Proteases
in
Staphylococcal Arthritis

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Staphylococcus aureus (S. aureus) is a ubiquitous bacterium. Due to an increasing prevalence of immunodeficiency states in the world population and the emergence of antibiotic resistant strains, the incidence of S. aureus septicaemia and its complications is increasing worldwide, as is the mortality thereof. S. aureus has an impressive capacity to adjust to the environment since it has developed a multitude of invasive and evasive mechanisms to cope with host defence. Many studies have been performed mapping S. aureus virulence determinants with the aim of improving our ability to combat S. aureus infections. So far, little is known concerning the impact of S. aureus extracellular proteases on virulence.

The aim of the first part of this thesis was to investigate the role of bacterial extracellular proteases as potential virulence factors in S. aureus induced septic arthritis. Inherent to this goal was to investigate whether there is a specific immune response to S. aureus extracellular proteases and if so, whether protease specific antibodies have any inhibitory function on the protease activity and thereby modulating the biological properties of the bacteria. The second part of this thesis aimed at shedding light on the impact of a host protease, matrix metalloproteinase 9 (MMP-9) on the development of septic arthritis.

S. aureus strain 8325-4 with a known high production of extracellular proteases and its mutants lacking extracellular proteases, Aur’, Ssp’ and SspB’ were compared concerning their capacity to induce arthritis and to prevail in kidneys and joints. Total serum levels of IgG and IgM were measured, as were antibodies specific for the deleted proteases. Silencing of the ssp, aur, or sspB genes did not affect the clinical nor the histological course of septic arthritis. Polyclonal B-cell activation was illustrated by auto-antibody production and a 10-fold increase in total IgG and a 50% increase in total IgM. Specific antibody response was demonstrated since only mice infected with bacteria expressing SspB and V8 responded with anti-protease specific antibody production. Functional capacity of the specific antibodies was illustrated by the fact that the V8 protease antibodies inhibited the activity of the enzyme in vitro.

To study the production of host protease MMP-9 over time, zymographic analyses were performed of spleen homogenates at different time points after bacterial inoculation. At day 9 after inoculation there was a four-fold increase of MMP-9 expression. To further investigate the role of MMP-9 in infectious arthritis, MMP-9 KO mice and their littermates C57Bl/6 wt mice, were inoculated i.v. with S. aureus. MMP-9 deficient mice showed more clinical arthritis and thrived less well. Importantly, the MMP-9 KO mice harboured significantly more bacteria in kidneys and joints than did their congenic controls, indicating MMP-9 as an indispensable molecule in the clearance of the infective agent.

Altogether this thesis shows that S. aureus proteases can evoke a specific immune response in the host, but that they are not essential in mediating bacterial arthritis. Furthermore, host MMP-9 contributes to innate immune responses during septic arthritis.

Keywords: Staphylococcus aureus, septic arthritis, proteases, matrix metalloproteinases
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.


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<td>Ab</td>
<td>antibody</td>
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<td>AbIA</td>
<td>Ab-induced arthritis</td>
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<td>agr</td>
<td>accessory gene regulator</td>
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<td>Aur</td>
<td>aureolysin</td>
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<td>CFU</td>
<td>colony-forming units</td>
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<td>CGD</td>
<td>chronic granulomatous disease</td>
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<td>CHIPS</td>
<td>chemotaxis inhibitory protein of staphylococci</td>
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<td>CIA</td>
<td>collagen induced arthritis</td>
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<td>CP</td>
<td>capsular polysaccharide</td>
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<td>CTAP-III</td>
<td>connective tissue activating peptide-III</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<td>EfbP</td>
<td>extracellular fibrinogen binding protein</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<tr>
<td>fMLP</td>
<td>formyl-methionine-leucine-phenylalanine</td>
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<tr>
<td>Fn-Bp</td>
<td>fibronectin binding protein</td>
</tr>
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<td>GBS</td>
<td>group B streptococcus</td>
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<td>GCSF</td>
<td>granulocyte-colony-stimulating-factor</td>
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<td>GRO-α</td>
<td>growth-related oncogene-α</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>Hsp</td>
<td>helicobacter surface protein</td>
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<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
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<td>IFN</td>
<td>interferon</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>IL</td>
<td>interleukin</td>
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<tr>
<td>kD</td>
<td>kilo Dalton</td>
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<td>KO</td>
<td>knock-out</td>
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<tr>
<td>LFA-1</td>
<td>Lymphocyte Function-Associated Antigen-1</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>LRP-1</td>
<td>lipoprotein receptor-related protein-1</td>
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<tr>
<td>Mac-1</td>
<td>leukocyte integrin CD11b/CD18</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MC</td>
<td>monocyte</td>
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<tr>
<td>MHCII</td>
<td>major histocompatibility complex class II</td>
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<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MRSAs</td>
<td>methicillin resistant <em>S. aureus</em></td>
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<td>MSA</td>
<td>murine serum albumin</td>
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<tr>
<td>MT-MMP</td>
<td>membrane-type MMP</td>
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<tr>
<td>NGAL</td>
<td>neutrophil gelatinase B-associated lipocalin</td>
</tr>
<tr>
<td>NMRI</td>
<td>Naval Medical Research Institute</td>
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<tr>
<td>OA</td>
<td>osteoarthritis</td>
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<tr>
<td>OG-domain</td>
<td><em>O</em>-glycosylated domain</td>
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<tr>
<td>PAMPs</td>
<td>pathogen associated molecular patterns</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PECAM-1</td>
<td>platelet/endothelial cell adhesion molecule-1</td>
</tr>
<tr>
<td>PF-4</td>
<td>platelet factor-4</td>
</tr>
<tr>
<td>PG</td>
<td>peptidoglycan</td>
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PGE2-cAMP  prostanoidE2-cyclicAMP
PKC  protein kinase C
PLG  plasminogen
PMNC  polymorph nuclear cell
RA  rheumatoid arthritis
rmMMP-9  recombinant murine MMP-9
Sag  superantigen
SAK  staphylokinase
sarA  staphylococcal accessory regulator
SCIN  staphylococcal complement inhibitor
scp  staphylococcal cysteine protease operon
ScpA  staphopain A
SDF-1  stromal-cell derived factor-1
SEA  staphylococcal enterotoxin A
spl  serine protease like proteins operon
ssp  staphylococcal serine protease operon
SspB  staphopain B
SspC  staphostatin C
TGF-β  transforming growth factor β
TIMP  tissue inhibitor of metalloproteinase
TNF-α  tumour necrosis factor-alfa
TSST-1  toxic shock syndrome toxin-1
V8, SspA  staphylococcal serine protease
VCAM-1  vascular cell adhesion molecule 1
VLA-4  very late antigen 4
VRSA  vancomycin resistant S. aureus
wt  wild-type
INTRODUCTION

Classification of proteases

Proteases are essential for the expression of all proteins, controlling protein composition, size, shape, turnover and ultimate destruction. Their actions are often exquisitely selective, each protease being responsible for splitting a very specific sequence of amino acids under a given set of environmental conditions. There are over 500 human proteases and proteases also occur in other vertebrates, as well as in plants, insects, marine organisms and in all microbes. Genes coding for proteases account for about 2% of the human genome and 1-5% of genomes of bacteria and viruses. Proteases play a central role in conception and birth, life, ageing, and death. An over- or under- abundance of a particular protease or altered levels of natural inhibitors/activators of proteases can lead to pathological conditions. Proteases are also essential in viruses, bacteria and parasites since they participate in their replication, spread and maintenance of infectious diseases within the human or animal hosts. Due to these factors proteases represent important potential targets for medical intervention [1].

