degradation of fibrin (Beesley et al., 1967) and extracellular matrices (Lahteenmaki et al., 1998) and promotes plague infection *in vivo*

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(Goguen *et al.*, 2000). Pla also cleaves the complement C3 component (Sodeinde *et al.*, 1992), modifies bacterium-produced Yops (Yersinia outer proteins) (Sodeinde *et al.*, 1988; Sample *et al.*, 1987), and proteolytically inactivates the physiological inhibitor of plasmin, α_2-antiplasmin (Kukkonen *et al.*, 2001).

Bacteria without endogenous PLG activators:

Unlike the above mentioned bacteria, certain pathogenic bacteria do not produce endogenous PLG activators. Rather, they depend on their host to activate surface-bound PLG. Many bacteria besides Borrelia are shown to bind to PLG and become capable of degrading protein components of the ECM. These bacteria include *Salmonella typhimurium, Haemophilus influenzae* and *Helicobacter pylori.* Plasmin enables *Salmonella typhimurium* to degrade laminin and a basement membrane-like preparation (Matrigel), (Korhonen *et al.*, 1997; Lahteenmaki *et al.*, 1995). Moreover, binding to plasmin increases the capacity of *H. influenzae* to degrade soluble components of the ECM, and enhances penetration through basement membranes (Virkola *et al.*, 1996). Another example is *H. pylori,* which colonizes the stomach mucosa leading to stomach and duodenal ulcers, gastritis, and other gastrointestinal disturbances. *H. pylori* relies on the degradation of the epithelial cells and sub-epithelial ECM as a main pathophysiologic process. Both the spiral and coccoid forms of this organism can bind PLG in a specific association which might be a requirement for the destructive process that leads to ulcer formation (Khin *et al.*, 1996; Ringner *et al.*, 1994).

B. burgdorferi is transmitted by Ixodid ticks and subsequently disseminates from the skin to affect secondary sites, including the heart, nervous system, and joints (Garcia-Monco and Benach, 1995; Steere, 1989). The dissemination of the organism from the site

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of entry requires penetration of the ECM and basement membrane of capillary endothelium both in entering and in exiting the blood vasculature in order to colonize distant sites. Moreover, migration would likely require the dissolution of fibrin deposits at the site of entry and inflammation. Penetration of the ECM is likely to involve the adhesion to and localized proteolytic breakdown of insoluble ECM components. The adhesion of *B. burgdorferi* to the ECM is evidenced by the strong affinity of *B. burgdorferi* for components of the ECM (Szczepanski *et al.*, 1990; Garcia-Monco *et al.*, 1989).

The presence of only few genes (Fraser, 1997) that could encode for recognizable virulence factors identified by homology, such as hemolysins, decorin-binding proteins, zinc proteases, a fibronectin/fibrinogen-binding protein, and drug efflux proteins, coupled with the inability of *B. burgdorferi* to exhibit a proteolytic activity against ECM-proteins on its own (Coleman *et al.*, 1995; Klempner *et al.*, 1995), provides added significance for the involvement of the PAS in *B. burgdorferi* invasion.

Accumulating evidence further supports the hypothesis that the PAS plays a role in the dissemination of *B. burgdorferi* in that the spirochetes can bind PLG as demonstrated by immunofluorescence (Coleman *et al.*, 1995; Klempner *et al.*, 1995; Fuchs *et al.*, 1994), radioimmunoassay (Coleman *et al.*, 1995; Hu *et al.*, 1995; Fuchs *et al.*, 1994), and immunoblotting (Coleman *et al.*, 1995; Fuchs *et al.*, 1994). Bound PLG could subsequently be converted to active plasmin by the simultaneous incubation with exogenous uPA or tPA (Coleman *et al.*, 1995; Hu *et al.*, 1995; Klempner *et al.*, 1995; Fuchs *et al.*, 1994) as demonstrated by cleavage of specific chromogenic plasmin substrates (Coleman *et al.*, 1995; Hu *et al.*, 1995; Klempner *et al.*, 1995; Fuchs *et al.*,

1994) and zymography (Hu *et al.*, 1995; Klempner *et al.*, 1995). Purified forms of PLG, Glu-PLG (Hu *et al.*, 1995) or Lys-PLG (Coleman *et al.*, 1995; Hu *et al.*, 1995), as well as the PLG in the plasma (Klempner *et al.*, 1995; Fuchs *et al.*, 1994), could bind to *B. burgdorferi*. Using affinity-blot analysis with radiolabeled PLG and *B. burgdorferi* whole-cell lysates, numerous polypeptides on *B. burgdorferi* that bind PLG (Coleman *et al.*, 1995) were identified. These included outer surface protein A (Fuchs *et al.*, 1994), a 31-kDa antigen that is abundantly expressed in cultured *B. burgdorferi*, and a higher affinity, 70-kDa protein identified in membrane detergent extracts (Hu *et al.*, 1995). The relevance of outer surface protein A as a spirochete PLG receptor is uncertain because it is downregulated in the mammalian host (Coleman *et al.*, 1997; Schwan *et al.*, 1995). Moreover, *B. burgdorferi* lacking the 54-kb linear plasmid that codes for outer surface protein A bind to PLG (Hu *et al.*, 1995) as well as do the wild-type bacteria.

The binding of PLG to *B. burgdorferi* involves the lysine-binding sites, kringle domains, of the PLG molecule and lysine rich surface proteins on Borrelia, as evidenced by data from inhibition experiments carried out with the lysine analogues tranexamic acid (Fuchs *et al.*, 1994) and ε-aminocaproic acid (Coleman *et al.*, 1995; Hu *et al.*, 1995). An important feature of plasmin bound on the surface of eukaryotic cells (Ellis *et al.*, 1991; Plow *et al.*, 1986) and bacteria (Lottenberg *et al.*, 1987) is the preservation of its enzymatic activity in the presence of the natural inhibitor of plasmin, α_2-antiplasmin. Similarly, plasmin bound on the surface of *B. burgdorferi* retains most of its enzymatic activity in the presence of physiologic levels of α_2-antiplasmin (Perides *et al.*, 1996; Coleman *et al.*, 1995; Fuchs *et al.*, 1994). In addition, the physiologic regulators, PAI-1 and PAI-2, are inefficient inhibitors of uPA-derived plasmin activity on the surface of the

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spirochete (Perides *et al.*, 1996). These results suggest that the fibrinolytic regulator system would not present a significant hindrance to the use of the host PAS by the spirochetes to enhance dissemination after transmission. In tick-feeding experiments with PLG-deficient mice, it has been found that the acquisition of PLG by *B. burgdorferi* occurs in the feeding tick and significantly enhances the systemic distribution of the spirochete to the tick hemolymph and salivary glands (Coleman *et al.*, 1997). The timing of events with a delay between the beginning of tick feeding and disease transmission ensures that the organism encounters the host in an environment characterized by tissue injury and inflammation. Surface acquisition of exogenous plasmin endows spirochetes with invasive qualities in ticks (Coleman *et al.*, 1997), the ability to degrade ECM components (Coleman *et al.*, 1999; Fuchs *et al.*, 1994) and enhanced capacity to penetrate through endothelial cell monolayers *in vitro* (Coleman *et al.*, 1999; Fuchs *et al.*, 1994). In addition, PLG enhances the penetration of *B. burgdorferi* across the blood-brain barrier (Grab *et al.*, 2005), facilitates the invasion of the kidney and brain by *Borrelia crocidurae* (Nordstrand *et al.*, 2001), and enhances invasion of brain and heart in murine relapsing borreliosis (Gebbia *et al.*, 1999).

Furthermore, PAs were present in the blood-meal contents of ticks allowed to feed on laboratory animals (Coleman *et al.*, 1997). Moreover, *in vitro* works showed that Pro-uPA (scuPA) (Fuchs *et al.*, 1996), as well as soluble and membrane-bound uPAR (Coleman *et al.*, 2001), are induced in peripheral blood monocytes and cultured monocytic cells after exposure to *B. burgdorferi*. uPAR was also shown to be present in the serum and cerebrospinal fluid of patients with bacterial and viral infection of the central nervous system (Garcia-Monco *et al.*, 2002).

Inaddition, binding of *B. burgdorferi* to plasmin initiates another pathway that results in the breakdown of the ECM. Borrelia-bound plasmin cleaves pro-matrix-metalloproteinases (MMPs) to active MMPs, further enhancing penetration. Moreover, *B. burgdorferi* induces the production of MMPs in mammalian cells (Gebbia *et al.*, 2001; Hu *et al.*, 2001; Kirchner *et al.*, 2000; Perides *et al.*, 1999) that in turn can degrade certain components of the ECM, particularly collagen IV.

Because of its relevance to understanding different mammalian physiological and pathological processes, the PAS is one of the most extensively studied systems. The goals of this dissertation were to further study the effects of *B. burgdorferi* on the expression levels and kinetics of the components of the PAS. Monocytes were tested *in vitro* as potential sources of uPA in ways that mimic inflammatory conditions. The effects of PAI-2 in the monocyte-potentiated penetration of *B. burgdorferi* were also addressed.

Since host immune cells use the PAS for migration to sites of inflammation, it was a part of the dissertation goals to investigate the effects of the kinetic modulation of the components of the PAS by *B. burgdorferi* on the inflammatory migration of monocytes. This dissertation examines how the system is utilized by the spirochetes to enhance their invasion while suppressing the migration of host inflammatory cells.

Finally, the dissertation addresses the *in vivo* roles of uPAR in the dissemination of *B. burgdorferi* and host inflammatory cells using uPAR deficient mice.

The specific aims of the dissertation were:

1. To examine the effects of *B. burgdorferi* on the production of urokinase plasminogen activator and its inhibitors, and assess their effects on the ability of Borrelia to penetrate the ECM.

2. To examine the overall effects of the induction of uPA and PAI-2 by *B. burgdorferi* on the invasive and adhesive properties of monocytic cells.

3. To investigate the *in vivo* roles of uPAR on the dissemination of *B. burgdorferi* and the infiltration of inflammatory cells.

Invasion

ECM degradation

pro-MMPs → MMPs

PLG → Plasmin

pro-uPA

pro-uPA-uPAR

uPAR

uPA-PAI-2 degradation

uPA-uPAR

Intergrin β_1 (Fibronectin)

Intergrin β_2 (Fibrinogen)

Intergrin β_3 (Vitronectin)

Proliferation

Adhesion

Migration

Recycling Endocytosis

PAI-2

uPA-uPAR-PAI-2

Vitronectin

$a_v\beta_5$
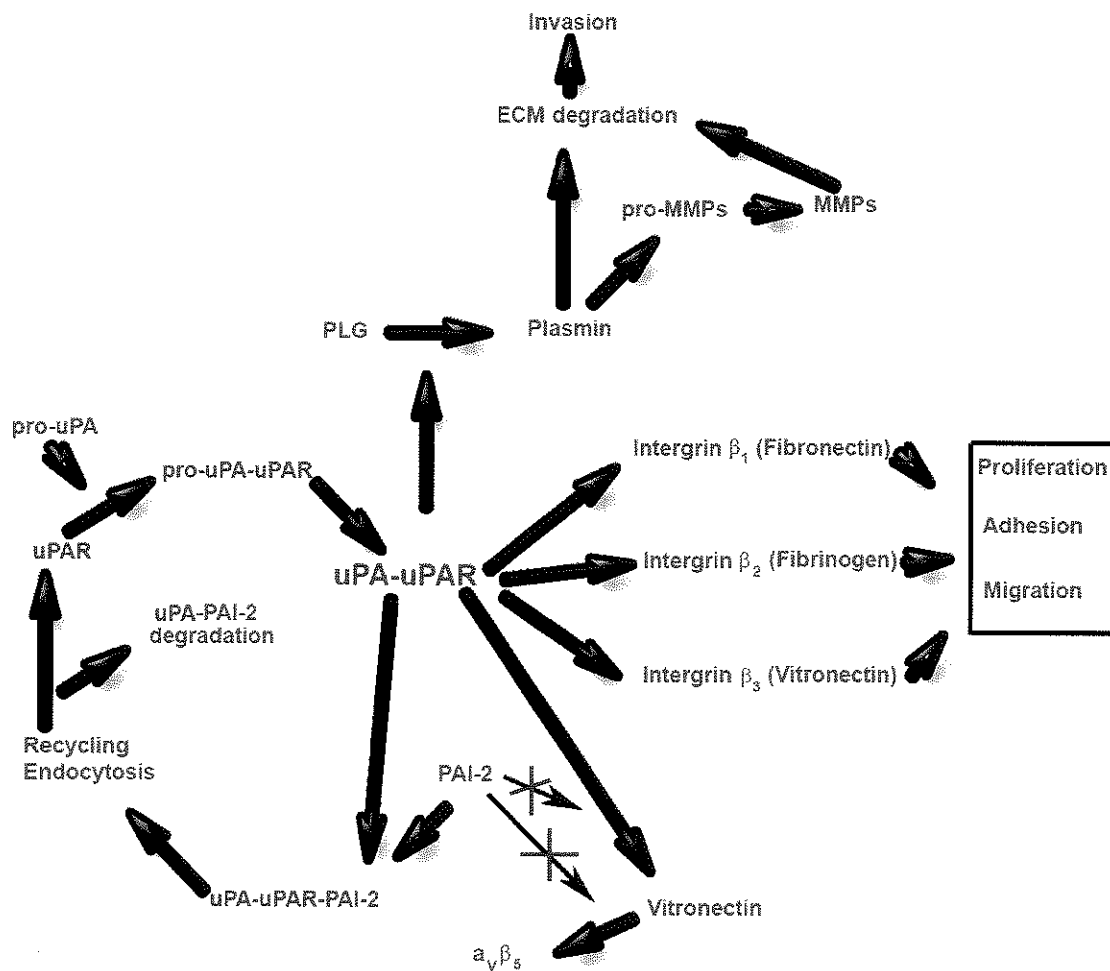
Figure 1.1: Schematic representation of uPAR mediated cellular events on the cell surface. Binding of pro-uPA to uPAR provides the cell surface with a potential plasmin dependent proteolytic activity determining matrix degradation. uPA bound uPAR interaction with PAIs influences endocytosis whereas uPAR interaction with vitronectin and subclasses of integrins results in modulation of cell adhesion and migration. (*Mohanam et al.*)

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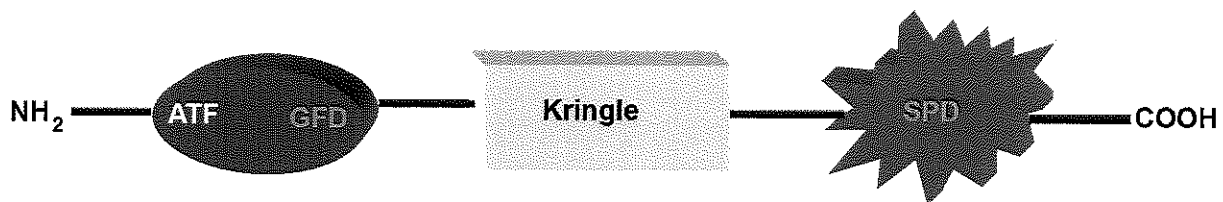
Figure 1.2: A cartoon representation of the molecular structure of pro-uPA: The domains from the N-terminus include: ATF, amino terminal fragment, (uPAR-binding structure with 16 amino acids, residues 14-30, within the GFD); GFD, growth factor domain; the kringle domain; SPD, serine protease domain

Chapter 2: The role of the PAS in the dissemination of *B. burgdorferi.*

Specific Aim I: To examine the effects of *B. burgdorferi* on the production of urokinase plasminogen activator and its inhibitors, and assess their effects on the ability of Borrelia to penetrate the ECM.

Rationale:

B. burgdorferi binds PLG and in the presence of exogenously added uPA generates plasmin on the bacterial surface. Moreover, *B. burgdorferi* induces production of uPA and uPAR in cultured monocytic cells. *In vivo,* PLG is ubiquitously available in abundance in the plasma and other body fluids but the source of uPA for the spirochetes is less clear. Since uPA circulates at a low concentration, its induction is necessary for its utilization by Borrelia. Earlier studies in our laboratory had shown the upregulation of uPAR on cells exposed to Borrelia. This suggested that receptor bound uPA could also be used to activate the bound PLG on the spirochetes. I examined this hypothesis by directly incubating monocytic cells with *B. burgdorferi* in transmigration chambers. By using different enzymes and inhibitors the exact involvement of PLG, uPA, uPAR and the PAIs could be determined. First, I looked into the kinetics of the production of uPA, uPAR, PAI-1 and PAI-2. Second, I examined how the difference in the kinetics of expression of the different components is leveraged by the spirochetes to maximize dissemination.

Experimental procedures:

Reagents:

Rabbit IgG antibody to human uPA, IMUBIND Total uPA and PAI-2 Strip-well ELISA kits, as well as purified uPA and recombinant PAI-2, were all purchased from American Diagnostica, Greenwich, CT. BSK-H medium and FITC-conjugated goat anti-

rabbit IgG were purchased from Sigma, St. Louis, MO. RPMI 1640, sodium pyruvate, and penicillin/streptomycin were purchased from Life Technologies, Grand Island, NY. Fetal Bovine Serum (FBS) low-endotoxin was purchased from HyClone, Logan, UT. Accudenz A.G. was purchased from Accurate Chemical and Scientific, Westbury, NY. Tissue culture plates (24-well) were purchased from Costar, Cambridge, MA.