The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) recommends that the term peptidase should be used for any enzyme that hydrolyses peptide bonds. Peptidases are recommended to be further divided into exopeptidases that act only near a terminus of a polypeptide chain and endopeptidases that act internally in polypeptide chains. Previously the term peptidase was restricted to the group of enzymes now denominated exopeptidases and therefore the original term protease is used in this thesis as an overall term including exo- and endo-peptidases. It should be noted that when speaking of bacterial enzymes the term exoprotease is commonly used for extracellular proteases although these enzymes according to the functional classification all are endopeptidases. Synonymously with endoprotease the term proteinase is used. The proteinases have so far been categorized into six catalytical classes according to the chemical group within the enzyme that participates in the cleavage of the substrate peptide bond. A peptide bond can be broken directly by amide hydrolysis in which a water molecule is added, as is the case with glutamic-, aspartic-, and metallo-proteinases. In the case of serine-, cysteine-, and threonine-proteinases the polarized amino acid is made nucleophilic, attacks the peptide carbonyl group in the substrate and forms an intermediate before the final hydrolysis. The enzymes studied in this thesis are all endoproteases and belong to the subclass families of cysteine proteinases, serine proteinases and metalloproteinases [2].
**Cysteine proteinases**

Papain originating from papaya is the best studied of all cysteine proteinases. It has been used for degrading meat fibres for thousands of years. Papain is also capable to cleave the Fc (crystallisable) portion from the Fab (antigen-binding) portion of immunoglobulins. Several mammalian intracellular proteinases like cathepsins B, L, K, and S belong to this family, as do caspases and calpains. Some parasitic proteinases and bacterial extracellular proteinases like the staphylococcal enzymes staphopain A (ScpA) and staphopain B (SspB) are also cysteine proteinases.

**Serine proteinases**

This class comprises two distinct families. The chymotrypsin family includes the digestive pancreatic enzymes such as chymotrypsin, trypsin and elastase and also other mammalian enzymes like plasmin, furin, and cathepsin G. Subtilisin is a serine protease in prokaryotes. Subtilisin is evolutionary unrelated to the chymotrypsin-families, but shares the same catalytic mechanism utilising a catalytic triad, to create a nucleophilic serine. This is the classic example used to illustrate convergent evolution, since the same mechanism evolved twice independently during evolution. The subtilisin family includes the bacterial enzymes such as subtilisin and staphylococcal serine protease (V8, SspA).

**Metalloproteinases**

The metalloproteinases exert their effect mainly extracellularly and at neutral pH, as do most of the serine proteinases. The metalloproteinases may be one of the phylogenetically older classes of proteinases and are found in bacteria, fungi as well as in all higher organisms. They differ widely in their sequence and their structure but have some highly conserved domains. The great majority of enzymes contain a zinc atom that is catalytically active. In some cases, zinc may be replaced by another metal such as cobolt or nickel without loss of the enzymatic activity. The metal ion usually serves to coordinate two to four side chains and is indispensable for the activity of the enzyme. Many of these enzymes are calcium dependent in that calcium is required for maintaining the molecule's conformation. Both the staphylococcal aureolysin (Aur) and the large group of mammalian matrix metalloproteinases (MMPs) are referred to this class of proteases.
Bacteremia and sepsis

Worldwide, bacteremia and its consequences constitute a great health problem. The frequency of bacteremia and sepsis is increasing, as is the mortality thereof. This is explained by the combination of the emergence of multiresistant bacterial strains and the growing cohort of immunocompromised individuals, disease-related (AIDS) or induced by cytotoxic treatment (e.g. in autoimmune and tumour diseases), an increasing use of prosthetic devices and indwelling catheters along with the ageing world population. A consensus conference defined sepsis as "the systemic inflammatory response syndrome that occurs during infection" [3]. The prevailing theory has been that sepsis represents an uncontrolled inflammatory response. Lewis Thomas popularized this notion when he in the seventies wrote: “The micro organisms that seem to have it in for us turn out to be rather more like bystanders. It is our response to their presence that makes the disease. Indeed, our arsenal for fighting bacteria is so powerful that we are more in danger from it than the invaders” [4]. However, sepsis may not be attributable solely to an “immune system gone haywire” but rather indicate an immune system that is severely compromised and unable to eradicate pathogens. Thus, efficient therapy should be directed at modulating the patient’s immune response, depending on genetic polymorphisms, the duration of disease, and the characteristics of the particular pathogen rather than providing antibiotics alone [5].

In the US, a nation wide study showed an overall hospital mortality rate in sepsis of 29 %, accounting for 215,000 deaths nationally per year, corresponding to almost 10% of all deaths and equalling the number of deaths following acute myocardial infarction [6]. A similar mortality rate was found in Great Britain in a study performed between 1997 and 2003. In this study it also was found that rates of S. aureus bacteraemia rose during the period [7]. In Australia more than 1,700 deaths are estimated to be associated with S. aureus bacteremia per year [8]. A European observational study documented sepsis in European intensive care units with mortality rates being 27%, rising to 50% mortality in patients with septic shock. Cultures were positive in 60% of patients with sepsis and S. aureus was the most common organism found [9]. The incidence of S. aureus sepsis, especially of methicillin resistant S. aureus (MRSA) and vancomycin resistant S. aureus (VRSA) has so far been lower in Scandinavia than in Great Britain, Australia and the USA, but the incidence is increasing and if one extrapolates Finnish data [10] to Sweden, the estimate would leave Sweden with approximately 400 deaths per year due to S. aureus bacteremia. The severe complications of S. aureus bacteremia include endocarditis, osteomyelitis, and septic arthritis.
Septic arthritis in man

The patient with septic arthritis usually presents with fever, malaise, and a swollen, painful medium sized or big joint with limited range of movement. The overall estimated incidence of acute septic arthritis in industrialized countries is about six cases per 100,000 in the general population and 60-70 cases per 100,000 per year among patients with rheumatoid arthritis (RA) or with prosthetic devices [11]. The majority of the bacterial arthritides are caused by gram-positive (G+) cocci whereof *S. aureus* is the most prevalent. In a French study of septic arthritis including all age groups, 38% of positive cultures consisted of *S. aureus* [12] and in Israel *S. aureus* was the most common pathogen isolated in a patient cohort with septic arthritis, making up 40% of all positive cultures [13]. *S. aureus* is also the microorganism that gives rise to the most severe sequelae. A retrospective study in Taiwan concerning septic arthritis in children showed that *S. aureus* was the most common etiologic agent (59.0%) and for almost 30% of the children the infection resulted in the development of sequelae including motion disability, limb-length discrepancy, chronic osteomyelitis, and abnormalities of bone growth [14]. Well-known risk factors for septic arthritis, in addition to a pre-existing joint disease, are infected skin ulcerations, psoriasis, eczema, diabetes mellitus, HIV infection and different immunosuppressive states. From the primary focus of infection the bacteria are spread to the blood stream [15] from where they extravasate to the joint. The infectious arthritis proceeds within days to a devastating destruction of cartilage and bone. This destruction takes place even if the bacteria have been successfully eliminated with antibiotics and irrigation. The explanation for this is the exaggerated host-response in which the inflammatory cascade releases harmful products. Thus early intervention with an antibiotic to which the organism is sensitive is not sufficient and vast efforts are being made to explore the intricate interplay between the invader and the host and the mechanisms by which the arthritis prevails with the ultimate goal of finding more efficient ways of treatment. Infectious arthritis is for obvious reasons, not readily studied and manipulated in humans. Therefore murine models of septic arthritis constitute valuable means for increasing our knowledge of the arthritic process.

Septic arthritis in mice

The experimental staphylococcal arthritis model mimics the spontaneously occasionally occurring bacterial arthritis that can be seen in mice [16, 17]. Circulating staphylococci disseminate to virtually all tissues but in less than a week they are cleared from the body with the great exceptions of kidneys and joints. The reasons for bacterial persistence in the joints
are mainly thought to be a relatively impaired extravasation of immune cells and plasma proteins due to the non vascularized hyaline cartilage mass in combination with tissue tropism mediated by bacterial adhesins that interact with ligands densely expressed in the joint cavity [18]. The characteristics of the murine model closely mirrors changes seen in human septic arthritis, especially with regard to periarticular bone erosivity [19]. Virulence factors, i.e. bacterial products that contribute either directly to the pathogenesis of the infectious disease or indirectly by triggering detrimental host response, have been thoroughly studied in the case of *S. aureus* induced arthritis [20].

**Staphylococci and immunity**

**Innate immunity**

One of the very first steps in the infection cascade of events is the activation of the complement system, which leads to the binding of C3 convertase (complexes C4b2a and C3bBb) to the bacterial surface [21]. The C3 convertase cleaves C3 and thereby initiates the opsonization of the bacteria with C3b and the release of the chemo-attractant C3a [22]. C3 convertase also takes part in activating C5 convertase to generate C5b and C5a [23]. The small chemo-attractants C3a, C5a and bacterial formylated peptides trigger neutrophil diapedesis, migration through the blood vessel wall, but also exert chemotaxis that is direct the movement of neutrophils to the site of infection in the tissue [24]. The protective role of the complement system against *S. aureus* has been attributed to its combined action on neutrophil migration and phagocytosis [25]. Phagocytosis, the uptake of staphylococci by neutrophils, is largely mediated by the binding of neutrophil receptors, i.e. complement receptor 1, 3, and the Fc receptor to opsonic molecules covering the bacterium (C3b, inactivated C3b and antibodies) [26]. The crucial role of neutrophils in *S. aureus* infection was demonstrated by the finding that eradicating neutrophils resulted in 100% septic mortality whereas all the control mice survived. Furthermore, neutropenic mice also showed a significantly increased arthritis frequency [27]. Staphylococci are finally killed in the phagolysosome by antimicrobial peptides and by oxygen radicals generated during the neutrophil respiratory burst [28]. During this process myeloperoxidase (MPO) released from azurophilic granules into the phagosome forms a powerful antimicrobial tool, shown by the fact that MPO-deficient mice display a severely reduced cytotoxic capacity towards bacteria and fungi [29]. MPO works in concert with NADPH oxidase to form the highly bactericidal hypochlorous acid which kills *S. aureus* [28, 30]. The role of NADPH oxidase is further
illustrated by the high susceptibility to staphylococcal infections among individuals suffering from chronic granulomatous disease (CGD) lacking functional NADPH oxidase [31]. During the oxidative burst, nitric oxide (NO), hydrogen peroxide (H₂O₂) and superoxide anion (O₂⁻) are formed, which are directly toxic to bacteria. In addition to this oxygen-dependent killing of bacteria, the innate immune system mediates oxygen-independent killing exerted both by anti-microbial peptides, so called defensins, and enzymes within the neutrophil phagolysosome. Defensins are cationic peptides that interact with the anionic components of bacterial cell membrane leading to depolarization, permeabilization, leakage of bacterial cell content and finally to bacterial death [32, 33].

Another important player in innate immunity is the coagulation system. Bacterial infection activates the coagulation cascade through the extrinsic pathway in which tissue factor and coagulation factor VII form a complex that through activation of factor IX and X activates thrombin which in turn converts fibrinogen to fibrin [34]. The formation of fibrin is considered as an attempt from the host to limit the spread of bacteria but in severe cases it may give rise to a fatal syndrome called disseminated intravascular coagulation (DIC). Infection can also trigger the coagulation cascade via the intrinsic pathway i.e. via the contact system. The contact system is composed of coagulation factor XI, XII, plasma kallikrein and the co-factor high molecular weight kininogen (HK). Processing of HK releases bradykinin (BK) which as a potent vasodilator is a key molecule in the development of sepsis induced hypovolemic shock [35]. Activation of the contact system at the surface of bacterial pathogens like *S. aureus* was recently found to result in further cleavage of HK resulting in a peptide with high capacity for bacterial killing [36], additionally pointing to the close relationship between coagulation and immune defence.

The cells of the innate immune system express germ-line encoded receptors that recognize pathogen associated molecular patterns (PAMPs), for example lipopolysaccharides (LPS) and peptidoglycans (PG). The macrophage mannose endocytic receptor recognizes PAMPs on the bacteria, mediates their uptake and delivery to lysosomes where they will be degraded, processed and presented be MHC molecules on the cell surface thereby constituting an important link between innate and acquired immunity.

**Acquired immunity**

Having gone through a staphylococcal infection does not usually render the patient any protection against reinfection. Approximately 25% of all healthy persons are symptom free *S.*
aureus carriers, and when hospitalized for any other reason they have an increased risk of developing nosocomial S. aureus septicaemia. Interestingly, their prognosis was found to be better than that of noncarriers. When comparing the antibody profiles for carriers and noncarriers toward S. aureus superantigens (see page 15), carriers were found to have high titers of neutralizing antibodies specific for those superantigens that were expressed by their colonizing strain. These results show that carriage status can confer strain-specific humoral immunity, which may contribute to protection during S. aureus septicaemia [37].

Studies in mice have failed to show beneficial effects of antibodies to capsular polysaccharide (CP) or whole cells of S. aureus for the protection against experimental septic endocarditis [38, 39]. However, if surface proteins expressed by staphylococci, like collagen adhesin, ClfA or truncated fibronectin adhesin are administered as recombinant proteins they will give rise to protective immunity in both systemic infection and arthritis [40-42].

CD4⁺ T cells invade the S. aureus infected joint after a few days, a majority expressing the Vβ11 TCR. These T cells respond to the staphylococcal superantigen TSST-1. Depletion experiments in mice of either the whole CD4⁺ cell compartment or just CD4⁺Vβ11⁺ cells clearly ameliorates both sepsis induced mortality and arthritis provoked by a TSST-1 positive S. aureus strain [43-45].

S. aureus’ strategies to evade host defence

The staphylococcus has developed a multitude of mechanisms to escape host defence. Its cell wall is resistant to lysozyme and it can survive within phagosomes. The bacteria’s own cell surface reduces the efficacy of host antimicrobial cationic peptides due to cell membrane phospholipids and cell wall teichoic and lipoteichoic acids that neutralize the cell surface negative charge [46]. A large number of human pathogens have evolved mechanisms of complement evasion and so has the staphylococcus as well. The staphylococcal extracellular fibrinogen binding protein (EfbP) inhibits complement-mediated opsonophagocytosis by blocking deposition of C3 on the surface of the microbe or by preventing further complement activation beyond C3b [47]. Staphylococcal complement inhibitor (SCIN) is found in 90% of clinical strains. It is a 10 kD protein that specifically interacts with bacterium-bound C3 convertase and thus efficiently prevents C3b deposition and phagocytosis [48]. Both SCIN and the chemotaxis inhibitory protein of staphylococci (CHIPS) are extracellular proteins, and in contrast to other staphylococcal extracellular proteins they are expressed early during growth, in exponential growth phase. CHIPS is produced by 65% of clinical S. aureus strains
and is a 14 kD protein that blocks neutrophil chemotaxis by binding the formylated peptide receptor and the C5a receptor on neutrophils [49-54]. Staphylokinase (SAK), a protein commonly released by *S. aureus*, activates plasminogen (PLG) to plasmin at the bacterial surface. This could create an ideal mechanism, from a microbe’s point of view, to modulate opsonization and thus increase bacterial survival. It has been demonstrated that SAK-activated PLG removes IgG as well as C3b from the bacterial surface, leading to impaired phagocytosis [55]. It was recently shown that SAK binds and counteracts the bactericidal effect of human defensins. A direct binding between staphylokinase and defensins was observed, leading to complex formation. The biological consequence of this interaction was an almost complete inhibition of the bactericidal effect of defensins [56]. *S. aureus* uses extracellular proteases to decrease its susceptibility to host antimicrobial peptide LL-37. Indeed, the secretion of aureolysin correlated well with the rate of LL-37 degradation monitored by mass spectroscopy analysis [57]. In *S. aureus*, 19 different superantigens (SAgs) have been described. SAgs bypass conventional antigen recognition by directly cross-linking major histocompatibility complex class II (MHCII) molecules on antigen-presenting cells with certain Vβ families within T cell receptors. This leads to massive T cell proliferation and cytokine release, which may end up in toxic shock syndrome [58]. Ultimately the balance between the host arsenal of defence measures and the invaders’ capacity to surpass them will decide whether the result will be an overt infection or not.