Bacteria and eukaryotic cell cultures:

B31 strain of *B. burgdorferi* was cultured at 33°C in serum-free BSK-H (Sigma) in all experiments. MM6 cells (Ziegler-Heitbrock *et al.*, 1988) were cultured in RPMI 1640 supplemented with 2mM L-glutamine (Gibco-Invitrogen, Grand Island, NY), 10% heat inactivated FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), 1% non essential amino acids (Gibco-Invitrogen), and 1 X OPI Media Supplement (Sigma). THP-1 (Tsuchiya *et al.*, 1980) cells were cultured in RPMI/10%FBS and 1% β-mercaptoethanol.

Isolation of monocytes from human donors:

Human peripheral blood monocytes were isolated by sedimentation from 60 ml whole blood collected from healthy volunteers was anticoagulated in 0.12% EDTA. Blood was mixed with 6% Dextran T500 (Pharmacia Fine Chemicals, Uppsala, Sweden) in pyrogen-free water at a ratio of 4 ml of dextran to 40 ml of blood. The mixture was allowed to stand for 45 min (females) or 60 min (males) at room temperature to sediment erythrocytes and granulocytes. After 1 h, the upper phase containing the leukocyte-rich plasma was centrifuged, and the cells were resuspended in one-third of the volume of the original plasma. Aliquots (2 ml) of resuspended cells were underlaid with 5 ml of Accudenz, followed by centrifugation at 650 x g, for 15 min at room temperature. The monocyte-rich band at the plasma-Accudenz interface was collected from each tube.

Monocytes were washed once at 400 x g for 3 min at RT in calcium and magnesium-free phosphate buffered saline (PBS), containing 1% low-endotoxin bovine serum albumin (BSA) (Sigma) and 3 mM EDTA (pH 7.4). The monocytes were washed and resuspended in PBS-BSA-EDTA several times to remove platelets. Monocytes were more than 90% pure as determined by their ability to take up three or more latex beads (0.82 μm diameter; Sigma) after 30 min at 37°C.

Spirochete/eukaryotic cell cocultures:

MM6 cells were washed twice with RPMI 1640. After viability was determined using trypan blue, the cells were counted and dispensed into 24-well polystyrene tissue culture plates (1 ml per well) at a density of 1 x 10^6 per ml. *B. burgdorferi* were washed twice in RPMI (10 min at 7000 x g at 25°C), enumerated, and added to the mammalian cells at multiplicities of infection of 10 and 100 spirochetes per cell. The final volume of each well was 1.5 ml. Also included for each experiment was a sham preparation to control for nonspecific stimulatory activity derived from the BSK-H medium. The sham was prepared at the same time as the spirochete preparation and consisted of an equal volume of uninoculated BSK-H medium that was subjected to the same manipulations.

Bacterial in vitro penetration assay:

Biocoat cell culture inserts for 24-well plates containing polyethylene tetraphalate membranes with pore size of 1 μm and pre-coated with fibronectin by the manufacturer were purchased from Becton Dickinson Labware, Bedford, MA. When inserted into a well in a 24-well plate, this membrane formed a barrier, making an upper and lower chamber. 5 x 10^5 MM6 cells or monocytes were delivered into the upper chamber in 500 μl of RPMI and allowed to adhere for 30 min, followed by the addition of 5 x 10^7 *B.*

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burgdorferi bound with PLG per well. The spirochete-PLG binding procedure has been described previously (Coleman *et al.*, 1995). Before placing the cell culture insert containing the monocytes and *B. burgdorferi* into the well, 500 μl of RPMI 1640 was added to the lower chamber. After 12 h, *B. burgdorferi* that had crossed to the lower chamber were quantified by dark field enumeration. In some experiments, α_2-antiplasmin, aprotinin, (Calbiochem, La Jolla, CA), or PAI-2 (American Diagnostica) were used as inhibitors at final concentrations of 30 μg/ml, 50 *μg/ml* and 25 *μg/ml* respectively. Monoclonal antibody against the amino-terminal fragment (ATF) of uPA (American Diagnostica) was used at a concentration recommended by the manufacturer (0.25 *μg/ml*). Purified uPA (American Diagnostica) was also used as a positive control at a final concentration of 4 *μg/ml*.

Real time PCR:

MM6 cells were cocultured with 0, 10 and 100 *B. burgdorferi* organisms per cell. Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. cDNA was then generated using RETROscript First Strand Synthesis Kit (Ambion, Austin, TX) according to the manufacturer's protocol using random decamers and 2 μg of the total RNA. The resulting cDNA sample was diluted 10 fold for use in the real-time RT-PCR. Specific primers to uPA, adapted from a previous work (Helenius *et al.*, 2001) were (F) 5' TCACCACCAAAATGCTGTGT 3' and (R) 5' AGGCCATTCTCTTCCTTGGT 3'. Cycle parameters were as follows: preincubation, 1 cycle at 95°C for 5 min; amplification (quantification), 40 cycles at 95°C for 10 s, 57°C for 10 s and 72°C for 9 s; melting curve, 95°C for 0 s, 65°C for 15 s, and 95°C for 0 s; and cooling, 1 cycle at 40°C for 30 s. LightCycler-FastStart DNA Master SYBR I Green

(Roche, Indianapolis, IN) reaction mix was used in the experimental procedure with the LightCycler (Roche, Indianapolis, IN), based on the manufacturer's protocol. 18S rRNA (primers supplied by Ambion) was used as an internal control to correct for sampling error.

Zymography:

To determine the possible involvement of tPA in the activation of PLG, zymographic analysis was carried out. Since the activity and molecular weight of the protease could be determined simultaneously, the involvement of tPA in the Borrelia induced activity could easily be discerned. MM6 cells were stimulated at multiplicity of infection (MOIs) of 1, 10 and 100 spirochete per monocytic cell for 2, 4 and 6 hours. Sham controls were also used for the time points stated. Supernatants were then subjected to electrophoresis on a non-reducing SDS gel that contained a final concentration of 1 mg/ml PLG and 1 mg/ml of casein. The gels were then washed with 2.5 % Triton X-100 for 20 min twice to wash the SDS, and transferred to a renaturation buffer (40 mM Tris, 0.2 M NaCl, 10 mM CaCl$_2$, 0.05% Brij-35 pH 7.4-7.5) and incubated at 37°C overnight (12 to 18 hours). After renaturation, the gels were stained with 0.2% Coomassie Brilliant Blue for 90 minutes while shaking at room temperature. Gels were finally destained with 20% methanol, 10% acetic acid and photographed with a Polaroid camera.

Immunofluorescent assay of surface uPA by flow cytometry and confocal microscopy:

MM6 cells were cocultured as described above. The cells were washed twice with RPMI and incubated with 100 μg/ml of non-specific human IgG to saturate surface Fc receptors. After washing the cells twice in medium, they were resuspended in 25 μl of a previously determined saturating concentration (10 μg/ml) of anti-uPA rabbit polyclonal

IgG and anti-uPAR mouse monoclonal IgG (American Diagnostica) in RPMI, incubated

for 30 min on ice, and washed twice in RPMI. The cells were then resuspended in 10

μg/ml of FITC-conjugated goat anti-rabbit IgG (Sigma) and 10 μg/ml of rhodamine-

conjugated sheep anti-mouse IgG (Sigma) for another 30 min. Cells were then finally

resuspended in 0.5 ml of Dulbecco's PBS containing 1% formalin. An irrelevant rabbit

IgG (American Qualex, San Clement, CA) was used as a negative control. FITC

fluorescence intensity for uPA was measured with a FACScan Flow Cytometer (Becton

Dickinson) in which the cells were gated with forward vs. side scatter. Labeled cells

were also viewed with confocal microscopy (Nikon, Eclips E 800).

Detection of soluble uPA, uPAR, PAI-1 and PAI-2 by quantitative ELISA:

Soluble uPA, uPAR, PAI-1 and PAI-2 in conditioned medium were measured by a

quantitative ELISA using the IMUBIND total uPA, uPAR, PAI-1 or PAI-2 Strip-well

ELISA Kits (American Diagnostica) according to the manufacturer's instructions. The

conditioned medium was routinely centrifuged for 15 min at 14000 x g before use to

remove the spirochetes.

Detection of surface PAI-2 using immunofluorescent assay:

For the detection of surface bound PAI-2, MM6 cells were stimulated with *B.*

burgdorferi for 5 h, a time point at which PAI-2 levels were the highest (see chapter one).

Cells were centrifuged and washed with PBS supplemented with 1% BSA and 0.1%

sodium azide to prevent internalization of the PAI-2/uPA/uPAR complex. MM6 cells

were then incubated with excess human IgG (100 μg/ml) on ice to block Fc receptors.

Anti-PAI-2 IgG (Sigma) (20 μg/ml) and the same concentration of irrelevant goat IgG

(Sigma) was then added to the experimental and control groups respectively and

incubated for 30 minutes on ice. After incubation, the cells were extensively washed and

incubated with 10μg/ml of FITC-conjugated rabbit anti-goat IgG (Sigma) for another 30

minutes on ice. Cells were again washed extensively to reduce non-specific interactions,

fixed using 1% paraformaldehyde and viewed under a fluorescent microscope.

Chromogenic substrate assay:

MM6 cells were cocultured with *B. burgdorferi* at multiplicities of infection of 10 and

100 spirochetes per cell, for 2, 4 and 6 h. Following incubation, the MM6 cells were

washed once with 1 ml of PBS and resuspended in 200 μl of PBS containing 100 μg/ml

of PLG and 500 μg/ml of chromogenic plasmin substrate, S2251 (H-D-Val-Leu-Lys-

pNA-2 HCL; Chromogenix, Milan, Italy). For the measurement of the activity of soluble

uPA, RPMI medium without phenol red was used for the incubation of the cells to avoid

color interference. Supernatant (100 μl) was then incubated with 100 μg/ml of PLG and

500 μg/ml of chromogenic plasmin substrate S2251 to a final volume of 200 μl of

solution. Plasmin activity was assessed by color development due to enzymatic

degradation of the chromogenic substrate measured at 410 nm in a Dynatech MR 700

microplate reader (Dynatech, Chantilly, VA) after 2 h of incubation at 37°C. MM6 cells

treated with sham preparations were used as negative control.

Statistical analysis:

Statistical analyses were performed for data that are presented as the mean or a

representative of three independent experiments done in triplicate. Analysis of variance

(ANOVA), followed by the Tukey post-test (InStat 3.0, GraphPad Software, San Diego,

CA) was used. Results that are statistically different from sham are denoted by *$p<0.05$,

**$p<0.01$ or ***$p<0.001$.

Results:

MM6 cells and human peripheral blood monocytes enhance the invasiveness of B. burgdorferi in vitro

We have shown previously that *B. burgdorferi*, in the presence of exogenous PLG and uPA, are capable of degrading and penetrating complex tissue barriers *in vitro* (Coleman *et al.*, 1999; Coleman *et al.*, 1995). *In vivo*, PLG is easily available to the spirochetes as it is ubiquitously present in blood and tissue fluids (Nordstrand *et al.*, 2001; Gebbia *et al.*, 1999; Coleman *et al.*, 1997). However, the source of PLG activators is less clear. We used functional assays to determine if *B. burgdorferi* could utilize inflammatory cells as sources of PLG activators. In migration assays using cell culture inserts, MM6 cells and freshly isolated human monocytes enhanced the penetration of PLG-bound *B. burgdorferi* through fibronectin (Figures 2.1.A and 2.1.B). At 12 h, the number of transmigrated spirochetes was significantly higher for the wells that had either MM6 cells or peripheral blood monocytes compared to the wells that had *B. burgdorferi* alone. To examine whether this was uPA-mediated induction, we tested if purified uPA alone could enhance PLG-bound *B. burgdorferi* transmigration. Purified uPA enhanced transmigration in a similar way (Figure 2.2.A). To examine the importance of plasmin, *B. burgdorferi*-MM6 cocultures were incubated without PLG. A partial but statistically significant reduction in transmigration was observed (Figure 2.2.B)

Different physiological inhibitors were used to determine the effects they have on the uPA-potentiated invasiveness of *B. burgdorferi*. α-2 antiplasmin produced partial inhibition, but it did not reach statistical significance (Figures 2.1.A & 2.1.B). PAI-2, however, had a minimal, if any, effect on the transmigration of *B. burgdorferi* enhanced

by either MM6 cells or purified uPA (Figures 2.2.A & 2.2.B). In contrast to α-2 antiplasmin or PAI-2, aprotinin completely inhibited monocyte-enhanced penetration of spirochetes (Figure 2.3.A.). Moreover, the addition of an antibody that blocks uPA binding to uPAR did not affect *B. burgdorferi* transmigration (Figure 2.3.B). Inter-experimental variation in the total numbers of transmigrated cells was due to variation in the thickness of fibronectin from one plate to another; however, the patterns of transmigration were always the same.

B. burgdorferi transiently induces the upregulation of uPA expression at the transcriptional level

We used quantitative real time RT-PCR to determine the transcriptional induction of uPA. Total RNA was extracted after 1, 4 and 6 h from MM6 cells exposed to different multiplicities of infection as described in the methods. Co-incubation of monocytic cells with Borrelia for 1 h resulted in a spirochete-dose-dependent increase in uPA mRNA up to nine fold. However, *B. burgdorferi* did not induce uPA mRNA at 4 and 6 h (Figure 2.4). Specificity of the amplification process was confirmed with gel-elctrophoresis of the PCR samples showing a single band at the right molecular size (Figure 2.5)

uPA is the only plasminogen activator secreted by MM6 cells

To examine the secretion of other proteases, such as tPA, in addition to uPA, we carried out zymographic analysis of the plasminogen activators. This technique can distinguish between the 70 kDa tPA and 55 kDa uPA because it separates proteins based on their molecular weight and enzymatic specificity. Only one band at the correct molecular weight for uPA appeared, confirming that only uPA is being secreted by the MM6 cells (Figure 2.6).

35

uPA induced by B. burgdorferi is bound to uPAR on the surface of MM6 cells

The enzymatic activity of uPA dramatically increases upon its binding to cell-bound uPAR (Ellis *et al.*, 1991). Therefore, we measured cell-bound uPA by confocal microscopy and flow cytometry following coincubation with *B. burgdorferi* for 4 h and 24 h. Both uPA and its receptor uPAR were elevated at 4 h on the cell surface in a bacteria-dose-dependent manner and highly colocalized (Figure 2.7). The increase in surface uPAR as a result of stimulation with *B. burgdorferi* is consistent with our previous findings (Coleman *et al.*, 2001). Flow cytometric analysis was in agreement with the microscopy results. uPA was elevated on the surface of MM6 cells after 4 h of coculture with *B. burgdorferi*, and the increase was dose dependent (Figure 2.8). At 24 h, however, there was no apparent elevation in uPA or uPAR levels induced by *B. burgdorferi* (Figure 2.9).

B. burgdorferi induces the release of uPA and uPAR from MM6 cells

Using capture-ELISA, we determined the kinetics of uPA and uPAR protein production and secretion by MM6 cells after stimulation with *B. burgdorferi*. As shown in Figure 2.10, uPA was elevated in the culture supernatant of Borrelia-stimulated MM6 cells compared to the sham-stimulated MM6 cell supernatant. Kinetic analysis revealed that *B. burgdorferi* induced uPA as early as 1 h, peaking at 3 h and declining past 5 h of incubation. Because the viability of MM6 cells was critical for this assay, given its quantitative nature, we limited the experimental samples to the time points when the cells were completely intact. Past 6 h, although the viability of cells was still above 90 percent, some cells started to change their morphology in response to the serum starvation and

bacterial load. However, secretion of soluble uPAR was induced at a longer time point (24 h) (Figure 2.11).

B. burgdorferi induces the release of PAI-1 by MM6 cells

There are two alternative possibilities that could account for the observed decrease in uPA levels (shown with ELISA and immunofluorescent assays) and activity (shown with chromogenic assays) at higher time points. The first explanation is that uPA levels could have decreased at the transcriptional level or at the protein level. This explanation, however, is not in agreement with both the q-PCR or ELISA results. The alternative explanation is the induction of the plasminogen activator inhibitors and the subsequent inhibition of uPA. To examine the induction of PAI-1, we tested several time points to see if PAI-1 is induced by B. burgdorferi and the kinetics of its induction. B. burgdorferi did not induce PAI-1 until after 24 h (Figure 2.12).

B. burgdorferi induces the release of PAI-2 by MM6 cells

Monocytic cells produce and secrete PAI-1 and PAI-2, which not only inhibit uPA, but also mediate its internalization (Ritchie and Booth, 1998; Nykjaer et al., 1997; Haj et al., 1995; Schwartz and Bradshaw, 1992; Wohlwend et al., 1987a; Wohlwend et al., 1987b). As PAI-1 induction could not account for the decreased levels of uPA proteolytic activity noted at 4 and 6 h time points in figure 2.12, we did a time course measurement of the production of PAI-2 in the conditioned media. As shown in figure 2.13, B. burgdorferi induced levels of PAI-2 at 3 h, which peaked at 5 h of stimulation in a concentration dependent fashion. The levels of PAI-2 started to decline after 5 h.

Measuring cell surface PAI-2 by immunofluorescent assay

PAI-2 binds uPA on the surface of cells, inhibits enzymatic activity and triggers its internalization in a uPAR mediated process. The induction of PAI-2 by *B. burgdorferi* should result in the elevated levels of PAI-2 on the cell surface provided the complex is not internalized. In agreement with previous work that showed labeling PAI-2 on the surface of monocytic cells is difficult (Liew *et al.*, 2000), labeled surface PAI-2 was not appreciably higher for the stimulated cells compared with sham treated cells. There was, nevertheless, a consistent increase in PAI-2 with the addition of *B. burgdorferi* (Figure 2.14).

The proteolytic activity of uPA decreases with longer periods of incubation with B. burgdorferi

To determine if the induction of uPA in MM6 cells results in the elevation of uPA-proteolytic activity, we conducted a functional assay. *B. burgdorferi*-stimulated MM6 cells were incubated in the presence of PLG and its chromogenic substrate, S2251, at 37°C. At 2 h of stimulation, cell-bound (Figure 2.15.A) and soluble (Figure 2.16A) uPA activities were elevated. At longer coincubation times (4 h and 6 h), however, there was a decrease in the proteolytic activity of cell-bound uPA (Figure 2.15.B & C) and soluble uPA (Figure 2.16.B & C). As ELISA and immunofluorescent assays for uPA and PAI-2 from the second chapter of this document show, the decrease in proteolytic activity of both bound and soluble uPA at 4 and 6 h contrasts with increased production of uPA at those time points but was concomitant with the induction of PAI-2 (Figure 2.17).

Discussion:

As summarized in figure 2.18, *B. burgdorferi* induces the production of uPA, PAI-1 and PAI-2. I showed that monocytic cells enhance the ability of PLG-bound *B. burgdorferi* to migrate through fibronectin *in vitro*. It was also shown using several approaches that *B. burgdorferi* transiently induces the production and release of uPA in monocytic cells. This suggests that *B. burgdorferi* could use monocytic infiltrates as sources of uPA to enhance its own dissemination in the early phases of infection. Mononuclear cells make up a great percentage of inflammatory infiltrates in Lyme disease (Salazar *et al.*, 2003), and they are primary uPA-producing cells (Saksela *et al.*, 1985). Therefore it is likely that they serve as sources of uPA for *B. burgdorferi* at sites of inflammation. Since PAI-2 can potently inhibit soluble and monocyte-bound uPA (Swartz *et al.*, 2004), the inability of monocyte-secreted or recombinant PAI-2 to inhibit uPA-potentiated spirochetal invasion suggests that secreted uPA is not available to the spirochetes in either form. In agreement with previous work, uPA is very likely to be bound to the surface of the spirochetes themselves (Klempner *et al.*, 1996) where it is much more resistant to inhibition by PAI-2 (Perides *et al.*, 1996). α-2 antiplasmin also did not have a significant inhibitory effect on the spirochete transmigration. This is likely due to the inefficiency of α-2 antiplasmin to inhibit membrane-bound plasmin (Perides *et al.*, 1996; Fuchs *et al.*, 1994). The partial reduction of transmigration of the spirochetes in the absence of plasminogen suggests the involvement of other serine proteases, possibly uPA and others, since aprotinin could completely reverse the effect of monocytes on Borrelia transmigration.

Using quantitative RT-PCR, we show that Borrelia-induced transcription of uPA in monocytic cells terminates after 4 h of coculturing with *B. burgdorferi*. This time frame of uPA transcriptional induction may require viable spirochetes since previous findings using lapidated OspA showed a delayed transcription of uPA that peaked at 4 h of stimulation (Fuchs *et al.*, 1996). The degree of induction of levels and activity of uPA by *B. burgdorferi* appears to be in the same order as in clinical situations where different concentrations can be associated with progression of disease and as an indicator of prognosis (Miyake *et al.*, 1999). Furthermore, tPA was not secreted showing that uPA is the primary protease involved in the enhanced penetration of *B. burgdorferi*.

The induction of uPA was followed by the upregulation of its physiological inhibitors, PAI-1 and PAI-2. PAI-2 is induced in MM6 cells in response to *B. burgdorferi* as early as 2 h, peaking at 4-5 h, and declines after 5 h. However, PAI-1 was not induced until 24 h of coculturing time. uPA proteolytic activity decreased simultaneous with the upregulation of PAI-2. The transcriptional downregulation of uPA and the concomitant induction of PAI-2 resulted in an efficient inhibition of uPA proteolytic activity in the supernatant and on the surface of the monocytic cells.

Previous reports showed that intact bacteria (Berk *et al.*, 2001), bacterial LPS (Suzuki *et al.*, 2000; Costelloe *et al.*, 1999) and human lipoprotein (Buechler *et al.*, 2001) could induce uPA and PAI-2. Consistent with our findings, it has been reported earlier that LPS induces uPA and PAI-2 in a sequential order (Costelloe *et al.*, 1999). This sequential induction of uPA and PAI-2 could be due to different induction pathways, which is supported by previous studies showing that the inductions of uPA and PAI-2 are driven by different cytokines (Gyetko *et al.*, 1993; Gyetko *et al.*, 1992). uPA might also

be required for the induction of the PAIs, as reported earlier (Shetty *et al.*, 2003; Dear *et al.*, 1997).

Functional assays of uPA on the surface of MM6 cells revealed that upon stimulation with *B. burgdorferi,* there was a temporary induction of uPA enzymatic activity on the surface of cells followed by a sharp decline after 4 h of coincubation. Paradoxically, the levels of both soluble and membrane associated uPA were elevated at 4 h as shown by immunofluorescence assay and ELISA. This apparent discrepancy is accounted for by the concomitant induction of PAI-2 at 4 h of coincubation as shown by ELISA. Although uPA levels were elevated, uPA was bound to the inhibitor so that its enzymatic activity was blocked. As the comparison of kinetics of induction of uPA and PAI-2 show, there is a slight delay in the induction of PAI-2 after the induction of uPA that results in the temporary elevation in uPA activity.

uPAR did not have an apparent effect on the penetration of *B. burgdorferi* across fibronectin. This was confirmed by an experiment using a blocking antibody to the amino terminal fragment (ATF) of uPA that is responsible for binding to uPAR. Blocking the binding of uPA to uPAR did not have any effect on the uPA-potentiated penetration of the spirochetes further confirming that uPA is bound to the spirochetes themselves. The production of uPAR could be a part of the inflammatory response by the mammalian cells as uPAR serves for the migration of inflammatory cells. The utilization of uPA bound to the surface of the bacterium as opposed to the monocyte may have several advantages. First, the binding of uPA and PLG to the surface of the spirochetes allows for a close proximity for the activation of PLG by uPA. Second, since PAI-2 is induced at longer time points, binding to the surface of the bacteria guarantees the perpetual

utilization of uPA by the spirochetes as they migrate and bind new PLG molecules. If *B. burgdorferi* used monocyte-bound uPA, it would be internalized after the induction of PAI-2. Third, the activation of the bacteria-bound PLG by monocyte-bound uPA would require a considerable physical contact between the monocytes and the spirochetes which would increase the risk of phagocytosis.
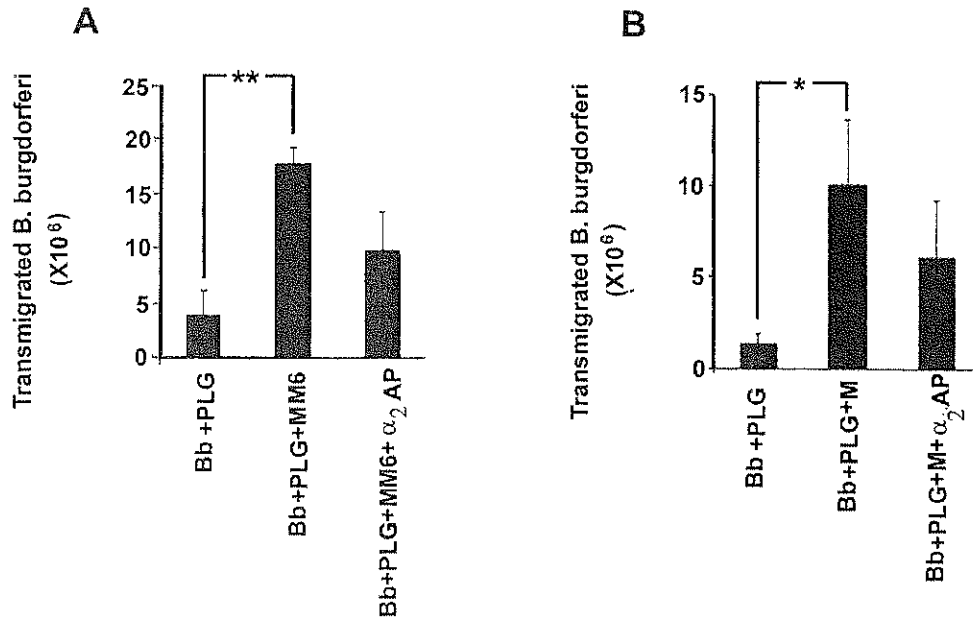
Figure 2.1: Coincubation of plasmin-bound B. burgdorferi with monocytic cells results in enhanced transmigration of B. burgdorferi across fibronectin-coated membranes.
B. burgdorferi were incubated with or without monocytic cells for 12 h, and their transmigration was assayed by direct enumeration of the bacteria in the lower chamber of biocoat plates. MM6 cells (A) and peripheral blood monocytes (B) were used for these experiments. α-2 antiplasmin was used as an inhibitor. Bb, *B. burgdorferi*; MM6, Mono Mac 6; M, peripheral blood monocytes; α-2 AP, α-2 antiplasmin; PLG, plasminogen. Bars show the mean values of a representative result from three independent experiments done in triplicate $\pm$ SD. *$p<0.05$, **$p<0.01$.
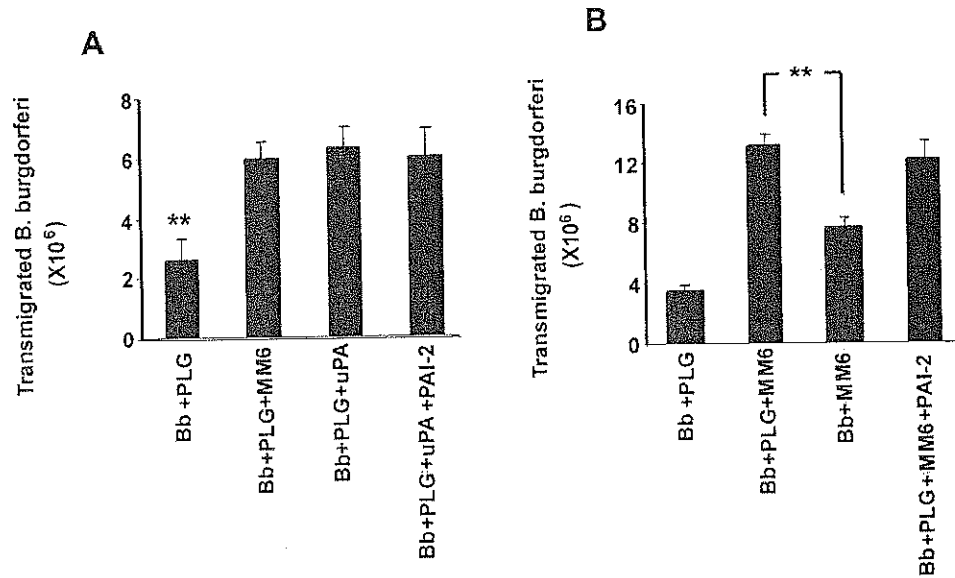
Figure 2.2: Monocyte-enhanced penetration of B. burgdorferi partly depends on PLG

and is not inhibited by PAI-2. B. burgdorferi were incubated with or without monocytic

cells and their transmigration was assayed by direct enumeration of the bacteria in the

lower chamber of biocoat plates. (A) uPA alone enhanced the penetration of B.

burgdorferi comparable to that of MM6 cell-enhanced penetration. The addition of PAI-2

did not inhibit bacterial penetration. (B) The absence of PLG resulted in a partial but

significant reduction in bacterial penetration. Moreover, PAI-2 did not inhibit MM6

enhanced bacterial penetration. Bb, B. burgdorferi; MM6, Mono Mac 6; PLG,

plasminogen; PAI-2, plasminogen activator inhibitor-2. Bars show the mean values of a

representative result from three independent experiments done in triplicate ± SD.

$**p<0.01$.
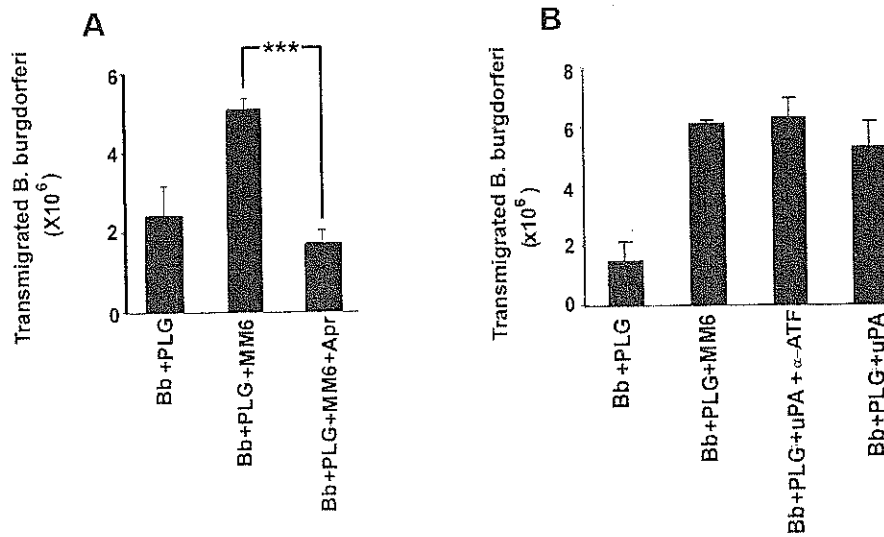
*Figure 2.3: Aprotinin completely inhibits uPA-potentiated transmigration of*

spirochetes while blocking uPA-uPAR interaction does not. B. *burgdorferi* were

incubated with or without monocytic cells and their transmigration was assayed by direct

enumeration of the bacteria in the lower chamber of biocoat plates. (A) The presence of

50 μg/ml aprotinin completely inhibited enhanced bacterial penetration through

fibronectin. (B) Blocking the binding of uPA to uPAR using an antibody against the ATF

(uPAR binding domain of uPA) did not affect uPA-potentiated transmigration of *B.*

burgdorferi. Bb, *B. burgdorferi*; MM6, Mono Mac 6; PLG, plasminogen; Apr, aprotinin.

Bars show the mean values of a representative result from three independent experiments

done in triplicate $\pm$ SD. ***$p<0.001$.
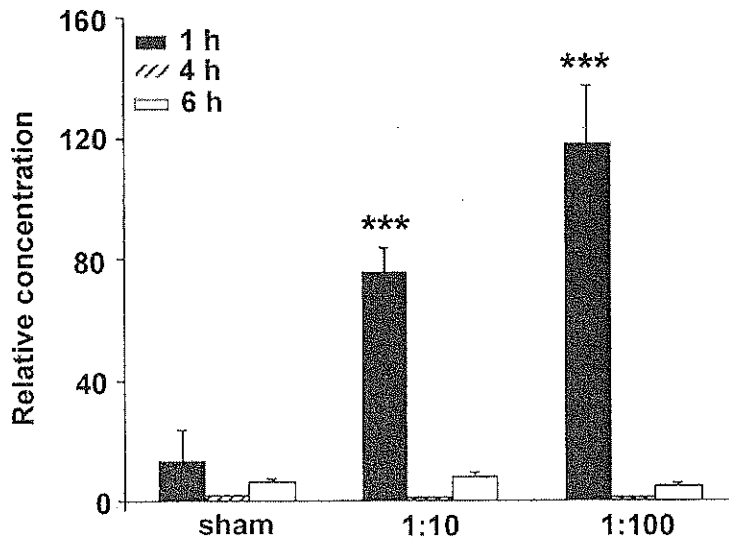
Figure 2.4: B. burgdorferi induces uPA at the trnascriptional level at early time points.

Equal numbers of MM6 cells (1×10^6) were incubated with 0 (sham), 10 or 100 *B.*

burgdorferi per MM6 for different time points. Real-time PCR analysis of uPA transcript

was carried out as described in the methods. Bars represent the mean values of three

independent experiments done in triplicate ± SD. ***$p<0.001$.
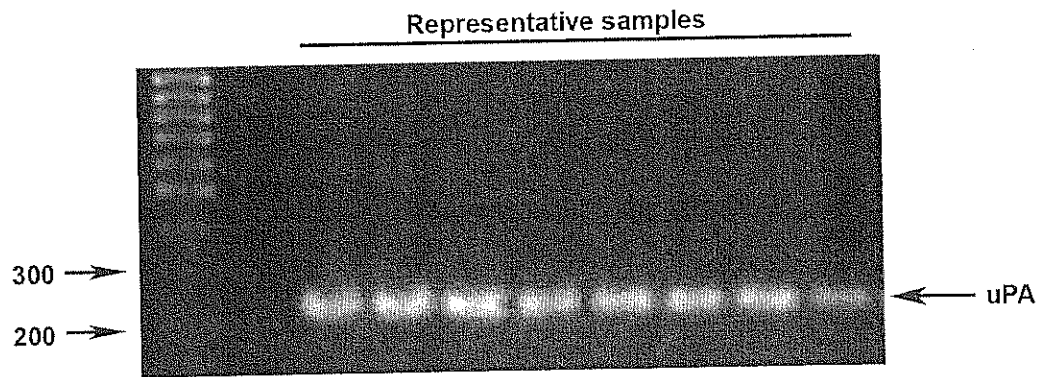
Figure 2.5: QRT-PCR resulted in the specific amplification of uPA gene. Gel

electrophoresis was performed on 8 representative samples of 27 PCR samples. The

presence of a single band at the right molecular size, 213 bp, confirmed specificity of

uPA primers.
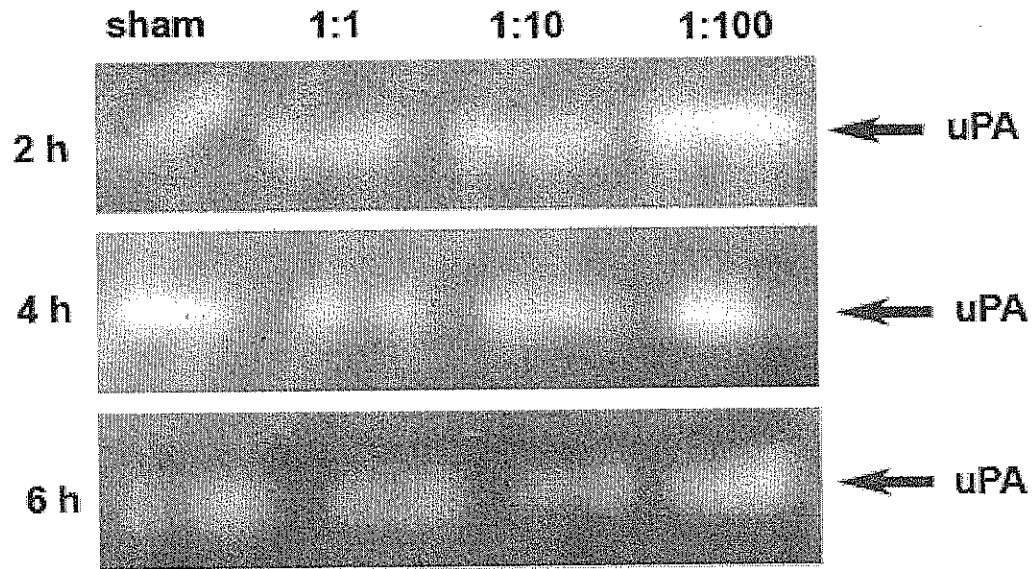
Figure 2.6: uPA is the only plasminogen activator secreted by MM6. MM6 cells were

co-incubated with or without *B. burgdorferi* for 2, 4 and 6 h as discussed in the methods.