**Staphylococcal extracellular proteases**

Until today a handful of extracellular proteases from *S. aureus* have been described. Their short denominations emanate from the operon by which they are encoded and not from their function as do their longer names which makes the nomenclature somewhat confusing. The genes of extracellular proteases are organized on the bacterial chromosome into four distinct operons: staphylococcal serine protease (ssp) operon, serine protease like proteins (spl) operon, staphylococcal cysteine protease (scp) operon, and aureolysin (aur) operon. Thus the V8 protease (SspA) and staphopain B (SspB) both are named by their encoding ssp operon although only SspA is a serine protease whilst SspB is a cysteine protease [59].

**Serine protease V8 (SspA)**

V8 protease was first purified from culture filtrate of *S. aureus*, strain V8 by Drapeau et al in 1972. Their studies revealed that V8 protease exclusively cleaves peptide bonds on the carboxyl-terminal side of either aspartic acid or glutamic acid [60]. Since then V8 protease
has been widely used for determining the primary structures of proteins. The V8 protease does not have a high degree of sequence identity with other serine proteases and does not contain any disulphide bridges, but its tertiary structure was found to have significant structure homology with, for example the staphylococci epidermolytic toxins A and B [61].

**Metalloprotease aureolysin (Aur)**

The primary and tertiary structures of aureolysin have been determined revealing a polypeptide chain of 301 amino acids which is folded into a β-pleated N-terminal domain and an α-helical C-terminal domain, a typical fold for the thermolysin family of metalloproteinases [62]. The diverse family of bacterial metalloproteinases encompasses several enzymes, which are acknowledged virulence factors, including *Pseudomonas aeruginosa* elastase, *Legionella pneumophila* and *Listeria monocytogenes* metalloproteinases, *Vibrio cholerae* hemagglutinin protease, *Staphylococcus epidermidis* elastase, and the lambda toxin of *Clostridium perfringens*. In contrast to these proteinases, however, little is known about the exact role of aureolysin in the pathogenicity of *S. aureus*. In vitro, aureolysin has been shown to cleave the plasma proteinase inhibitors, α1-antichymo-trypsin and α1-proteinase inhibitor. Aureolysin cleaves the oxidized form of α1-proteinase inhibitor faster than it cleaves the native inhibitor, suggesting that bacteria which secrete these metalloproteinases may specifically take advantage of the host defense oxidative mechanism to accelerate elimination of α1-proteinase inhibitor and consequently increase tissue degradation by neutrophil elastase [63]. The structure of the aureolysin gene is strongly conserved among *S. aureus* strains. This argues in favor of the likelihood that the enzyme may have an important house keeping function [64].

**Cysteine proteases Staphopain B (SspB) and Staphopain A (ScpA)**

The cysteine proteases exhibit, with their papain like features, potent elastinolytic activity which has been considered to be of importance for bacterial spread within tissues and also for the forming of abscesses [65]. Kinin-releasing cysteine proteinases have been reported from various microbes. From *S. aureus* a submicromolar concentration of ScpA generated large amounts of kinin from human plasma within 5 minutes from exposure and in a guinea pig experimental model, ScpA in concert with SspB induced vascular leakage and lowering of blood pressure. Thus staphopain mediated vascular leakage was proposed to be involved in the oedema formation at the infected sites as well as in the induction of septic shock [66].
Regulation of *S. aureus* extracellular proteases

The transcription of most of the extracellular protease genes occurs maximally at post-exponential-phase, being positively regulated by the accessory gene regulator (agr) and negatively regulated by the staphylococcal accessory regulator (sarA). The enzymes are excreted as zymogens. Using mutations in each protease gene the proteolytic cascade of activation has been analyzed. Aureolysin catalyzes the activation of the SspA zymogen although it is not the sole agent capable of conducting this process. Activated SspA in turn activates the SspB pro-enzyme [59]. Aureolysin has not been shown to be capable to undergo auto-proteolysis to achieve activation. Thus it is still not proven what triggers the start of the activation of the extracellular enzymes. The regulation of protease activity is also under control already within the bacteria. Staphostatin C (SspC), the inhibitor of SspB, protects intracellular proteins against proteolytic damage by prematurely activated Staphopain B [67].

**Host matrix metalloproteinases (MMPs)**

MMP activity was first described in the tail of metamorphosing tadpoles, subsequently identified as interstitial collagenase (MMP-1) [68]. Since then, a total of 24 vertebrate MMPs have been described [69], all containing a zinc-binding catalytic domain. Collectively, MMPs are able to degrade all components of the extracellular matrix (ECM) and they share extensive substrate overlap. MMPs can be classified broadly by substrate specificity into collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, -11), elastases (MMP-7 and -12) and membrane-type MMPs (MT-MMPs, MMP-14, -15, -16 and -17), which are surface anchored by either a transmembrane domain or a glycosyl-phosphatidylinositol anchor. The catalytic domain is highly conserved and determines substrate specificity, which may be enhanced by the haemopexin-like domain [70].

**Modulation of active MMP availability**

MMPs are rarely stored but instead require increased gene transcription to drive secretion, the exceptions being neutrophil MMP-8, -9 and -25, which can be released instantly. MMPs are secreted as pro-enzymes that undergo proteolytic cleavage for activation, releasing a cysteine residue in the pro-peptide domain from the catalytic zinc, a process known as the cysteine switch [71]. After secretion, MMPs are compartmentalized in close proximity to the cell [72, 73]. MMP activity is regulated by secretion of specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), which bind in a non-covalent manner to the catalytic domain [74]. MMPs are also systemically, and unspecifically inhibited by the global protease
inhibitor α2-macroglobulin. The balance between matrix catabolism and anabolism is further complicated by the ability of MMPs to degrade non-specific protease inhibitors such as alfa-1 anti-proteinase [75, 76]. MMPs are secreted by inflammatory cells in response to both exogenous insults like bacterial lipopolysaccharide (LPS), peptidoglycan (PG) or helicobacter surface protein (Hsp) and inflammatory cytokines such as tumour necrosis factor-alfa (TNF-α) [77] and interleukin 1-beta (IL-1β) [78]. MMP secretion is down regulated by diverse cytokines including interferon-gamma (IFN-γ), IL-4 and IL-10. Cell contact dependent signaling may drive MMP up-regulation [79, 80]. Intracellularly, signal transduction pathways involve mitogen-activated protein kinase (MAPK) and prostaglandin E 2/cyclic AMP pathways [81, 82].

**The MMP-9 molecule and its domains**

The signal peptide contains 24 hydrophobic residues directing the MMP to the Golgi apparatus for secretion. The pro domain, located immediately after the signal peptide, comprises a free cysteine, which interacts with the essential Zn ion of the catalytic domain to block the active site and maintain the proteinase in an inactive state.

![Figure 1. Schematic drawing of the MMP-9 molecule and its domains.](image)

1. Signal peptide
2. Prodomain
3. Cysteine switch
4. Zn$^{2+}$ in active site
5. Fibronectin type II repeats
6. Catalytic domain
7. Glycosylated spacer region-“OG domain” (CollagenV-like domain)
8. Hemopexin domain

The amino acid sequences surrounding both the critical cysteine residue in the prodomain and the active site region containing the histidine zinc-binding ligands are highly conserved in all
of the MMPs. Latent MMPs can be activated in multiple ways, all of which result in the dissociation of the cysteine residue from the Zn-atom. The C-terminal hemopexin domain is preceded by a unique linker sequence of 64 amino acid residues often denoted as the collagenV domain. The collagen type V domain only has limited homology with collagen V. Van den Steen et al recently demonstrated that this sequence, which connects the active site and the hemopexin domain, is an extensively glycosylated domain with a compact three-dimensional structure and therefore proposed it to be named the O-glycosylated domain (OG-domain). The active site of MMP-9 can be inhibited by interaction with the N-terminal domain of the tissue inhibitors of metalloproteinases (TIMPs) [83]. In addition, the C-terminal part of TIMP-1 binds to the hemopexin domain of both the pro- and active MMP-9, leading to a high affinity interaction [84]. The OG domain was shown to influence the binding of TIMP-1 to the haemopexin domain, as the deletion of the OG domain resulted in 10-fold decreased affinity for TIMP-1 [84, 85]. The fibronectin type II module within the MMP-9 molecule is approximately forty residues long and has a cavity in the side of the module made up of aromatic and hydrophobic residues, in conjunction with a charged serine. It is thought to bind specific leucine and/or isoleucine sequences in collagen. MMP-2 and MMP-9 have three fibronectin type II repeats in the so-called fibronectin domain, which is responsible for their capacity to bind gelatin, laminin, and collagen type IV. Gelatinolysis by gelatinases is more than 100-fold enhanced by the gelatin-binding fibronectin domain [86].