Borrelia-treated or untreated (sham) MM6 cell culture supernatants were subjected to

electrophoresis in a non-reducing SDS gel that had casein and PLG incorporated in it.

After they have been immersed in renaturation buffer overnight, gels were Coomassie-

stained. Clear bands, only at molecular weight of 55 kDa, show the absence of protein

(casein) as it was degraded by plasmin that was activated by uPA.

Isotype

1:10

sham

1:100
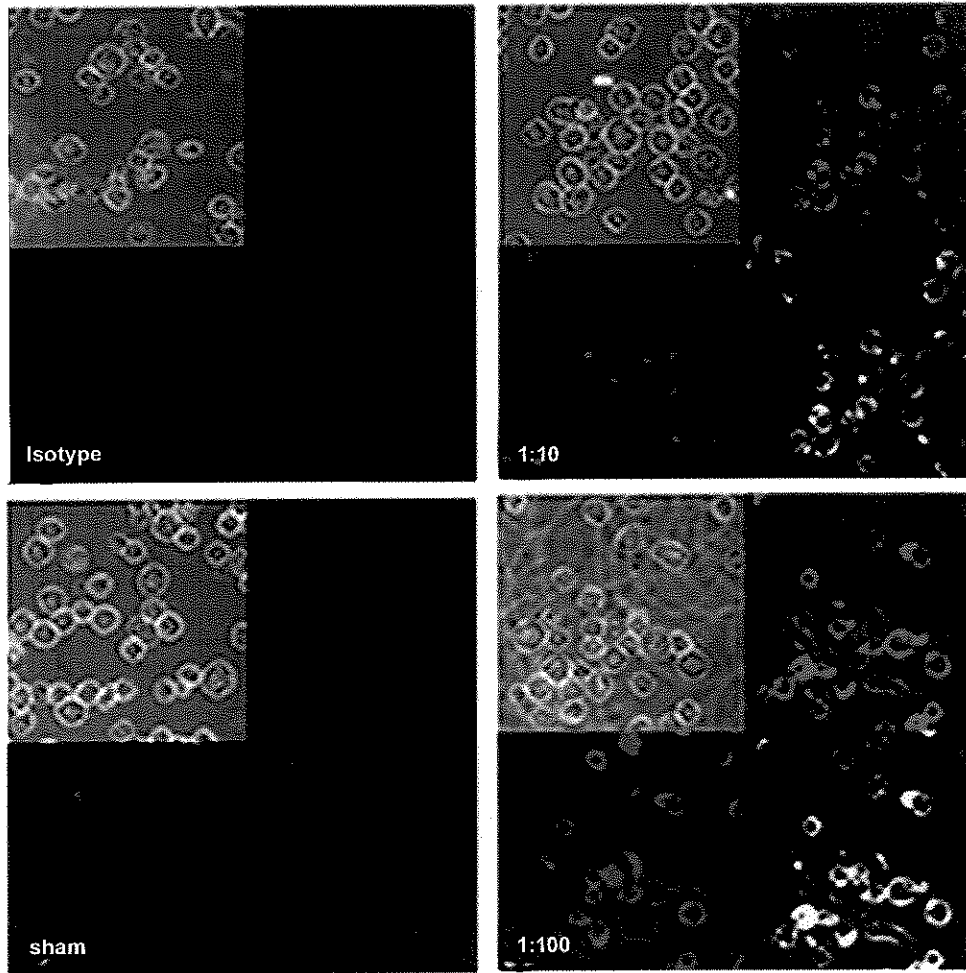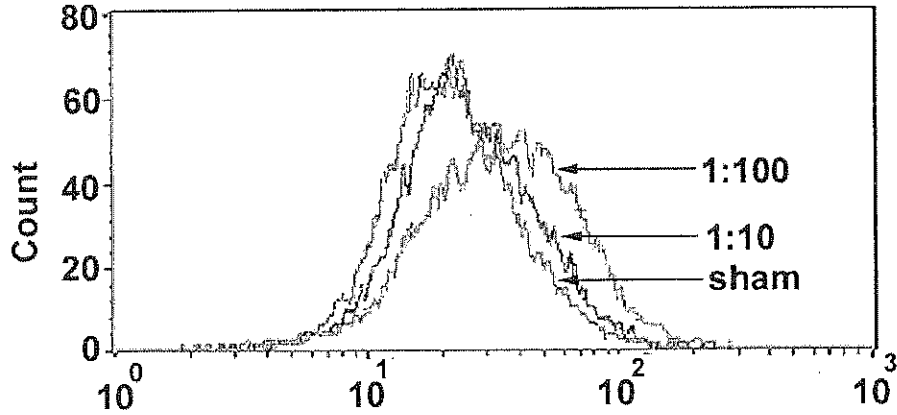
Figure 2.8: Flow cytometry analysis confirmed the confocal images. Flow cytometry

analysis for cells labeled with FITC-conjugated anti-uPA IgG was carried out after

incubating monocytic cells with sham preperations or with *B. burgdorferi* at MOIs of 10

or 100 bacteria per cell for 4 h. *Borrelia burgdorferi* induced elevation in levels of cell-

bound uPA in MM6 cells in a bacterial dose dependent manner.
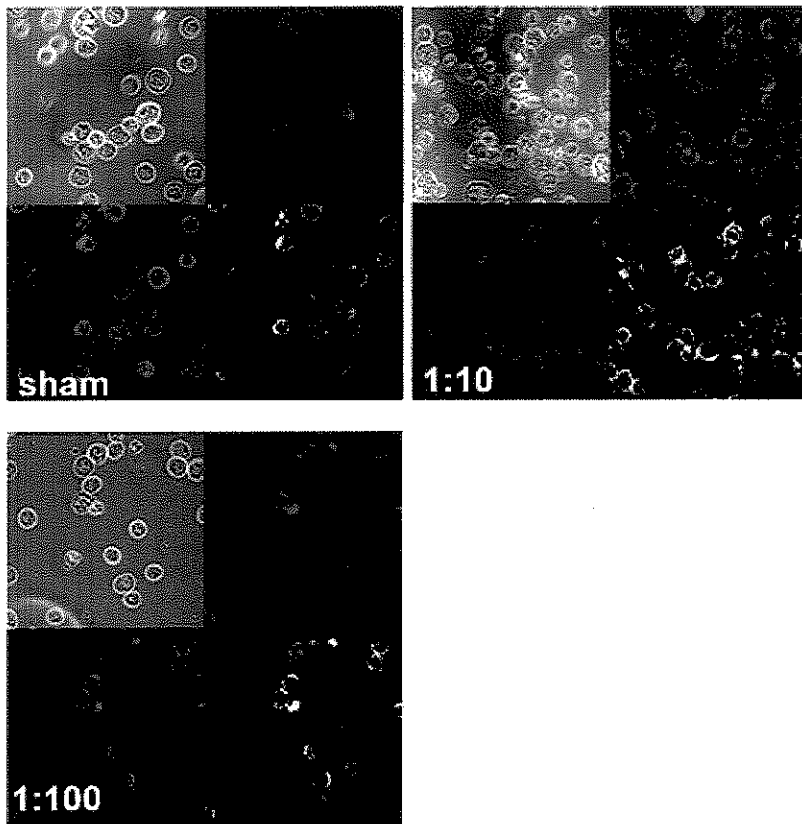
Figure 2.9: At 24 h, surface-uPA and uPAR returned to basal levels. Confocal images

showing MM6 cells labeled with FITC-conjugated anti-uPA (green) and Rhodamine-

conjugated anti-uPAR (red) antibodies, after incubation MM6 cells with sham

preperations or with *B. burgdorferi* at MOIs of 10 or 100 bacteria per cell for 24 h. An

irrelevant isotype matched control antibody was also used. Phase-contrast images are

shown as well as the colocalization of uPAR and uPA (yellow
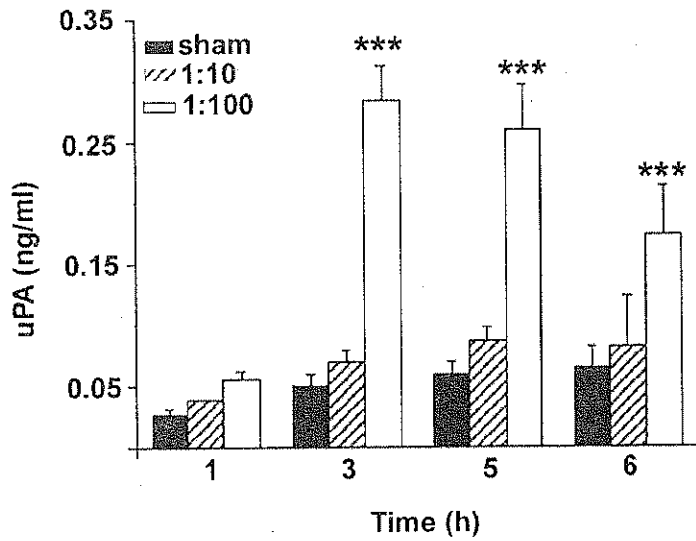
Figure 2.10: B. burgdorferi induces uPA protein levels at early time points. Equal

numbers of MM6 cells (1×10^6) were incubated without (sham) or with 10 or 100 *B.*

burgdorferi per MM6 cells for different time points. Conditioned media were harvested

and the levels of soluble uPA were assayed by quantitative ELISA. Bars represent the

mean values of three independent experiments done in triplicate ± SD. ***$p < 0.001$.
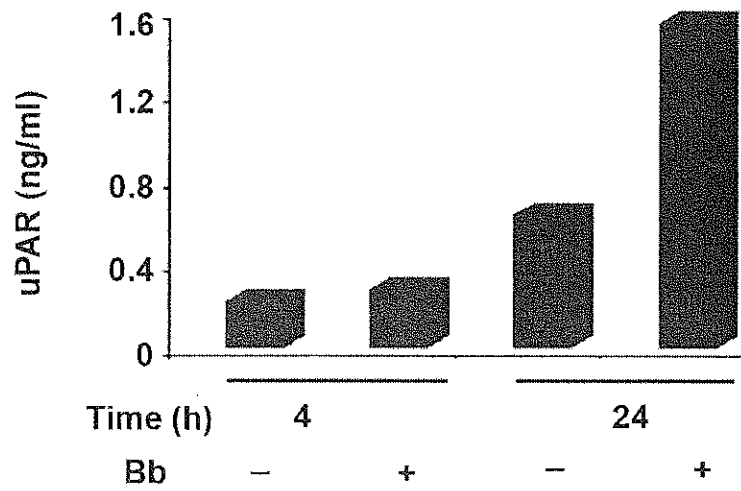
Figure 2.11: uPAR production is elevated in MM6 cells in response to Borrelia stimulation. MM6 cells were coincubated with *B. burgdorferi* for 4 h or 24 h in serum-free RPMI. The supernatants were assayed for soluble uPAR by ELISA. Bars represent the mean values of one experiment of two independent experiments done in duplicate.
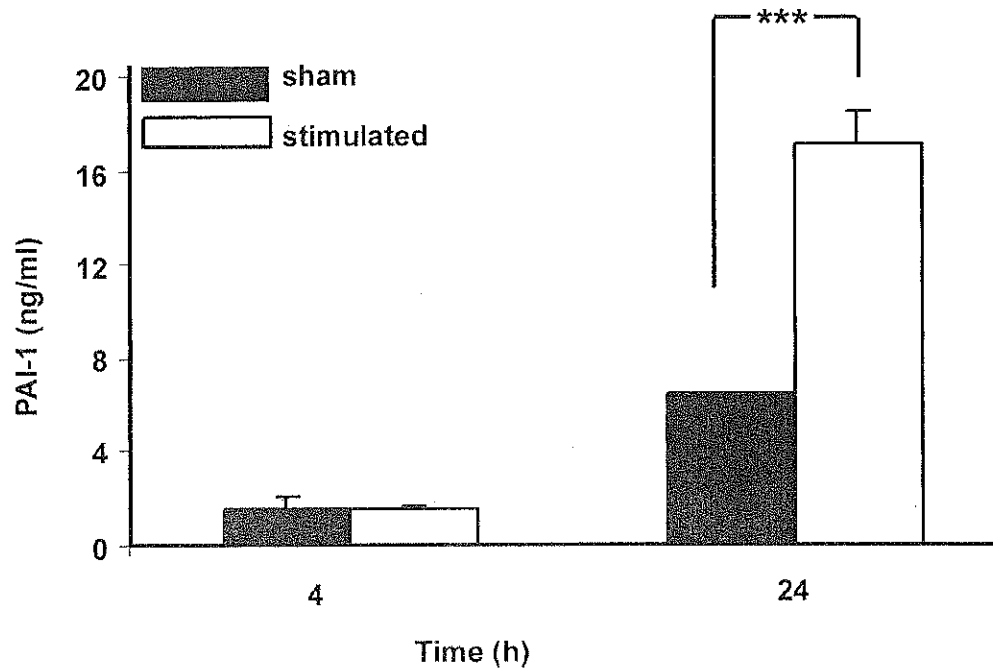
Figure 2.12: B. burgdorferi induces the production of PAI-1 in monocytic cells. Equal

numbers of MM6 cells (1×10^6) were incubated with 0 (sham), or 100 *B. burgdorferi* per

MM6 for different time points (4 or 24 h) and PAI-1 protein levels were assayed in the

supernatant samples of the MM6 cell cultures using capture ELISA. PAI-1 was induced

at 24 h. Bars represent the mean values of three independent experiments done in

triplicate $\pm$ SD. ***$p<0.001$.
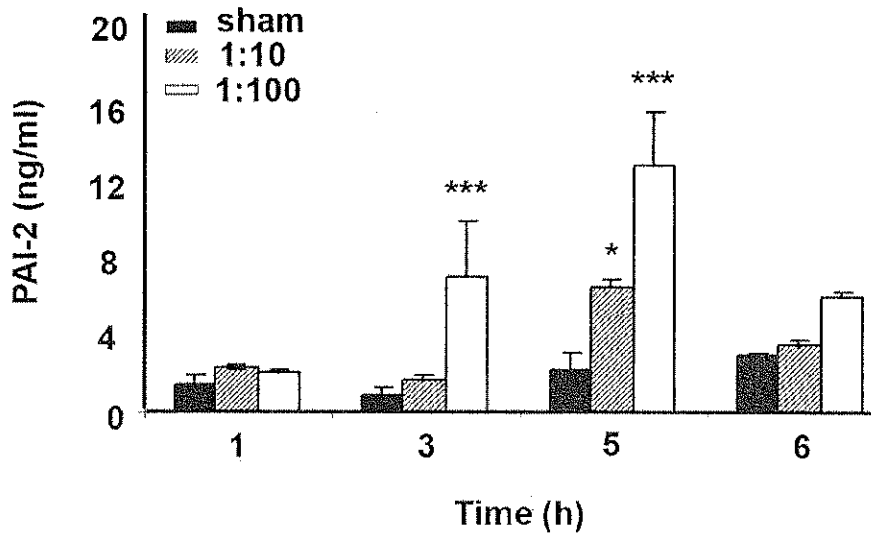
Figure 2.13: B. burgdorferi induces the production of PAI-2 in monocytic cells. Equal

numbers of MM6 cells (1×10^6) were incubated with 0 (sham), 10 or 100 *B. burgdorferi*

per MM6 for different time points and PAI-2 protein levels were assayed in the

supernatant samples of the MM6 cell culture using capture ELISA. Bars represent the

mean values of three independent experiments done in triplicate ± SD. *$p<0.05$,

***$p<0.001$.
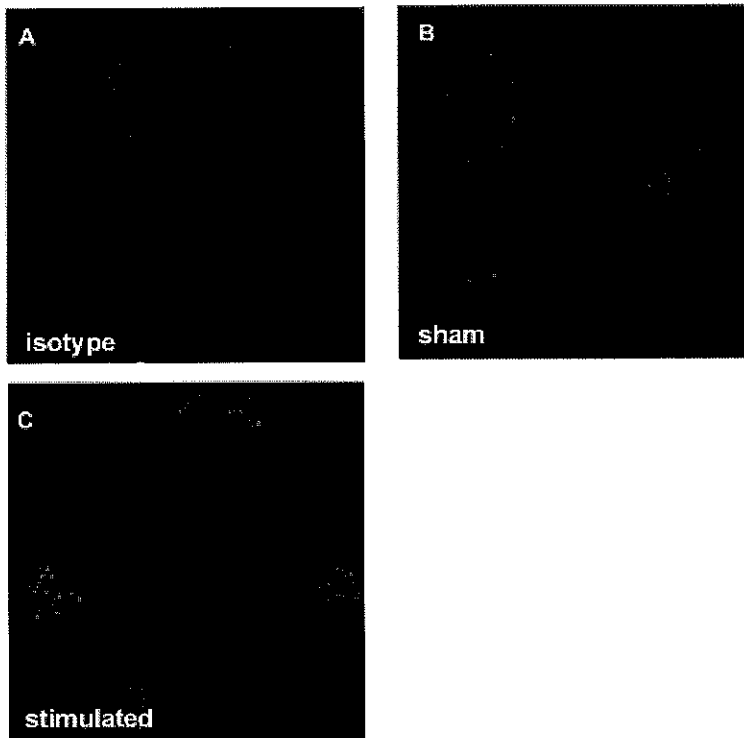
Figure 2.14: Surface PAI-2 increases with Borrelia stimulation: Surface-bound PAI-2 was measured with immunofluorescent assay. After MM6 cells were incubated with sham or *B. burgdorferi* at MOI 100 bacteria per cell for 5 h, they were stained with goat anti-PAI-2 IgG, counterstained with FITC conjugated anti-goat IgG and viewed with a fluorescent microscope and photographed. An irrelevant isotype matched antibody was used to control for non-specific antibody interaction. The above picture represents one of two independent experiments with consistent results.
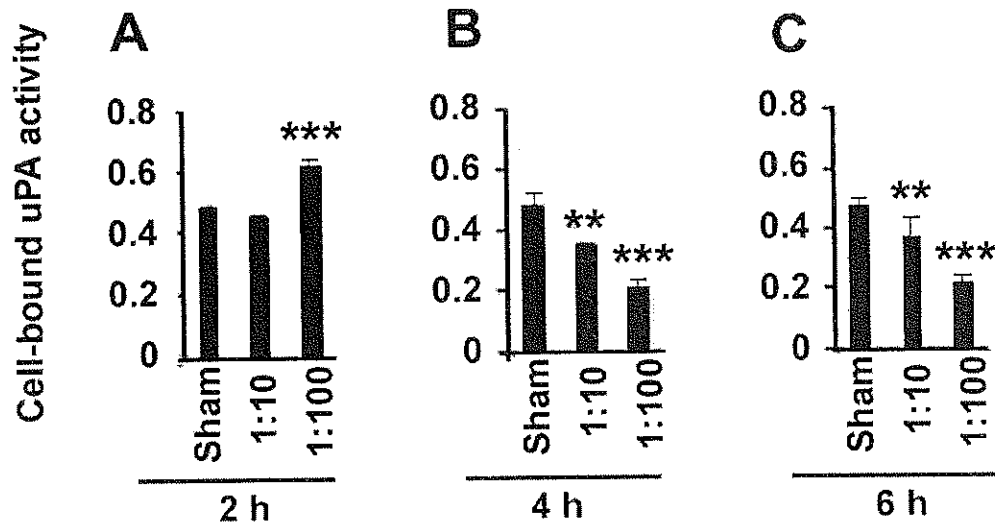
Figure 2.15: B. burgdorferi induces surface uPA activity at early time points and subsequently decreases it. MM6 cells were coincubated with *B. burgdorferi* at MOI of 10 or 100 bacteria per cell for (A) 2 h (B) 4 h and (C) 6 h. As described in the methods, an indirect plasmin chromogenic assay was performed to assess uPA activity. Bars show the mean values of a representative experiment of three independent experiments done in triplicate ± SD. $**p<0.01$, $***p<0.001$.
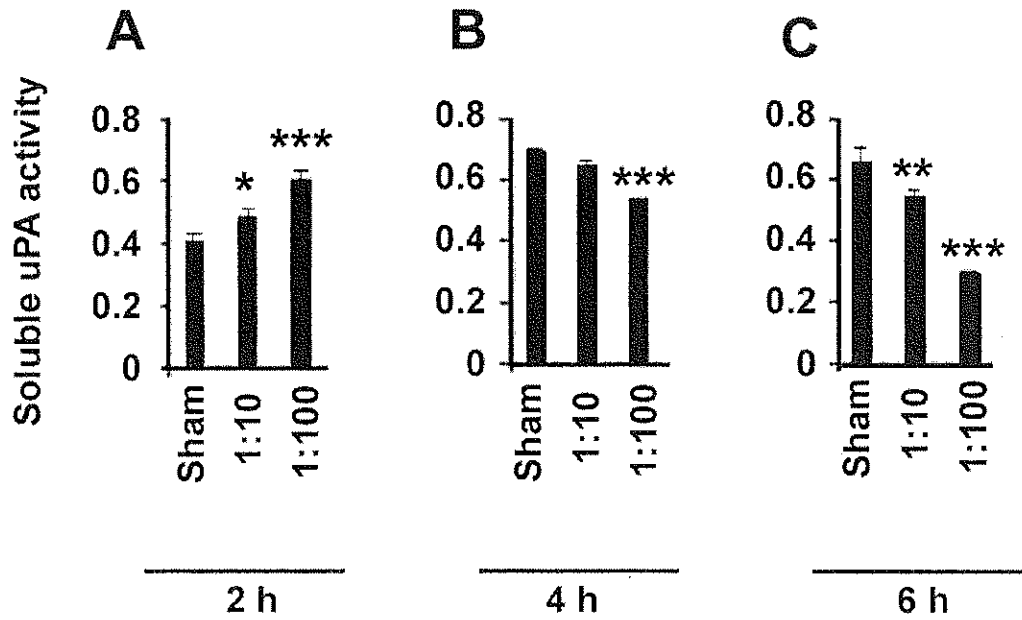
Figure 2.16: Parallel to cell surface uPA, B. burgdorferi induces soluble uPA activity at early time points and subsequently decreases it. MM6 cells were coincubated with *B. burgdorferi* at MOI of 10 or 100 bacteria per cell for (A) 2 h (B) 4 h and (C) 6 h. As described in the methods, an indirect plasmin chromogenic assay was performed to assess uPA activity. Bars show the mean values of a representative experiment of three independent experiments done in triplicate ± SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$.
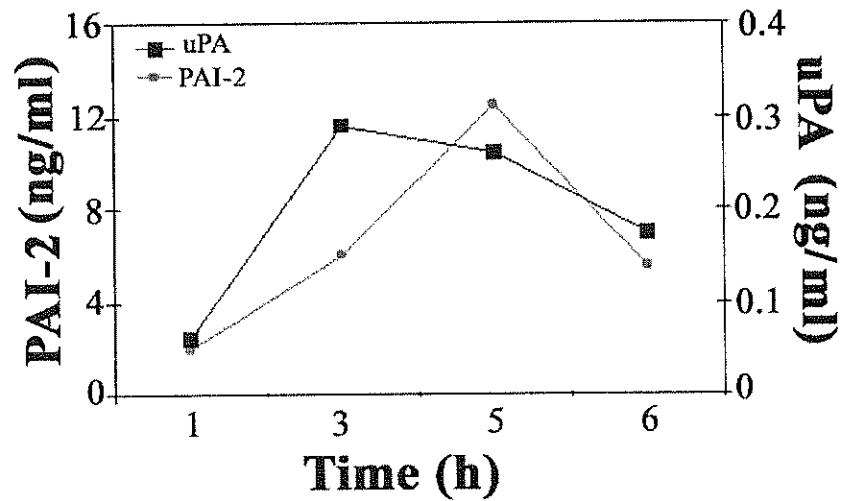
Figure 2.17: Superimposed ELISA graphs showing the comparison of the kinetics of induction of uPA and PAI-2 in MM6 cells: *B. burgdorferi* induced uPA prior to the induction of PAI-2. This resulted in a time-window in which uPA activity is not inhibited which accounts for early uPA activity in the culture supernatant and on cell surface.
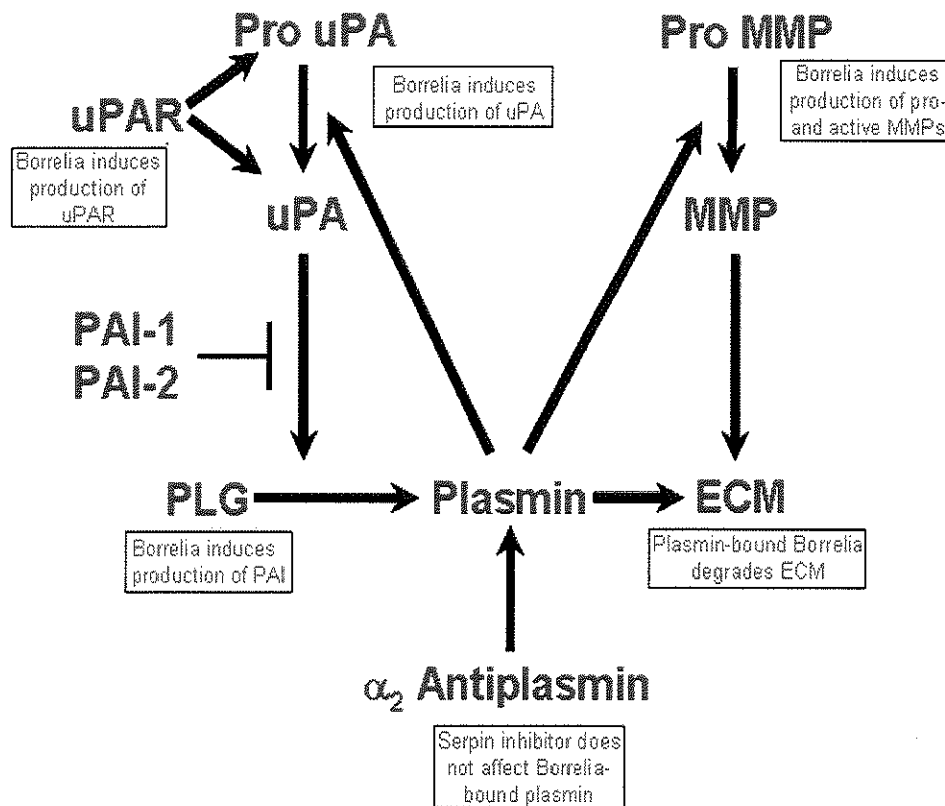
Figure 2.18: The complex interactions of Borrelia burgdorferi and the mammalian host's proteolytic systems: A summarized picture showing the complexity of the interaction between *B. burgdorferi* and the PAS. It was previously shown in our laboratory that Borrelia binds PLG. Borrelia also induces the MMP system as well as uPA receptor. In this work, it was further shown that Borrelia induces uPA, PAI-1 and PAI-2 in a kinetically regulated manner.

Chapter 3: The PAS and monocytic cells

Specific Aim II: Examine the overall effects of the induction of uPA and PAI-2 by *B. burgdorferi* on the invasive and adhesive properties of monocytic cells.

Rationale:

The ECM plays a double role in the migration of cells. It serves as a substratum for cells to migrate upon but also poses a physical barrier blocking cell migration. Thus, the PAS is the system of choice for the migration of inflammatory cells because it provides them with both the vehicle to move on the ECM and a proteolytic activity to degrade the physical barrier posed by the ECM. The uPA/uPAR system engages the cytoskeleton for the attachment and movement of the cells on the ECM and the same system activates PLG to degrade the ECM and open up a passage for the cells to migrate through, hence playing the double role.

Because of the roles the PAS plays in cell migration, it is suggestive that the multiple interactions of *B. burgdorferi* with the components of the PAS would have an impact on the migratory behavior of monocytic cells. This dissertation addressed the above hypothesis using different monocytic cell lines with differential expression of PAI-2, stimulating them with *B. burgdorferi* and comparing their migratory behavior. A key to this approach was the utilization of THP-1 cells, which lack functional PAI-2 but express normal levels of other components of the PAS and other adhesive molecules (Table 3.1). Thus THP-1 cells have been utilized as a PAI-2 deficient monocytic cell line to study different functions of PAI-2. By comparing THP-1 with MM6 cells, it was possible to show the distinct roles of the individual components of the PAS in the migratory response to Borrelia stimulation.

The molecular basis of the PAI-2 deficiency in THP-1 cells:

THP-1 cells have an intact PAI-2 gene as genomic DNA analysis by southern blot revealed but have a truncated transcript that is translated into a deficient PAI-2 protein (Katsikis *et al.*, 2000). The transcript lacks 6 out of the 8 exons that results in complete functional abrogation (Katsikis *et al.*, 2000).

	Cell line	
	THP-1	MM6
Adhesion Molecules		
LFA-1 (CD11a/CD18)	+	+
Mac-1, CR3 (CD11b/CD18)	+	+
ICAM-1	+	+
CD29 (B1)	+	+
VLA-4	+	+
Components of the PAS		
uPA	+	+
uPAR	+	+
tPA	+	+
PAI-1	+	+
PAI-2	–	+

Table 3.1: A comparison of surface proteins, proteins of the PAS and differentiation markers between THP-1 and MM6 cells: Both of these cell lines are considered to be mature monocytic cell lines (Yamada *et al.*, 1997; Erl *et al.*, 1995).

Experimental procedures:

Detection of uPA-PAI-2 complex by ELISA:

uPA-PAI-2 complex was detected using PAI-2-ELISA wells to capture the complex and then detected with anti-uPA primary antibody (American Diagnostica). In this assay, the complex is captured at the bottom of the well because PAI-2 from the complex in the culture supernatant binds the immobilized anti-PAI-2 antibody, and uPA bound to PAI-2 is detected with an anti-uPA antibody. Using this technique, only the complex was detectable.

Eukaryotic cell invasion assay:

For the invasion assay of MM6, THP-1 and peripheral blood monocytes, BD biocoat matrigel invasion chambers were used (BD Labware, Bedford, MA). These invasion chambers consist of a Falcon companion plate with Falcon cell culture inserts containing an 8 μm pore size membrane with a layer of matrigel. The matrigel matrix serves as a reconstituted basement membrane *in vitro*. The matrigel layer occludes the pores of the membrane, blocking non-invasive cells from migrating through the membrane. After placing 750 μl of RPMI supplemented with 10% FBS (HyClone) as a chemoattractant in the bottom chamber, 5 x 10^5 of MM6 or THP-1 cells were seeded in the top chamber, in the presence of 100 μg/ml of PLG and at a final volume of 500 μl. Monocytic cells were then incubated with or without 5 x 10^7 *B. burgdorferi*. In some experiments, excess concentrations of purified uPA (40 μg/ml) and recombinant PAI-2 (1 μM) were added to the MM6 cell culture. After 10 h, cells in the lower chamber were directly enumerated using hemacytometers and cells that were adherent to the bottom of the membrane were stained and viewed under the microscope according to the manufacturer's instructions.

Monocytic cell adhesion assay

MM6 or THP-1 cells (5×10^5 cells/ml) were stimulated with 5×10^7 *B. burgdorferi* in RPMI 1640 in 24 well plates at 37°C for 4-5 h in flat-bottomed polystyrene wells (Costar). Non-adherent cells were then washed off by rinsing three times with RPMI media and adherent cells were fixed on the bottom of the wells using 50% methanol. To some cultures, excess exogenous uPA (10 μg/ml) (American Diagnostica) and PAI-2 (1 μM) (American Diagnostica) were added to examine their roles in the Borrelia-induced change of adhesive properties of the cells.

Detection of soluble PAI-2 in peripheral blood monocytes by quantitative ELISA:

Soluble PAI-2 in conditioned medium was measured by a quantitative ELISA using the IMUBIND total PAI-2 Strip-well ELISA Kit (American Diagnostica) according to the manufacturer's instructions. The conditioned medium was routinely centrifuged for 15 min at 14000 x g before use to remove the spirochetes.

Viability assay:

To examine the effect of *B. burgdorferi* on the viability of monocytic cells, cell viability was tested using the trypan blue staining method. Cell culture supernatant (10 μl) is mixed with 10 μl of 0.4 % trypan blue solution, loaded on the counting chambers of a hemocytometer, left for 1-2 minutes to stain and counted. Unstained cells represent the viable cells as dead cells take up the blue stain due to the compromised membrane integrity.

Statistical analysis:

Statistical analyses were performed for data that are presented as the mean or a representative of three independent experiments done in triplicate. ANOVA, followed by

the Tukey post-test (InStat 3.0, GraphPad Software, San Diego, CA) was used. Results

that are statistically different from sham are denoted by $*p<0.05$, $**p<0.01$ or

$***p<0.001$.

Results:

B. burgdorferi induces the formation of uPA/PAI-2 complex

We measured the amount of uPA-PAI-2 complex in the cell culture supernatants harvested directly from the invasion chambers showed in figures 3.2 and 3.3. *B. burgdorferi* treated MM6 cells showed elevated levels of the complex compared to the non-stimulated ones (Figure 3.1). uPA-PAI-2 complex remained very low in the supernatants of THP-1 cells with or without stimulation with *B. burgdorferi* (Figure 3.1).

B. burgdorferi and PAI-2 inhibit the transmigration of MM6

The interaction of *B. burgdorferi* with multiple components of the PAS is suggestive that *B. burgdorferi* may affect the invasive properties of monocytes. To investigate this, we used Matrigel invasion chambers (Boyden chamber analogs), where monocytic cells were made to migrate from one chamber to another under the stimulus of a chemoattractant across a Matrigel-coated membrane. *B. burgdorferi* caused more than a five-fold decrease in the invasive capability of MM6 cells compared to the MM6 cells that received no spirochetes (Figure 3.2). Similarly, inhibition was observed when the MM6 cells were treated with recombinant PAI-2 alone (Figure 3.2). To analyze whether the binding of PAI-2 to uPA is necessary for this inhibition, we treated Borrelia-stimulated MM6 cells with excess purified uPA. There was a partial but significant restoration of invasiveness of the MM6 cells (Figure 3.2).