**MMPs in neutrophils**

In many cell types, MMP-9 is produced as a mixture of monomers and homodimers and is often found in complex with TIMP-1. The bodies largest store of MMP-9 is in tertiary granules of neutrophils where it’s found as a heavily glycosylated protein. Approximately 85% of the glycans in natural MMP-9 are O-linked, and the remaining 15% are N-linked sugars [87]. The tertiary granules also contain MMP-8 and MMP-25. MMP-9 covalently complexed with neutrophil gelatinase B-associated lipocalin (NGAL) is found in secondary granules [88]. However, neutrophilic granulocytes do not harbour MMP-2 nor TIMP-1.

**Release of neutrophil granulae**

Several mediators have been shown to induce MMP-9 release from neutrophils including LPS, formyl-Met-Leu-Phe (fMLP), PG, IL-8, TNF and granulocyte-colony-stimulating-factor (GCSF). Unlike responses to other soluble stimuli, neutrophil responses to TNF are usually dependent on signalling by cell-surface integrins. TNF can however, induce MMP-9 release
from tertiary granule from human neutrophils also in the absence of beta 2-integrin signaling, and protein kinase C (PKC) isoforms seem to be critical in regulating MMP-9 release in response to TNF stimulation [89]. IL-8-mediated tertiary granule release is independent of intracellular calcium changes whereas primary and secondary neutrophil granule release depends on increased intracellular calcium.

Cell migration and MMPs

The proteolysis of matrix proteins can contribute to cell migration in several ways: (1) it can simply clear a path through the matrix; (2) it can expose cryptic sites on the cleaved proteins that promote cell binding, cell migration, or both; (3) it can promote cell detachment so that a cell can move onward, or (4) it can release extracellular signal proteins that stimulate cell migration. In mouse mammary carcinoma, MMP-9 was shown to degrade collagen IV and to activate the proform of transforming growth factor β (TGF-β). This observation was observed only if MMP-9 was bound to the cell surface by CD44 after which active TGF-β triggered neovascularization [90]. T cell and dendritic cell migration has been shown, in vitro, to be in part MMP-9-dependent [91-93]. In mice, MMP-9 activity is required for lymphocyte transmigration across high endothelial venules into lymph nodes [94].

Leukocyte extravasation, initiated by exposure to a local inflammatory trigger, starts with the low affinity cell-cell contact between the leukocytes and the endothelial cells and is mediated by selectins. Subsequently, leukocytes cease to roll along the endothelium and start to adhere more firmly through integrins, including VLA-4, LFA-1 and Mac-1. An irreversible process starts whereby leukocytes migrate through the endothelial membrane. This involves integrins and cell adhesion molecules of the Ig superfamily, such as ICAM-1, VCAM-1, and PECAM-1. Cellular invasion depends on the cooperation between adhesive and proteolytic mechanisms. To move through the ECM, cells must first adhere to it via cell-ECM contacts. The first primordial contacts are highly labile, present at the cell front heading in the direction of migration, and involve anchoring onto the basement membrane collagen type IV constituent. These contents are rapidly remodeled through the specific degradation of collagen type IV by the proteolytic action of enzymes, such as MMP-2 and MMP-9. Migrating cells subsequently establish new focal contacts with the fibronectin of the ECM. Both MMP-2 and MMP-9 are actively involved in this cell migratory process [95] and co-localize with the β1 integrin that is incorporated into focal contacts [96].

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MMPs in inflammation and infection

MMPs are involved in many steps of defence against infectious organisms, ranging from the recruitment of leukocytes to the site of infection, breaking down extracellular matrix, modulating cytokines and chemokines and participating in the final resolution of inflammation and healing of tissue. One of the important MMPs is MMP-9 as briefly presented in figure 2.

**Figure 2.** Schematic drawing on the triggering factors for MMP-9 synthesis/release and major functions of MMP-9.

The migration of immune cells to sites of inflammation from the blood stream requires proteolysis of the basement membrane being mediated by MMP-9. In addition to opening a path through the ECM for cell migration, MMPs modulate the chemokine and cytokine gradients that drive inflammatory cell recruitment. MMP-1, -2, -3, -7, -9 and -12 can all
release active TNF-α from the membrane-anchored precursor by a similar mechanism to that of TNF-α converting enzyme [97]. MMP-9 plays an important role in chemotaxis by truncating IL-8 to a ten-fold more potent chemoattractant molecule [98]. MMPs can also both activate pro-IL-1β proteolytically and cleave the activated form of IL-1β to an inactive form, thereby providing both positive and negative regulation [99, 100]. MMP-9 is capable of degrading the chemokines GROα and PF-4 [101], providing a negative feedback loop that dampens inflammatory cell influx and helps resolution of inflammation.

**MMP-9 and endotoxin shock**

LPS is known to stimulate neutrophils to MMP-9 secretion directly and indirectly through cytokine induction [102] and to up-regulate MMP-1, -7 and -9 production and secretion by monocytes and macrophages [103-105]. LPS has been found to stimulate increased production of MMP-9 through a PGE2-cAMP-dependent pathway and it can also regulate gene transcription of MMP-9 by modifying mRNA stabilization.

The inflammatory cascade may be exacerbated by the catecholamines released in septic shock amplifying monocyte MMP-9 production [106]. LPS endotoxaemia in mice induces multiple MMPs and causes increased gelatinolytic activity in affected organs [107]. In patients with endotoxic shock, the serum levels of MMP-9 parallel the severity of illness [108]. Increased MMP-9 levels are found in bronchoalveolar lavage fluid from patients with the acute respiratory distress syndrome, one of the main complications of endotoxaemia [109]. The functional importance of MMP-9 activity in the case of endotoxin shock is suggested by the observation that MMP-9-deficient mice have reduced mortality in endotoxin shock compared to wild-type animals [110]. Although MMP-9 in many cases act as a tuner of immune responses in favour of the host, in the case of endotoxic shock it appears to exacerbate pathology [111].

**MMP-9 and Gram-positive bacteria**

Staphylococcal PG was shown to significantly increase MMP-9 levels in plasma, lung and liver in the rat within one hour after the exposure [112]. Addition of PG to whole human blood caused enhanced levels of MMP-9 after 1 h of incubation assay. In neutrophil cultures, MMP-9 values increased significantly after 30 min of incubation with peptidoglycan, whereas muramyl dipeptide had no effect. The total MMP-9 values (the sum of intracellular and secreted MMP-9) before and after stimulation were mainly unaltered, reflecting the fact that the MMP-9 that was measured emanated from the prestored pool in neutrophils [113].
Bacterial proteases and MMPs

Proteases of the thermolysin family secreted by *Pseudomonas aeruginosa* and *Vibrio cholera* activate pro-MMP-1, -8 and –9 [114]. Serine proteases associated with LPS preparations also activate MMP-9 [115], while proteases from the oral pathogen *Porphyromonas gingivalis* activate MMP-1, -3 and –9 [116]. *Streptococcus pneumoniae*, the major cause of severe pneumonia, secretes a zinc metalloproteinase ZmpC, which cleaves MMP-9 in vitro.

MMPs in animal models of arthritis

It was long proposed that stromelysin-1 (MMP-3) is one of the major degradative matrix metalloproteinases responsible for the loss of cartilage in rheumatoid arthritis (RA) and osteoarthritis (OA). This hypothesis was tested on MMP-3 deficient mice in a model of collagen induced arthritis (CIA) but the disruption of the MMP-3 gene neither prevented nor reduced the cartilage destruction associated with CIA [117]. The impact of MMP-2 and MMP-9 on arthritis was tested in a model of Ab-induced arthritis (AbIA) in which MMP-2 deficiency was shown to exacerbate arthritis while the MMP-9 deficient mice showed reduced arthritis indicating a role for the MMP-9 molecule in the progression of arthritis. However, cartilage erosion is mild in AbIA, even in severely arthritic mice and therefore no conclusions concerning the role of MMP-2 and MMP-9 in cartilage erosion could be drawn [118]. The significance of MMP-7 has been studied in the model of *S. aureus* induced septic arthritis with rapidly progressing destruction of joint cartilage and periarticular bone. MMP-7 wild-type mice showed a more destructive course of septic arthritis than the knock-outs (KO) despite the fact that they were more successful at clearing bacteria [119].

Considerable circumstantial evidence implicates MMPs in the pathogenesis of arthritis, but more clues are needed to “convict” individual enzymes. In order to identify specific MMP targets for joint protective therapy, the role of each MMP in the pathogenesis of arthritis needs to be determined.
AIMS OF THE STUDY

• To investigate the role of bacterial extracellular proteases as potential virulence factors in *S. aureus* induced septic arthritis

• To examine whether there is a specific antibody response to *S. aureus* extra-cellular proteases in addition to the polyclonal antibody response during *S. aureus* infection

• And if so, whether these antibodies have any inhibitory function on the protease activity and thereby might modulate the biological properties of the bacterial products

• To explore the impact of MMP-9 expression on joint inflammation and destruction in a model of bacterially induced septic arthritis
MATERIALS AND METHODS

Mice

For the first and second paper, as well as for the intra-articular experiments in the third paper, female NMRI mice 5–8 weeks old were obtained from B&K Universal AB (Sollentuna, Sweden). For in vitro assays with spleen cells and macrophages, in the third study, C57Bl6 (B&K) were used.

MMP-9 knock-out mice

The mice in the in vivo experiments in the third study were MMP-9 KO mice, constructed and bred in the lab of prof. G Opdenakker, Belgium. A targeting vector was constructed with the herpes simplex virus thymidine kinase gene in front of the mouse MMP-9 gene in which the exons and corresponding introns 3-7 and about half of exon 8 were deleted and replaced by the neomycin resistance gene. The resulting targeting vector contained 10,7 kbp of the mouse MMP-9 gene in which most of the catalytic domain (exons 3-4) and the zinc-binding domain (exon 8) are deleted. The latter 2 domains are essential for enzymatic activity, and homologous recombination should result in complete lack of MMP-9 activity in homozygote knockout animals. The result of the knock-out was confirmed by Southern blot, PCR and zymography, showing a complete absence of both the gene and the protease activity of MMP-9 [120].

The mice were kept in the animal facility of the Department of Rheumatology and Inflammation Research. They were housed 10 per cage under standard conditions of temperature and light. They were fed standard chow and water ad libitum.

Bacterial strains

In the first study, in which the aim was to answer the question whether staphylococcal proteases act as virulence factors, we used the staphylococcal strain 8325-4, known for its high production of extracellular proteases, and it’s mutants devoid of certain proteases, we used wild-type *S. aureus* 8325-4 and three of its mutants. The mutant AK1, the *ssp*² strain, lacks both the V8 protease (SspA, a serine protease) and staphopain B (SspB, a cysteine protease). AK2, the *aur*² strain, is negative for the metalloprotease aureolysin whereas the *sspB*– mutant lacks expression of staphopain B. *S. aureus* 8325-4 has a deletion in the rsbU gene, the sigB activator protein, resulting in an increased level of protease production [121]. The *aur*² and *ssp*² mutants were constructed by insertional inactivation technique as previously
described [122], as was the sspB\textsuperscript{−} mutant [59]. The bacterial strains were cultured on blood agar plates for 24 h and then reincubated on blood agar for another 24 h. Thereafter, bacteria were harvested and kept frozen at −20°C in phosphate-buffered saline (PBS) containing 5% w/v bovine serum albumin and 10% v/v dimethyl sulfoxide until use.

In the second study, in addition to \textit{S. aureus} 8325-4 and its mutants, we used \textit{Streptococcus agalactiae}, group B streptococcus (GBS) strain 6313, a clinical isolate belonging to serotype III for comparison. The streptococci were grown in Todd-Hewitt broth, washed, resuspended and kept frozen in the same manner as the staphylococci.

In the third study the arthritogenic strain \textit{S. aureus} LS-1 was used to induce haematogenously spread arthritis as well as local arthritis of the knee joint. The bacteria were cultured and prepared according to the same protocol as for the \textit{S. aureus} 8325-4.

**Bacterial proteases and superantigens**

Aureolysin, the V8 protease, and mature staphopains A (ScpA) and B (SspB) were purified from \textit{S. aureus} by ammonium sulphate precipitation, followed by combination of hydrophobic and ion-exchange chromatography as previously described [65, 123-125]. Prostaphopain B (proSspB) was expressed in \textit{E. coli} and purified as described before [126]. Toxic Shock Syndrome Toxin-1 (TSST-1) was obtained from Toxin Technology (Sarasota, FL), and Staphylococcal Enterotoxin A (SEA) was obtained from Sigma, (St. Louis, Mo).

**Experimental protocol**

The arthritis model encompasses haematogenously spread of the \textit{S. aureus} bacteria to the joints, followed by a destructive course of the disease in situ. Before i.v. administration, the bacterial solutions were thawed and washed in PBS. For i.v injections each mouse was injected with 0.2 ml bacterial suspension in the tail vein, corresponding to 4x10\textsuperscript{7} or 3 x10\textsuperscript{7} (the latter amount in the experiment with SspB\textsuperscript{−}) bacteria. After each inoculation procedure, viable counts were performed to check the actual number of administered bacteria. The mice were sacrificed on day 14, on which sera were obtained and kept frozen at −20°C until analyze. In each experiment, the mice were coded to ensure that the observer was blinded. The mice were weighed and evaluated for signs of arthritis regularly after inoculation. At day 14, they were sacrificed.
Clinical evaluation of arthritis

All four limbs of each mouse were inspected visually at day 3, 6–7, 10–11, and 14. Arthritis was defined as erythema and/or swelling of a joint. For each group, the frequencies of synovitis and destruction of cartilage and/or bone, was reported. To evaluate the intensity of arthritis, a clinical scoring system of 0–3 for each limb was used. Score 1 represents mild swelling and/or erythema, 2 moderate swelling, and 3 marked swelling. The arthritic index was constructed by adding the scores from all four limbs in each animal.

Histological examination

In three of the experiments, all four limbs of each mouse were fixed in paraformaldehyde, decalcified and paraffin embedded. In the fourth experiment, one front paw and one hind paw were prepared for histological analysis, while the other two paws were used for bacterial culture. Tissue sections were stained with haematoxylin and eosin. All sections were coded before examination. Preparations were evaluated with respect to synovitis and destruction of cartilage and/or bone. The number of mice with synovitis and cartilage/bone destruction was reported as a frequency for each group. Synovitis was also graded, where 1 represents mild synovial hypertrophy with more than two cell layers, 2 represents moderate hypertrophy with infiltration of inflammatory cells, and 3 marked hypertrophy and abundance of inflammatory cells. As different amounts of joints where visible, depending on the section, the synovitis score was counted per judgeable joint in each animal.

Bacteriological examination of infected animals

The kidneys were aseptically removed, and both kidneys from each mouse were pooled, homogenized, diluted serially in PBS and transferred to agar plates containing 5% v/v horse blood. Bacteria were grown for 24 h and then counted as colony-forming units (CFU). When joints were checked for bacterial growth, the talocrural and the radiocarpal joints were first dissected aseptically. Thereafter bacterial samples were collected with charcoaled sticks and cultured on horse blood agar plates for 48 h before counting.