B. burgdorferi enhances THP-1 cell invasion

To further confirm PAI-2 mediated inhibition of monocytic transmigration, we used another monocytic cell line, THP-1, which does not make functional PAI-2 but produces

uPA and uPAR. If inhibition of transmigration of MM6 cells is caused by Borrelia-induced PAI-2, THP-1 cells should not exhibit a reduction in transmigration. As predicted, *B. burgdorferi* did not have an inhibitory effect on the transmigration of THP-1 cells across the matrigel. Instead, a marked increase in transmigration was observed upon treatment with *B. burgdorferi* (Figure 3.3.A, 3.3.B. and 3.3.D), which was significantly decreased with the addition of recombinant PAI-2 (Figure 3.3.C and 3.3.D). These results collectively suggest that PAI-2 is responsible for the Borrelia-induced decrease of MM6 invasiveness across the matrigel.

B. burgdorferi enhances adhesive properties in THP-1 cells but not in MM6 cells

As described in detail in the first chapter, the uPA/uPAR system plays a role in cell adhesion through the interaction with the cytoskeleton. This interaction is counteracted by PAI-1 and PAI-2. To examine the effects of *B. burgdorferi* on the adhesive property of the monocytic cells, we conducted cell adhesion assays. *B. burgdorferi* significantly induced adhesiveness in THP-1 cells (Figures 3.5.A, 3.5.B and 3.5.D), but did not affect the adhesive properties of MM6 cells (Figures 3.4.A, 3.4.B and 3.4.D). The addition of excess exogenous uPA (10 μg/ml) to the co-culture did not have any significant effect on the adhesiveness of the Borrelia-stimulated THP-1 cells (Figure 3.5.C and 3.5.D). On the other hand, although *B. burgdorferi* alone had no effect on the adhesiveness of MM6 cells, the addition of 10 μg/ml of exogenous uPA significantly increased the adhesiveness of these cells (Figure 3.4.C). Exogenously added PAI-2 partially decreased the adhesiveness of Borrelia treated THP-1 cells (Figure 3.6).

Stimulation of THP-1 cells with B. burgdorferi results in the induction of uPA

To examine the causes for the increased invasive and adhesive properties of THP-1 cells, we used ELISA to measure the elevation of uPA with Borrelia treatment. Our results confirmed that *B. burgdorferi* induces the elevation of uPA in THP-1 cells (Figure 3.7).

Peripheral blood monocytes express high levels of PAI-2

Peripheral blood monocytes expressed very high levels of PAI-2 with or without *B. burgdorferi* stimulation (Table 3.2). Using ELISA, it was observed that peripheral blood monocytes express PAI-2 levels as high as 4.5 times that of stimulated MM6 cells (compare Table 3.2 with Figure 2.13). Transmigration assay of the peripheral blood monocytes failed to show any migration of the cells across matrigel-coated filters (data not shown).

Discussion:

The uPA/uPAR system is involved in the regulation of cell adhesion and cell migration both through plasmin mediated proteolysis and integrin assisted cell adhesion (Chapman, 1997). In light of earlier *in vitro* results showing the induction of the uPA/uPAR system in monocytic cells presented in the previous chapter, I looked at the effects of *B. burgdorferi* on the cell adhesion and cell migration of monocytic cells. I chose two monocytic cell lines that are different only in the expression of PAI-2 as far as adhesion and migration molecules are concerned, namely, THP-1 cells (which do not express PAI-2) (Katsikis *et al.*, 2000; Gross and Sitrin, 1990) and MM6 cells (expressing PAI-2).

To confirm that THP-1 cells do not express PAI-2 in response to Borrelia, I used a double-ELISA approach to detect uPA-PAI-2 complex in the conditioned media of MM6 cells and THP-1 cells that were incubated with Borrelia. There was no uPA/PAI-2 complex that was detected in the supernatant from the Borrelia-THP-1 cell coculture whereas uPA-PAI-2 complex was detected from the MM6-Borrelia coculture. This technique detected only the uPA-PAI-2 complex since uPA from Borrelia-THP-1 cell coculture could be detected by ELISA. Thus, although uPA levels in THP-1 cells were high, the fact that uPA-PAI-2 complex was not detected was indicative of the specificity of the technique. These results provided further evidence that THP-1 cells do not express PAI-2 in response to Borrelia.

Upon stimulation of THP-1 cells with *B. burgdorferi,* cell adhesion to a plastic substratum markedly increased. Unlike THP-1 cells, *B. burgdorferi* did not increase cell adhesiveness of MM6 cells. However, addition of exogenous uPA considerably increased

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MM6 cell adhesion, suggesting that the uPA/uPAR system is involved in Borrelia-induced monocytic cell adhesion. The adhesion of THP-1 cells to the substratum is likely to be due to increased expressions of uPA and possibly also uPAR. uPAR mediates cell adhesion through two pathways. First, uPAR binds ECM protein component, vitronectin, directly and increases cell adhesiveness to the ECM (Deng *et al.*, 1996; Kanse *et al.*, 1996; Wei *et al.*, 1994). Second, uPAR binds to and forms a complex with integrins and increases cell adhesion to the substratum through the rearrangement of the cytoskeleton (Degryse *et al.*, 2005; Chapman, 1997; Sitrin *et al.*, 1996). uPAR mediated adhesion induced by Borrelia is likely to involve the latter pathway that utilizes the integrins, since monocytic cells adhered to plastic substrata not coated with vitronectin in our adhesion assays. Indeed, it has been shown that deletion of uPAR decreases Mac-1 (β2 integrin) dependent adhesion to plastic surfaces in monocytic cells (Nusrat and Chapman, 1991). Thus Borrelia induces uPA and uPAR and increases cell adhesiveness in an integrin dependent manner.

The fact that MM6 cells and THP-1 cells both express monocyte adhesion molecules (Yamada *et al.*, 1997; Erl *et al.*, 1995), as well as uPA/uPAR system (Katsikis *et al.*, 2000; Gross and Sitrin, 1990) (also see table 3.1), suggests that the difference in cell adhesiveness of the two cell lines in response to Borrelia could be attributed to PAI-2 expression. Excess uPA induced adhesiveness in MM6 cells comparable to that of stimulated THP-1 cells, implying that uPA/uPAR may have been blocked by the inhibitor. The prediction is that in the absence of PAI-2, MM6 cells would display an increase in adhesiveness since both uPA and uPAR are induced upon stimulation with Borrelia (see results in chapter two). PAI-2 could decrease cell adhesiveness through a

number of possible ways. First, the binding of PAI-2 to the uPA/uPAR complex results in the rapid internalization of the complex which could decrease uPAR availability to interact with the integrins. Second, the binding of PAI-2 to uPA on the surface of uPAR could sterically hinder uPAR interaction with integrins resulting in diminished adhesion. This hypothesis is supported by an earlier observation that uPAR specific antibodies can block association of uPAR with Mac-1 via steric hindrance resulting in decreased cell adhesion (Sitrin *et al.*, 1996). Intracellular PAI-2 may also be involved in affecting cell adhesion possibly through uPAR mediated signal transduction, as THP-1 cells that endogenously expressed PAI-2 showed more reduction in adhesiveness (Yu *et al.*, 2002) compared to exogenously added PAI-2 in my experiments. PAI-2 mediated decrease in adhesiveness is likely not to be through the inhibition of uPA enzymatic activity since the amino-terminal fragment of uPA (the domain that is responsible for the binding to uPAR but lacks the active site) can inhibit cell adhesion as effectively as intact uPA (Chapman, 1997).

Cell migration is the result of cell adhesion and pericellular proteolysis (Chapman, 1997). Since Borrelia increased cell adhesiveness in THP-1 cells, it was consistent that it considerably increased THP-1 cell migration across a reconstituted basement membrane. Addition of exogenous PAI-2 significantly decreased Borrelia-induced migration of THP-1 cells suggesting that invasion is mediated by the uPA/uPAR system. MM6 cell-migration was also consistent with the adhesion assays, in that upon stimulation with Borrelia, MM6 cell invasion significantly decreased below that of the controls (non-stimulated MM6). In agreement with our adhesion assays, transmigrated THP-1 cells were predominantly adhered to the filter while MM6 cells were mostly in suspension.

The difference in the migration pattern between THP-1 cells and MM6 cells is likely to be due to PAI-2 expression in MM6 cells. This was supported by the observation that the addition of recombinant PAI-2 to the non-stimulated MM6 cells resulted in a similar inhibitory effect on the MM6 cell invasion. Furthermore, addition of purified uPA reversed Borrelia-induced inhibition in MM6 cell migration. These experiments provide further evidence that PAI-2 is critical in the Borrelia induced inhibition of invasion.

The addition of PAI-2 to THP-1 cells had a more pronounced effect on their transmigration compared to cell adhesion. This could be due to the fact that in cell invasion, both the proteolytic as well as adhesive effects of uPA/uPAR complex are needed (Chapman, 1997) making the uPA/uPAR system more vulnerable to inhibition because PAI-2 efficiently blocks uPA activity on cell surfaces. The same reason may apply for the more pronounced effect of Borrelia on the migration of MM6 cells compared to cell adhesion. Interestingly, the presence of PLG did not have a significant effect in the Borrelia induced migration of monocytic cells (data not shown). This could be due to one of two reasons. First, uPA may play a direct proteolytic role in the degradation of the matrigel. Second, cell adhesion might be the primary mechanism of migration rendering PLG irrelevant. Indeed, it was shown that cell migration chiefly depends on the optimal level of adhesiveness in cells (Palecek et al., 1997). Since cell adhesion can not be permanent, otherwise it will be impossible for cells to detach and move, uPA may play a role in the detachment process by cleaving cell-attached ECM structures at the rear of the cells and freeing them for movement.

Peripheral blood monocytes secreted very high levels of PAI-2 compared to MM6 cells. Although PAI-2 is normally below detection limits in vivo (Kruithof et al., 1995),

PAI-2 expression was constitutive in these cells *in vitro*, in that it was expressed with or without Borrelia stimulation. This could be due to the rigor of the isolation procedure that could have induced one or more mediators that regulate PAI-2 production in monocytes (Kruithof *et al.*, 1995). Indeed, mechanical stimulation has been shown to induce the PAS (Chu *et al.*, 2006). Contrary to MM6 cells, treatment with Borrelia seemed to have slightly reduced secretion of PAI-2 in peripheral blood monocytes. This could be due to antagonistic effects of some transduction pathways that upregulate PAI-2 individually (Kruithof *et al.*, 1995). Peripheral blood monocytes did not display a difference in migration between Borrelia-stimulated and non-stimulated cells, as there were no observable migrations through the matrigel (data not shown). This is likely to be due to the high levels of PAI-2 produced in these cells.

The results described in this chapter along with the data presented in the second chapter could be used to speculate on the overall impact of the PAS in the dissemination of Borrelia, in that the interaction of *B. burgdorferi* with the PAS could benefit the spirochetes in two ways. First, elevation of uPA has an advantage for *B. burgdorferi* dissemination, at least *in vitro*, by directly degrading biological barriers and activating PLG in a localized manner, which in turn can degrade the ECM directly or indirectly through the activation of matrix metalloproteinases (Gebbia *et al.*, 2001). In addition, anti-coagulant activity of uPA at the sites of inflammation could also enhance bacterial mobility (Moir *et al.*, 2004; Sun *et al.*, 2004). However, it is not to be ruled out that although PAI-2 does not appear to inhibit uPA-assisted penetration of *B. burgdorferi in vitro*, it may have a negative effect on the dissemination of *B. burgdorferi in vivo* due to its pro-coagulatory effects (Sun *et al.*, 2004). The second way the PAS could assist the

invasion of Borrelia is through the induction of PAI-2, which decreases the inflammatory migration of host cells, an observation supported by previous reports (Varro *et al.*, 2004; Nakamura *et al.*, 1992). This could partially help the spirochetes to evade the first line of defense and disseminate to target tissues.
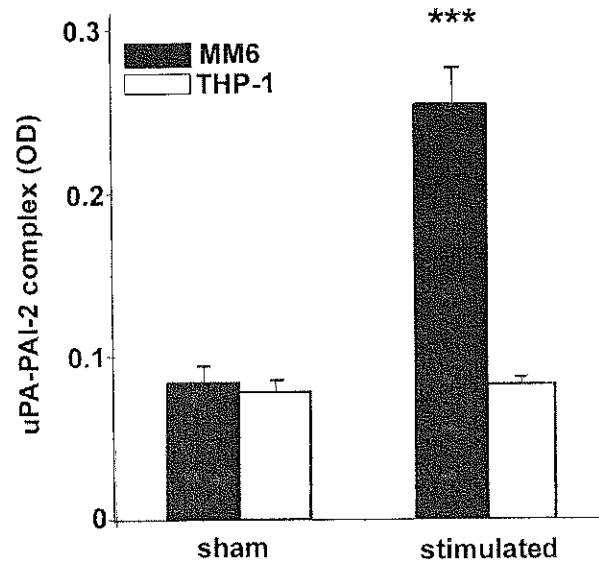
Figure 3.1: B. burgdoferi caused elevation of uPA-PAI-2 complex in MM6 but not THP-1 cells. uPA-PAI-2 complex was measured in the supernatant sampled from transmigration chambers of the stimulated or non-stimulated MM6 and THP-1 cells using ELISA at 450 nm. uPA-PAI-2 complex was captured using α-PAI-2 antibody-coated wells and detected using α-uPA antibody. Bars are the mean values ± SD of a representative experiment of three independent experiments done in triplicate. ***$p<0.001$.

A.
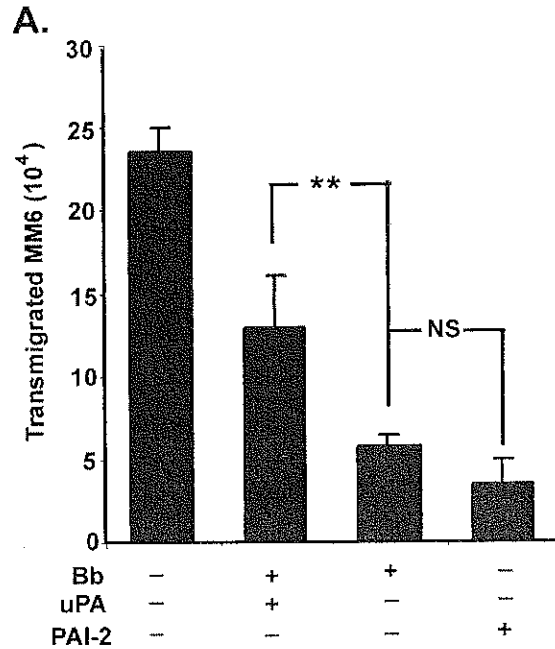
Figure 3.2: Co-incubation with B. burgdorferi results in the decreased transmigration of MM6 cells across a matrigel layer. MM6 cells were placed in the the upper chamber of biocoat inserts and were made to migrate to the lower chamber in response to the concentration gradient of serum in the bottom chamber. In the top chamber, MM6 cells were coincubated with sham preparation or with *B. burgdorferi* at MOI 100 bacteria per cell. In some conditions purified uPA and recombinant PAI-2 were used. Bars show the mean values of a representative experiment from three independent experiments done in triplicate ± SD. **$p<0.01$.