In vitro stimulation of splenocytes

Spleens were aseptically teased with forceps and passed through a nylon sieve. The cells were suspended in PBS and centrifuged at 515xg for 5 min. The pelleted cells were resuspended for 10 min in Tris-buffered 0.83% ammonium chloride to lyse erythrocytes. After two washings in PBS, the mononuclear spleen cells were counted. The cells were incubated at a
concentration of 1x10⁶ cells/well in 24-well flat-bottomed microtitre plates in 500 µl medium at 37°C in 5% CO₂ and 95% humidity. The culture medium used was Iscove’s medium supplemented with 10% foetal calf serum, 2 mM glutamine, 50 µg/ml gentamycin and 50 µM mercaptoethanol (complete medium). Either of the following mitogens or bacterial constituents were added as a stimulus to the cell culture; TSST-1 at a concentration of 2 µg/ml, Concanavalin A (ConA) 1.25 µg/ml, staphylococcal cell walls 1x10⁷/ml or staphylococcal PG 10 µg/ml. Supernatants from the stimulated cells were harvested following 24 and 48 h of culture for analysis. To assess proliferative responses spleen cells were incubated in triplicates with mitogens/bacterial constituents, as described above, in 96-well flat-bottomed microtitre plates and incubated for 72 h. During the final 18 h of culture, 1 µCi [³H] thymidine was included in each well. The cultures were harvested into glass fibre filters, processed and counted in a β-counter for assessment of proliferation responses.

**IL-6 analysis**

The cell line B13.29, which is dependent on interleukin 6 (IL-6) for growth, has been described previously [127, 128]. For IL-6 determinations, the more sensitive subclone B9 was used. B9 cells were harvested from tissue culture flasks, seeded into microtiter plates (Nunc, Roskilde, Denmark) at a concentration of 5000 cells/well, and cultured in Iscove’s medium supplemented with 5x10⁻⁵ M 2-mercaptoethanol, 10% FCS (Seralab, Sussex, UK), gentamycin (50 µg/ml) and L-glutamine. The samples were added for 72 h and [³H] thymidine was added 4 h prior to harvesting. Each sample was tested for IL-6 in a series of twofold dilutions and compared with a recombinant IL-6 standard. B9 cells do not react with recombinant cytokines such as IL-1a, IL-1b, IL-2, IL-3, IL-5, granulocyte-macrophage colony-stimulating factor, TNF-α, and γ-interferon and have only weak reactivity with IL-4.

**MMP extraction and analysis**

In the second paper NMRI mice were inoculated with 1x10⁷ colony-forming units (CFU) of *S. aureus* LS-1 and killed at days 4 and 9 for zymographic analysis of MMP-9 in spleens. Proteins were extracted from the spleens and analyzed using gelatin substrate gel sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) zymography as previously described [129]. Briefly, spleens were thawed in a 1% saponin solution, homogenized and centrifuged to remove the insoluble material. Total concentration of soluble proteins was determined using a protein assay kit (Bio-Rad). Twenty-five micrograms of soluble proteins

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from each sample was diluted with non-reducing sample buffer and the samples were separated in a 7.5% polyacrylamide gel containing 1 mg/ml of gelatin. One nanogram of recombinant murine MMP-9 (rmMMP-9) (R&D systems) was used as a positive control. A separate essay was run with different amounts of rmMMP-9 to ascertain a linear relationship between concentration and measured intensity in the actual interval used in this study. Gels were subjected to electrophoresis at 120 V for 60-90 min and then incubated in 2.5% Triton X-100 Tris-buffered saline for 30 min followed by 20 h at 37°C in buffer containing 100 mM CaCl₂. Bands were visualized with 0.2% Coomassie blue and the intensity of each sample band determined (using Scion Image v4.0.2, Maryland, USA) relative to the rmMMP-9 band, and expressed as relative MMP-9 activity per 100 µg of protein.

Serological analyses

Total serum levels of IgG and IgM were measured by radial immunodiffusion [130]. Antisera and immunoglobulin standards were purchased from Sigma (St. Louis, Mo). Levels of IgG and IgM antibodies specific for Aur, V8 protease and staphopains (proSspB, SspB and ScpA) were estimated by an enzyme linked immunosorbent assay (ELISA). Microplates were coated with each bacterial protease diluted to 5µg/ml in PBS and left at 4°C over night. After washing with PBS, the plates were incubated for 30 min with 1% paraformaldehyde to abrogate the proteolytic activity of the enzymes. After another wash, non-specific binding sites on microplates were blocked with 0.1% BSA for 30 min. The sera, diluted 1:50 and 1:200 in PBS supplemented with 1%-BSA, were added in duplicates and plates were left over night at 4°C. Next day, the plates were washed and incubated with 100 µl/well of biotinylated goat anti-mouse antibodies of either IgG or IgM class, (purchased from Sigma, St. Louis, Mo) for 2 hours. After washing, plates were incubated with avidin-HRP (0.5 µg/ml) for one hour, washed again and incubated with H₂O₂ and chromogen (100 µl/well). The reaction was read at 405 nm using spectrophotometer (Spectramax, Molecular devices).

Test of inhibitory activity

In the second study, sera pooled from mice infected with wt S. aureus strain and the ssp⁻ and aur⁻ mutants were used. Immunoglobulin fraction was isolated from each pool by standard protein A affinity chromatography and dialyzed against PBS. In parallel, immunoglobulins from sera of mice infected with Streptococcus agalactiae strain 6313 were isolated by the same procedure. Isolated Ig from mice infected with S. aureus 8325-4 wt, aur⁻, ssp⁻ or with Streptococcus agalactiae was preincubated with the V8 protease (30 ng) for 30 min in 150 µl
PBS. Thereafter the residual activity of the protease was determined as the rate of hydrolysis of a fluorescent substrate (Z-Leu-Leu-Glu-7-amido-4-methylcoumarin, 60 µM final concentration.)

RESULTS AND DISCUSSION

Staphylococcal proteases and host responses

With respect to measures of general health such as survival and weight loss, there was no statistical difference between the groups of mice infected with wild-type S. aureus 8325-4 and either of the protease deficient mutants. Indeed, 80% of the mice that had received wild-type strain survived compared with 90% and 82%, respectively, for those inoculated with aur and ssp mutated strains. In the separate experiment with SspB, the survival was 75% in both the wild-type group and the cysteine mutant group. In both experiments the mice inoculated with wild-type bacteria showed almost the same maximum weight loss as those given mutants, and the mice in all groups regained weight in a similar pattern. In the light of latter findings that SepA and SspB together mediate vascular leakage and lowering of blood pressure [66] one could have expected a higher survival rate among the Ssp negative inoculated mice compared with wild-type. But the overall survival rate was high as the bacterial dose was titrated to induce arthritis rather than septic death. It is possible that using a higher bacterial dose, differences as to mortality caused by S. aureus strains differing in protease expression might become apparent.

In order to analyze the impact of proteases on bacterial persistence, bacterial growth in kidneys and joints was analyzed. We found that there was no significant difference regarding bacterial growth in kidneys and joints, irrespective of the inoculated strain. Several bacteria including S. epidermidis produce metalloproteinases being known virulence factors. This fact together with in vitro data showing that S. aureus metalloproteinase aureolysin can cleave plasma proteinase inhibitors [63], talks in favour of the hypothesis that aureolysin acts as a virulence factor. Our data could not prove this. However, the fact that cultures from radiocarpal and talocrural joints showed growth of the intravenously inoculated bacteria in 3/10 mice given wild-type strain and in none of the joints in the mouse groups given Aur⁻ mutants or Ssp⁻ mutants suggests that there might have been a significant difference in bacterial growth if the mice had been kept longer.