A   B   C

D

Transmigrated cells per field (40 X)

**

160

120

80

40

0

THP-1+Bb   THP-1+Bb+PAI-2   THP-1 alone

80

A sham

B MM6+Bb

C MM6+Bb+uPA

D.

cell number per field (40 X)

***

sham   MM6+Bb   MM6+Bb+uPA

A. sham **B.** THP-1 +Bb **C.** THP-1+Bb+uPA
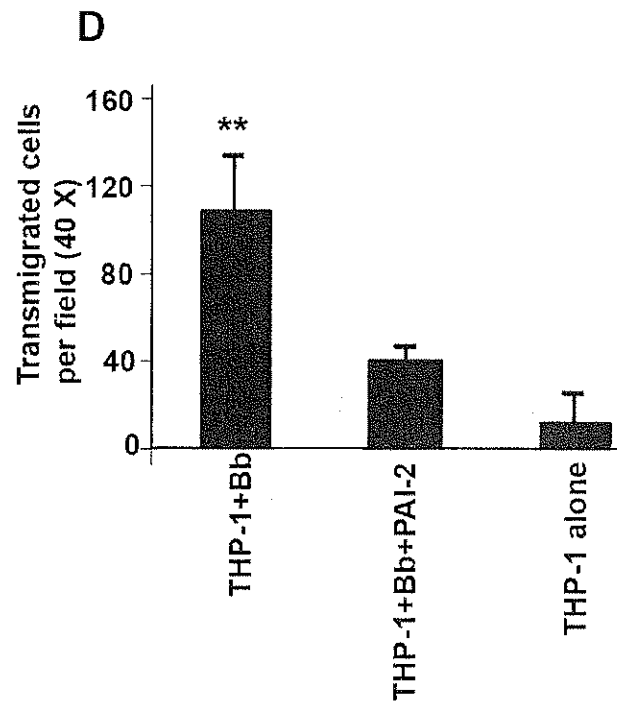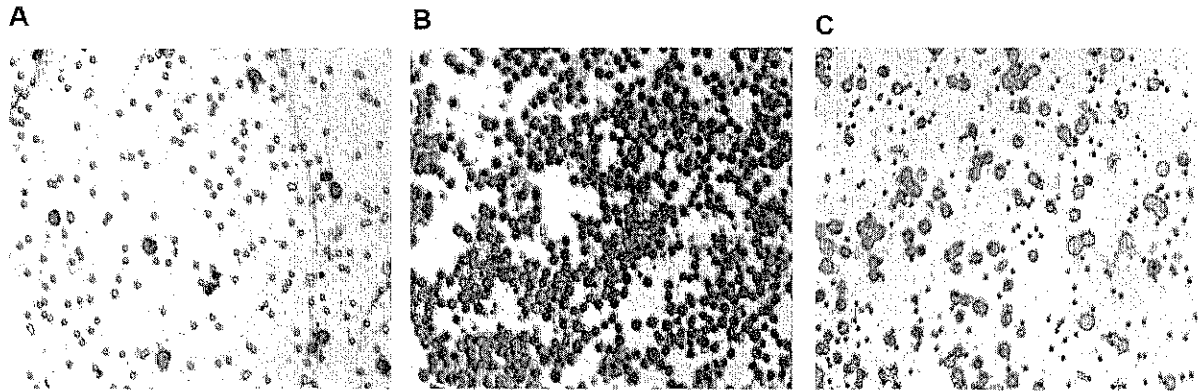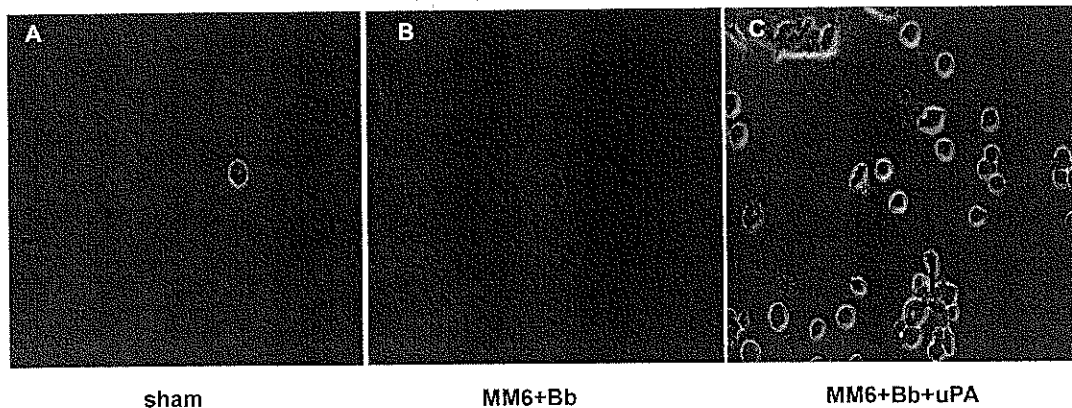
D.
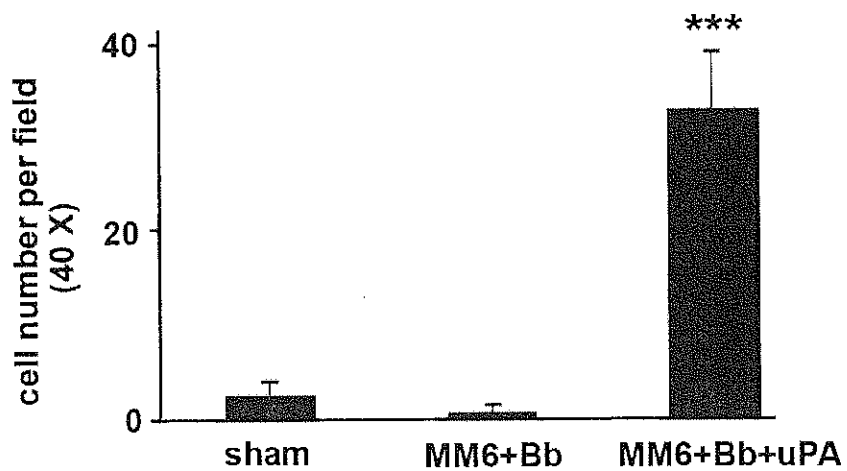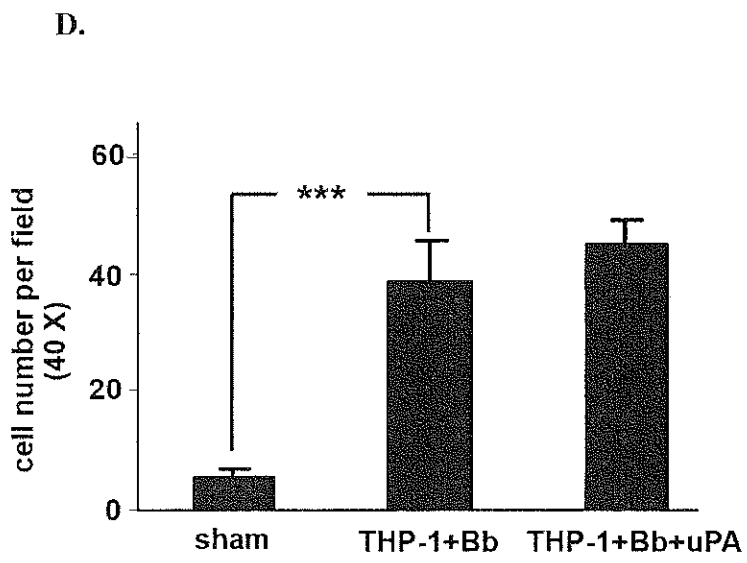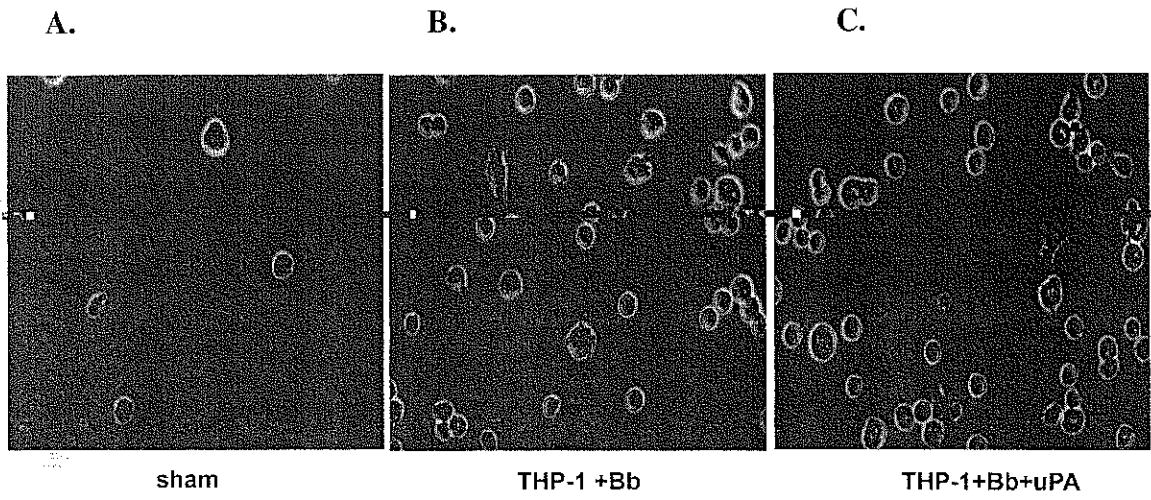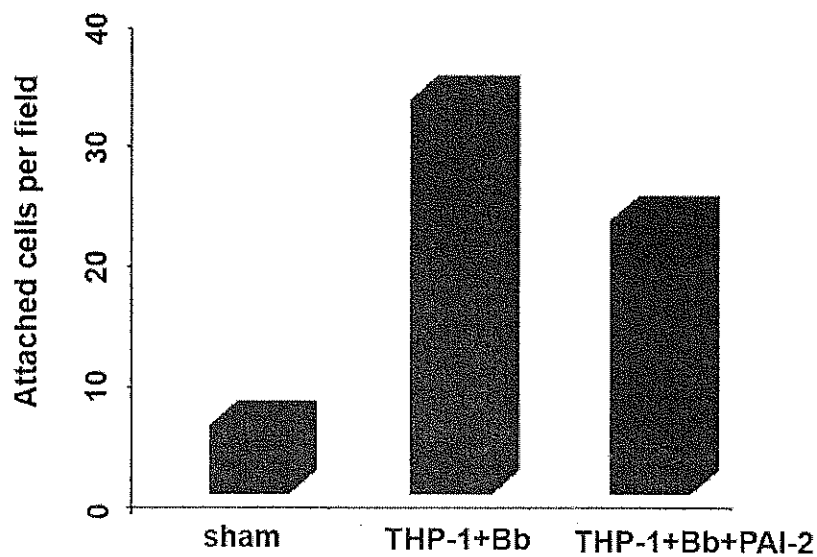
Figure 3.6: the addition of exogenous PAI-2 moderately decreases THP-1 adhesive property. THP-1 cells were stimulated with *B. burgdorferi* for 4 h in the presence or absence of PAI-2. Non-adherent cells were washed off and the adherent cells were fixed with methanol. Adherent cells were counted under the microscope (40 X). Sham preparations were used as negative controls. Bars represent the mean value of one experiment from two independent experiments done in duplicate.
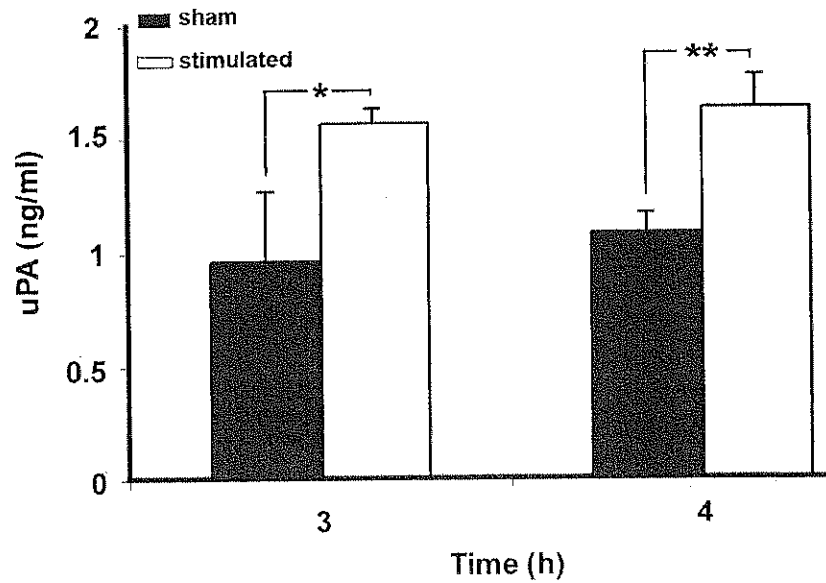
Figure 3.7: THP-1 cells secrete more uPA in response to B. burgdorferi: THP-1

cells were stimulated with *B. burgdorferi* for 3 and 4 h at a cell to bacteria ratio of

1:100. There was a significant induction of uPA compared to the unstimulated

(sham) THP-1 cells. Bars show the mean values of a representative experiment

from three independent experiments done in triplicate ± SD *p<0.05, **p<0.01.

Peripheral blood monocytes PAI-2 production

	sample	PAI-2 (ng/ml)	
		+	−
Bb	1	63	69.6
	2	57	64
	3	59	63
Mean		59.7	65.5
STDV		3.05	3.5

Table 3.2: A very high production of PAI-2 by stimulated and non-stimulated monocytes: Peripheral blood monocytes were isolated and incubated for 4 h with or without *B. burgdorferi* and the conditioned media were assayed for secreted PAI-2 by ELISA. Table is a representative of two experiments performed in triplicate.

Chapter 4: The *in vivo* roles of uPAR

Specific Aim III: To investigate the *in vivo* roles of uPAR on the dissemination of *B. burgdorferi* and the infiltration of inflammatory cells.

Rationale:

To study the *in vivo* effects of uPAR upon bacterial dissemination and inflammatory infiltration, we used uPAR deficient mice. These mice grow normally and have no phenotypic aberrations under non-pathological conditions (Bugge *et al.*, 1995). The rationale for selecting uPAR as a gene of interest is that uPAR plays a central role in the migration of inflammatory cells. As detailed in earlier chapters, uPA mediated proteolysis and cell adhesion that lead to increased cell migration are dependent upon the binding of uPA to uPAR. PAI-1/PAI-2 induced regulation and internalization of surface uPA is mediated by uPAR as it interacts with transmembrane proteins. uPAR also plays a role in chemotaxis of inflammatory cells apart from its ligand uPA.

The induction of uPAR by *B. burgdorferi* was suggestive that uPAR might be involved in either bacterial dissemination, infiltration of inflammatory cells or both, depending on whether the bacterium induces it for its benefits or it is the response of the host to control infection. We addressed both questions using experimental approaches that measure bacterial dissemination by way of addressing bacterial load and inflammatory cell migration by measuring heart tissue infiltration.

Experimental Procedures:

Infection of mice and ankle size measurement:

Sixteen C57BL/6, 8 uPAR-/- and 8 WT, mice were used to test the *in vivo* effects of uPAR in the dissemination of *B. burgdorferi* and the infiltration of monocytes to sites of

inflammation. Transgenic mice were a gift from the laboratory of Dr. Thomas Bugge, NIH. Briefly, 8 uPAR +/+ and 8 uPAR-/- mice were sedated with the help of the Division of Laboratory Animal Resources (DLAR) staff. Tibiotarsal joints of both rear legs were measured using a digital micrometer to establish baseline joint sizes. Mice were subsequently shaved at the base of the tail and injected intradermally with 2×10^3 *B. burgdorferi* strain N40 in 0.1 ml BSK medium (complete) into the shaved back of each mouse. Tibiotarsal joint measurement was continued during the course of the infection at 7, 14 and 21 days post infection to assess arthritis development.

Qualitative measurement of bacterial dissemination by direct culturing method:

Mice were sacrificed at 21 days post infection in a CO_2 chamber and subsequently exsanguinated by cardiac puncture into 0.1 M trisodium citrate and 0.5 M disodium EDTA. Five different tissues (one ear, 4 mm of skin, one fourth of the apical region of the heart, a whole bladder) and 100 μl of citrated blood were cultured in complete BSK media at 33°C. The ventral side of each mouse was immersed in 70% ethanol for 20 min. Tissues were then removed and immersed in BSK with 50 μg/ml rifampicin (Sigma-Aldrich) for another 20 min. The tissues cultured in rifampicin-free BSK medium and cultures were examined twice a week for spirochete growth for 21 days.

Histopathology of the heart:

Hearts were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The sections were examined by three investigators without knowledge of the section identity and were scored according to the degree of inflammatory infiltrate, with 0 being the lowest degree and 4 being the highest degree.

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Measurement of bacterial burden using QRT-PCR:

Total DNA was isolated from the specimens by use of the DNeasy Tissue Kit (Qiagen) and quantitated by spectrophotometry. DNA samples were then amplified using real-time PCR using the Rec A gene for *B. burgdorferi* DNA. To control for sampling error, a host gene (Nitogen-1) was used. Specific primers and probes to Rec A and Nidogen-1 are given in Table 4.1. For the PCR step, taqman universal PCR master mix (Roche) was used. Cycle parameters were as follows: preincubation, 1 cycle at 50°C for 2 min, and 95°C for 10 min; amplification (quantification), 40 cycles at 95°C for 15 s and 60°C for 1 min.

Results:

Ankle swelling

We measured the effects of uPAR deficiency on the severity of Borrelia-induced arthritis in mice. We determined ankle swelling over the course of the infection by measuring ankle size using a digital micrometer at 7-day intervals. The results indicated that there were no differences in ankle swelling between the two groups of mice (Figure 4.1).

Histopathology

We compared the inflammatory infiltration of innate immune cells in the hearts of the two groups of mice 21 days post infection using histological staining. There were no significant differences between the uPAR deficient and the wild-type mice (Figure 4.2).

uPAR does not affect dissemination of B. burgdorferi in mice

Our results showed that uPAR deficiency does not significantly affect the dissemination of spirochetes in mice. After injecting uPAR-/- and wild type mice with equal numbers of spirochetes, we assessed dissemination of *B. burgdorferi* to distant tissues using direct culturing and quantitative RT-PCR. After directly culturing tissue sections from the skin, blood, heart, spleen and ear, we observed that there was bacterial dissemination virtually to all the tissues tested in both the wild-type and the uPAR deficient mice in comparable proportions (Table 4.2). To measure the degree of dissemination using quantitative PCR, we chose to examine the heart as colonization of the heart is a major mark of disseminated infections. There was no significant difference in the number of copies of bacterial DNA between the two groups of mice (Figure 4.3).

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Discussion:

Leukocyte activation and adhesion to the endothelium and the subsequent transendothelial migration are pivotal steps in the recruitment of cells to inflamed tissue. Intact uPAR binds uPA as well as the adhesive protein *vitro*nectin with high affinity (Kanse *et al.*, 1996), and thereby plays a critical role in pericellular proteolysis and modulation of cellular contacts in adhesion and migration (Chapman, 1997). Previous works have implicated uPAR as a regulator of cell recruitment to sites of inflammation *in vivo* (Paul *et al.*, 2005; Mondino and Blasi, 2004; Blasi and Carmeliet, 2002; Rijneveld *et al.*, 2002; Gyetko *et al.*, 2000; May *et al.*, 1998). Therefore, I investigated whether uPAR deficiency affects cell migration during *B. burgdorferi* induced inflammation using uPAR-/- mice.

Using comparative histopathology, it was revealed that uPAR deficiency did not affect leukocyte recruitment to the heart in Borrelia infection. In light of previous works showing that uPAR is required for the leukocyte recruitment in inflammation (Rijneveld *et al.*, 2002; Gyetko *et al.*, 2000), our results were surprising. However, our *in vitro* invasion assays demonstrate that PAI-2 interferes with the uPAR-mediated migration of monocytic cells. Thus, the induction of PAI-2 could partially account for why uPAR did not appear to affect the recruitment of inflammatory cells. Another plausible explanation is the long period of infection of the mice in our experiments, which was 21 days. In support of this line of reasoning, it was reported that in accordance with the known role of β_2 integrins for the acute and early inflammatory responses, reduced leukocyte infiltration in uPAR-/- mice was observed after short-term inflammation for 4 or 24 h (May *et al.*, 1998). However, the total number of migrated leukocytes in uPAR-/- mice

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was not affected after long-term inflammation for 3 d (Dewerchin *et al.*, 1996). This may be due to the fact that prolonged inflammation upregulates and activates several other adhesion receptor systems on leukocytes and endothelial cells apart from β_2 integrins; such as β_1 integrins, vascular cell adhesion molecule 1, or addressins, all of which contribute to leukocyte recruitment (Springer, 1994). Thus, effects of uPAR on leukocyte recruitment in short term Borrelia-induced inflammation should be investigated.

Recent studies have demonstrated that uPAR plays a role in the immune response to bacterial infection. uPAR-/- mice displayed strongly decreased neutrophil recruitment to the pulmonary compartment after induction of *Pseudomonas* or pneumococcal pneumonia, and this decreased recruitment was associated with an impaired antibacterial defense (Rijneveld *et al.*, 2002; Gyetko *et al.*, 2000). Direct tissue culturing experiments did not reveal a significant difference between uPAR-/- mice and the WT littermates in Borrelia infection. Although there was a noticeable difference in the timing of the appearance of spirochetes in the tissue cultures between the two groups of mice, in that there was, as a general trend, a faster appearance of the bacteria in the tissue cultures from the uPAR-/- mice, there was a comparable number of mice from both groups that showed dissemination of the bacteria to distant tissues. Moreover, quantitative PCR did provide further evidence that there is no significant difference in the bacterial load, at least in the heart. Since bacterial load was associated with leukocyte recruitment (Rijneveld *et al.*, 2002; Gyetko *et al.*, 2000), the inability of uPAR to affect bacterial dissemination may be due to the unaltered recruitment of leukocytes in both the uPAR-/- and WT mice. Furthermore, uPAR is likely not to affect bacterial dissemination directly since a uPA specific antibody that blocks binding of uPA to uPAR did not affect Borrelia

invasion *in vitro,* suggesting that uPAR is not directly involved in bacterial spread. As hypothesized in chapter two, uPA is likely to bind to the surface of the spirochetes to provide proteolytic advantage.

uPAR-/- mice and their WT littermates showed no differences in ankle swelling. In fact, there was no noticeable increase in ankle size for both groups over the course of the infection. The genetic background of the mice that were used in these experiments, in that the C57BL/6 mice show moderate levels of arthritis, could partially account for our results (Ma *et al.,* 1998; Barthold *et al.,* 1990).

	Rec A	Nido-1
Primer		
FWD	TTGAGCCCGATCTAGTTACTTGTTC	AGGGCCCAGCAGCAG
REV	CACTACCGGTGGGAATGCTTTAA	GGAATTTGAGGAGGCCAGAACAG
probe	AAGTCTAAGAGATGAATAAAATT	AAGGCAAGAGAAACACG

Table 4.1: Primer and probe sequences for Rec A and Nidogen-1 used for

quantitative real time PCR to determine bacterial load in the heart

	Blood	Heart	Ear	Bladder	Skin	Spleen
WT	0/8	6/8	4/8	5/8	6/8	6/8
uPAR-/-	2/8	7/8	8/8	6/8	7/8	6/8

Table 4.2: Borrelia dissemination measured by the direct culturing of tissues:

Twenty-one days post-infection, mice were sacrificed by CO_2 inhalation and subsequently exsanguinated by cardiac puncture. Five different tissues (one ear, 4 mm of skin, a fourth of the apical part of the heart, a whole bladder and 100 μl of blood) were aseptically cultured in complete BSK-H media at 33°C. Cultures were monitored by dark field microscopy. Fractions represent the number of mice that were positive for bacteria in the specified organ per total of eight mice.
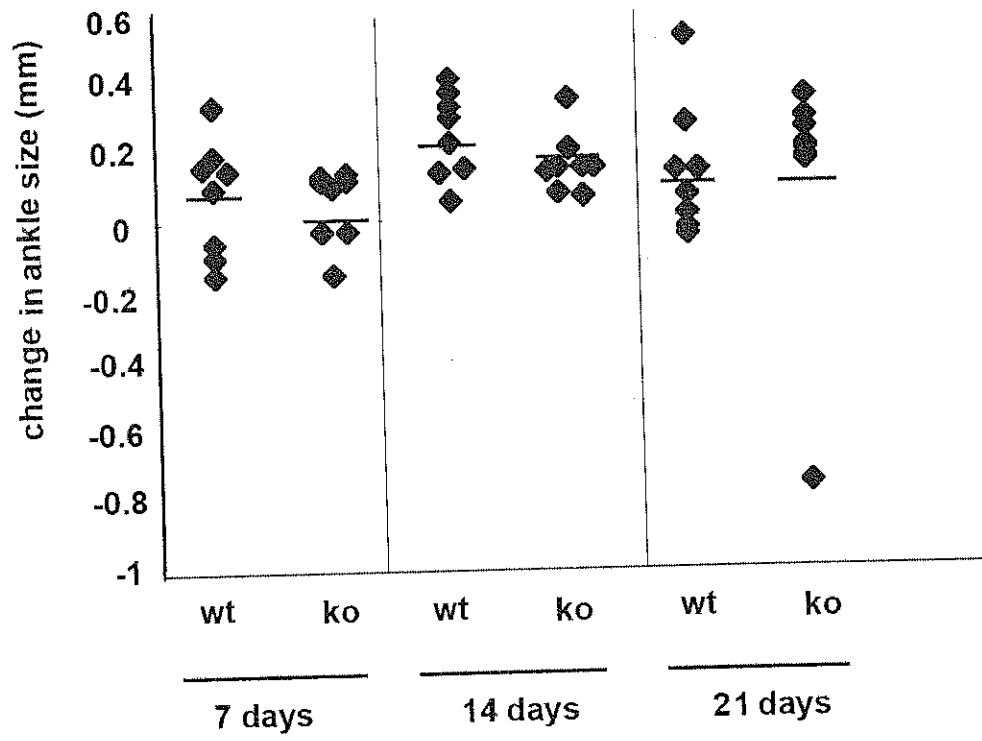
Figure 4.1: No significant differences in ankle swelling were noted between wild type and uPAR -/- mice. Ankle size was measured at day zero (prior to injection of bacteria) and 7 days, 14 days and 21 days after bacterial injection with a digital micrometer (n = 8).
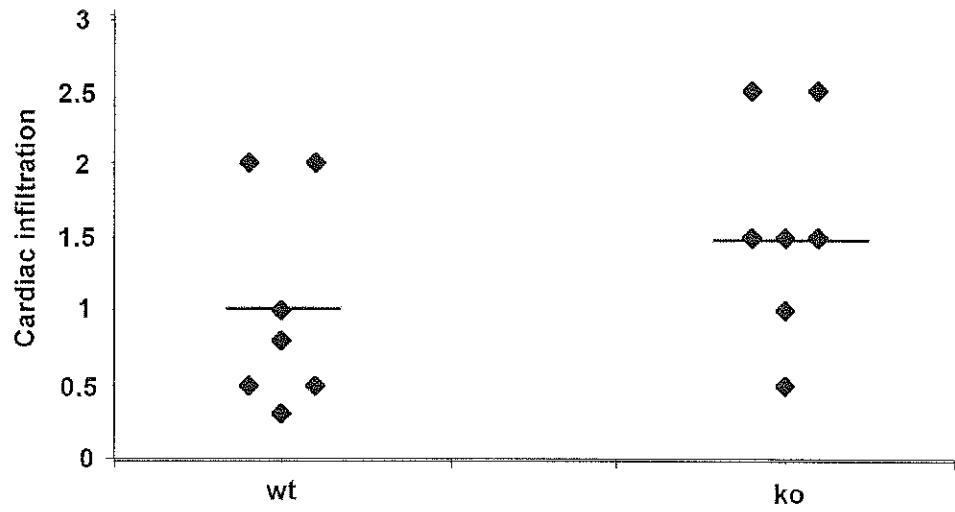
*Figure 4.2: Inflammation was comparable between wild type and uPAR -/-
mice.* Cardiac sections were stained and examined with a microscope. Cardiac

infiltration was rated blindly by three individuals and assigned numbers 0-4

(n = 8).
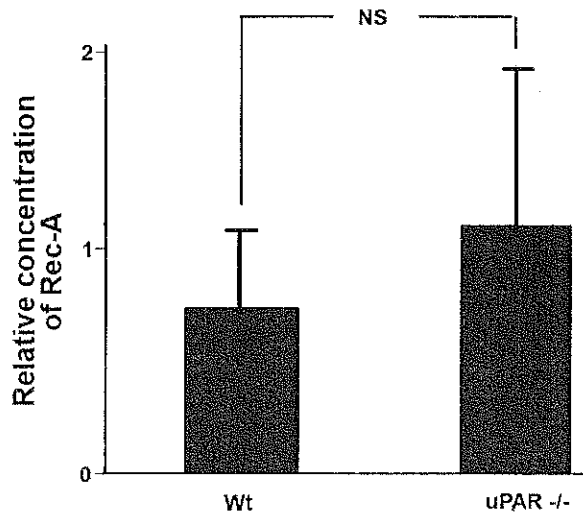
Figure 4.3: uPAR does not affect dissemination of B. burgdorferi in mouse.

Heart tissue was analyzed for spirochetal burden using qRT-PCR. Total DNA was extracted according to the protocol in the methods and bacterial Rec A gene was amplified and normalized to nidogen-1. There was no statistical difference in the levels of Borrelia gene between WT and uPAR-/- deficient mice (n = 8).

Conclusions:

In conclusion, the findings in this dissertation can be summarized as follows.

1. *B. burgdorferi* transiently induces production of uPA in monocytic cells, both at the transcriptional level and the protein level.

2. The induction of uPA is shortly followed by the induction of PAI-2, which in turn is followed by the induction of PAI-1 and uPAR.

3. The coincubation of *B. burgdorferi* with monocytic cells directly enhances the invasive ability of *B. burgdorferi* across ECM components (fibronectin) *in vitro*. This enhanced invasion is mediated by uPA, and it is not inhibited with physiological inhibitors of uPA and PLG.

4. Borrelia inhibits the invasion of monocytic cells across matrigel barriers. This inhibition is mediated by PAI-2 upregulation and can be partially restored with excess exogenous uPA.

5. uPAR does not play a major role in the dissemination, pathogenesis and inflammatory cell recruitment *in vivo* in the infection of Borrelia in long-term infections.

Based on our data, we propose a model (Figure 4.4), representing the *in vivo* events in the infection of and inflammatory response *to B. burgdorferi*.

monocyte

B. burgdorferi

↑ uPA, uPAR, PAI-1, PAI-2

PLG

uPAR uPA PAI-2

uPA

uPA PLG

PAI-2

ECM

Distant organs
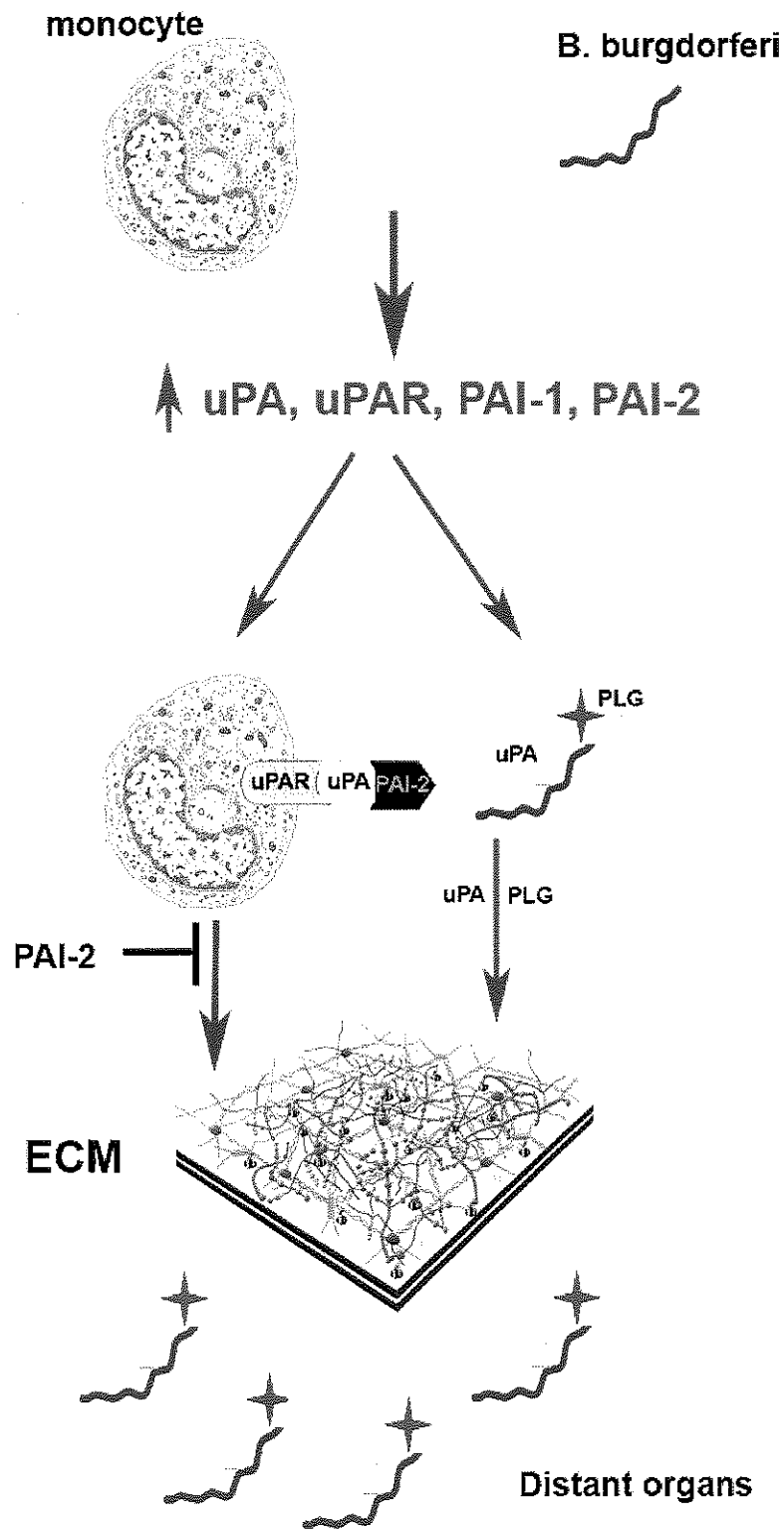
Future directions:

Previous works with other bacteria showed that uPA is not required for the infection of *Pseudomonas aeruginosa* (Gyetko *et al.*, 2000). However, our *in vitro* data show that uPA binds to the surface of the spirochetes and renders them invasive through the ECM. Thus it would be important to test the *in vivo* roles of uPA in Borrelia infection using uPA-/- animal models.

The role of PAIs also needs to be investigated further. Previous work has shown that PAI-1 does not play a role in the uPAR mediated recruitment of inflammatory leukocytes in *Pneumococcal pneumonia* (Rijneveld *et al.*, 2003). Our *in vitro* data suggest that PAI-2 is involved in the invasion of monocytic cells across reconstituted basement membrane. This may mean that PAI-2 and not PAI-1 is important in inflammatory cell recruitment, an observation supported by previous work (Nakamura *et al.*, 1992). Moreover, the relevance of these inhibitors may depend on the infecting pathogen and the course of infection it follows. Using PAI-2-/- mice one could study the exact role of this inhibitor in the infection of Borrelia.

Our explanation for the failure of uPAR-/- mice to show any phenotypic difference from the WT is that we performed our experiments after long-term Borrelia infection that would allow for other adhesion and migration molecules to be utilized. Short-term infections (4 h and 24 h) should be used to see the effects of uPAR upon short time inflammatory migration of leukocytes. The induction of PAI-2 was also offered as an additional explanation for the ineffectiveness of uPAR in Borrelia infection. This can be addressed using double knockout mice, deficient in both uPAR and PAI-2. If PAI-2 is indeed rendering uPAR inefficient in the recruitment of inflammatory cells, uPAR+/+

mice on a PAI-/- background will show increased invasion of leukocytes compared to the uPAR-/- on PAI-2-/- background revealing the importance of uPAR.

Based on our data, we propose that PLG and uPA are in close proximity to each other allowing uPA to activate PLG. Thus, it is important to perform further experiments to identify uPA binding protein(s) on the surface of Borrelia. Moreover, further studies should be done to address the biological explanation for the resistance of Borrelia-bound uPA to PAI-2 inhibition. Binding assays could be carried out to see if there is a binding competition between PAI-2 and bacterial surface proteins for binding sites on uPA.

In conclusion, this dissertation addressed the multiple involvements of the mammalian PAS in infection by *B. burgdorferi*. We show the complex nature of the interaction between the PAS and *B. burgdorferi* in that *B. burgdorferi* not only upregulates uPA and its receptor uPAR, but also the physiological inhibitors PAI-1 and PAI-2. We also show that the kinetics of the induction of these proteins plays a role in the overall effects of the PAS on both the dissemination of the *B. burgdorferi* and the inflammatory response of the host.

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