The frequency of clinical arthritis varied between 50% and 60%, with no differences between the mice inoculated with wild-type strain 8325-4 and those injected with the mutants lacking
functional genes for Aur, SspA and SspB or SspB alone. Signs of clinical arthritis were observed in similar frequency for all groups, with 50–54% for the wild-type, 60% for the aureolysin (Aur) negative, 55% for the serine and cysteine protease (Ssp) negative, and 50% for the cysteine protease (SspB) negative group. Assessing severity of clinical arthritis, we found the same development over time, and at day 14, only a negligibly more severe disease was seen in the wild-type group. In the cysteine protease (SspB) experiment, the arthritis score for the wild type and the cysteine mutants was similar. The histopathologically analyzed frequencies of arthritis were much alike when comparing all groups. As expected the overall histological scores for arthritis were somewhat higher than the corresponding clinical scores. This has two explanations. Firstly, it is possible to detect a milder degree of inflammation using the microscopy than with the naked eye and secondly certain affected joints like knees and elbows were covered with fur hindering an optimal macroscopic inspection. Of the mice inoculated with the wild-type strain 77% showed arthritis histologically as compared to 85% of those given the Aur\textsuperscript{−} mutant, and 72% of those given the Ssp\textsuperscript{−} mutant. The frequencies of either cartilage and/or bone destruction were similar, with 60% in the wild-type, 66% in the Aur\textsuperscript{−}, and 62% in the Ssp\textsuperscript{−} group. The scoring for severity of histopathological changes did not either show any significant differences between the groups analyzed. Thus, silencing of certain bacterial extra cellular protease genes did not influence the development of arthritis in our model. This was somewhat surprising in the light of what is known about bacterial protease effects in general. A possible interpretation of our findings is that what we see is the net effect of a diminished protease production and an increased expression of adhesin proteins in vivo. Indeed, the phenotypes of the \textit{aur} and \textit{ssp} 8325-4 mutants do not only lack the actual proteases but simultaneously express three times more Fn- BPs and protein A on their cell surface, compared with the wild-type strain when grown in vitro [122]. Decreased amounts of cell surface proteins on \textit{sarA} mutant bacteria were found to be due to proteolytic degradation [131], especially by staphylococcal serine protease. Also, inactivation of the protease genes of the \textit{sarA} mutant of the 8325-4 has been shown to increase the amount of cell bound protein A 10–20 times [122].
This points to an inverse relationship between the production of extracellular proteases and bacterial surface adhesins in the phenotype fitting the concept of proteases as players in the transition of bacteria from adhesive to invasive phenotype. Since a number of adhesins act as virulence factors, including e.g., ClfB and protein A, the outcome of the study possibly represents the net effect of increased density of adhesin expression, on the one hand, and lack of extracellular proteases, on the other.

**Polyclonal and protease-specific B-cell responses during staphylococcal infection**

Mice inoculated with the wild-type *S. aureus* strain 8325-4 produced specific antibodies to Aur, proSspB, SspB, the V8 protease and ScpA. In contrast, the mice that had been inoculated with the *S. aureus* 8325-4 mutant devoid of the ssp operon, coding for the V8 protease (SspA) and staphopain B (SspB) lacked IgM antibodies specific for the V8 protease. Furthermore, sera from the latter animals, despite having high levels of total IgG as well as of IgG recognizing proSspB were devoid of SspB specific IgG-class antibodies and most spectacularly totally lacked IgG antibodies reactive with the V8 protease. However, the mice that had been infected with the Aur-null mutant also displayed increased serum levels of aureolysin-specific IgG.
Notably all infected mice showed significantly higher levels of auto-antibodies specific for endogenous albumin as compared to naive mice. Antibodies specific for superantigens TSST-1 and SEA were found to be significantly increased in all infected mice irrespective of inoculated strain. Since the inoculated strains do not express these toxins, we interpret occurrence of these antibodies as a part of polyclonal B-cell activation. The 10-fold increase of total IgG and the 50% increase of total serum IgM levels also reflect this phenomenon.

We demonstrate that staphylococcal infection gives rise to production of specific antibodies directed against mature SspB and the V8 protease in mice inoculated with S. aureus strains expressing these antigens. In contrast, mice infected with the ssp operon-deficient mutant, despite polyclonal B-cell activation, did not produce any SspB- and V8 protease-specific antibodies at all. The latter finding indicates that the SspB and V8 protease antibodies detected in mice inoculated by the S. aureus strain with the functional ssp operon truly represent antigen specific antibody responses. Specific anti-V8 protease antibodies were found in quantities high enough to test their potential inhibitory activity. Isolated mouse Ig from wt S. aureus inoculated mice inhibited V8-protease activity in a dose dependent manner and at a concentration of 0.6 mg/ml 50% of the V8-protease activity was inhibited. In contrast, Ig from mice infected with the ssp' strain or streptococci, lacking anti-V8 protease specific antibodies, did not affect the rate of hydrolysis of the substrate at all. Ig from mice infected with the aur' strain showed an intermediate inhibition of the V8-protease. This probably reflects the fact that the proform of V8-protease is activated not only by aureolysin. This finding suggests that the host despite the overwhelming polyclonal B cell activation is able to raise antigen-specific and thus potentially protective B-cell responses.

**Impact of host MMP-9 on the outcome of staphylococcal infection**

Host matrix metalloproteinase MMP-9 has been implicated in the pathogenesis of arthritis mainly based on findings of high levels of MMP-9 in synovial fluids of RA patients with levels correlating to the severity of the disease [132-134]. High levels of MMP-9 has also been detected in plasma already within half an hour after intravenous exposure to LPS in different models of sepsis indicating an important role in host defence of bacteraemia. We wanted to study the impact of MMP-9 expression on joint inflammation and destruction in our model of bacterially induced septic arthritis. For the purpose of examining whether exposure to S. aureus triggers increase in MMP-9 in vivo, mice were injected intravenously with S. aureus followed by zymography of spleen and kidney tissues. On day 9 following bacterial inoculation, zymography revealed a four-fold increase of spleen levels of MMP-9.
I.v. inoculation of live staphylococci to MMP-9 KO mice and to congenic healthy controls initially gave rise to similar patterns of arthritis in both groups. However, on day 10 following bacterial inoculation, the MMP-9 knock-out mice showed more severe arthritis and by day 14 the KO mice showed a significantly more severe arthritis measured as arthritis score as well as a higher overall frequency of arthritis. Importantly, MMP-9 deficient mice harboured significantly higher amounts of staphylococci both in joints (p < 0.02) and in kidneys (p < 0.03) as compared to congenic wild-type mice. In contrast, the histologic examination did not reveal any significant difference between the MMP-9 KO mice and their congenic controls regarding bone or cartilage destruction.

To analyze if intra-articular delivery of MMP-9 affects the course of infectious arthritis, knee joints of NMRI mice were injected intra-articularly with \textit{S. aureus} with or without recombinant MMP-9. Regardless of whether bacteria were administered alone or together with recombinant murine MMP-9, the inflammation and joint erosivity were similar. Injection with MMP-9 alone did not induce arthritis to any greater extent than the vehicle itself. By the same token, intra-articular injections of staphylococcal peptidoglycans or live \textit{S. aureus} into knee joints of MMP-9 KO mice and congenic controls did not show any difference between the groups with respect to synovitis or tissue destruction.

MMP-9 deficiency did not affect the in vitro cellular response to staphylococcal constituents that were assessed in this study. When IL-6 in the supernatants from the stimulated spleen cells was analyzed the two groups had similar levels of this cytokine. Finally, to make sure that MMP-9 did not have a direct killing effect on \textit{S. aureus} we performed an in vitro assay, which showed no apparent bactericidal activity of MMP-9.

MMP-9 has been implicated as an important molecule in the destructive process of arthritic joints, so why protection against joint destruction in mice devoid of MMP-9 is absent? One explanation that needs to be taken into account is of course the possibility that the knock-out construct leaks i.e. still can produce MMP-9. This has been thoroughly checked and has not been found to be the case. Our results indicate an important protective role for MMP-9 in clearance of \textit{S. aureus} as a part of infection defence. This is well in line with a study of Streptococcus pneumoniae-induced meningitis, peritonitis and sepsis, in which conditions MMP-9-deficient mice were found to harbour higher titers of bacteria in blood and in spleen [135]. Furthermore, a study on \textit{E. coli} peritonitis showed that MMP-9 KO mice display an enhanced bacterial outgrowth in the peritoneal cavity and increased dissemination of the infection [136]. MMP-9 has been shown in vitro to have the capability to degrade collagen
type IV in basement membranes. This property has been regarded as an important factor for enabling leukocytes to migrate to the site of infection. In our study the MMP-9 knock-out mice did not clear the infection as well as the controls, harbouring significantly more bacteria in joints and in kidneys. This explains a more severe inflammation in the joints of MMP-9 KO mice and suggests the importance of this molecule in surveillance process.
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