Biologics in Staphylococcus aureus Arthritis

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Cover illustration: Micro-CT image of a healthy mouse wrist joint vs. a wrist joint with S. aureus septic arthritis.

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To my family
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ABSTRACT

The emergence of new type of drugs known as biologics has led to rapid disease improvements in many autoimmune arthritic patients. Nevertheless, most of these biologics are immunomodulators that may consequently increase the susceptibility of patients towards infections, such as septic arthritis. Septic arthritis is still considered a major public health challenge due to its rapidly progressive disease character with poor prognosis regarding joint functions. It is mainly caused by *Staphylococcus aureus* and despite optimal antibiotic treatment, nearly half of patients have permanent joint dysfunction.

The main aim of this thesis was to investigate the inflammatory response of the host to living as well as antibiotics-killed *S. aureus* and to study the effect of biologics on the course of staphylococcal infections. The role of host inflammatory response on post-infectious joint dysfunction using antibiotic-killed *S. aureus* was the subject of Paper I of this thesis. The main focus of Paper II and III were to study the effects of different biologics treatments on *S. aureus* induced septic arthritis and sepsis.

We demonstrated that antibiotic-killed *S. aureus* is capable of inducing and maintaining destructive arthritis. By using different knockout mice, we showed that this type of arthritis was mediated through TLR-2, TNFR1 and RAGE receptors. Furthermore, we found that insoluble cell debris was a key initiator of this type of arthritis. Finally, anti-TNF therapy attenuated the arthritis caused by antibiotic-killed *S. aureus*.

All the biologic treatments tested (including anti-TNF therapy, CTLA4-Ig and IL-1 Ra) aggravated *S. aureus* infections but had different clinical manifestations. Both CTLA4-Ig and IL-1 Ra therapy significantly increased the susceptibility to *S. aureus* induced septic arthritis in mice. Anti-TNF
therapy on the other hand resulted in more severe weight loss and impaired the bacterial clearance ability of the host.

In conclusion, antibiotic-killed *S. aureus* induced chronic destructive arthritis and anti-TNF therapy attenuated this type of joint inflammation. In the living *S. aureus* induced septic arthritis, all tested biologics complicated the disease course. Therefore, the potential dangers associated with biologics should be taken into account and patients with high risk of *S. aureus* bacteremia might be considered to refrain from them.

**Keywords:** *Staphylococcus aureus*; CTLA4-Ig; IL-1 Ra; anti-TNF therapy; mouse; septic arthritis

SAMMANFATTNING PÅ SVENSKA

Människokroppen exponeras ständigt för olika former av mikroorganismer. En del av dessa kan vara skadliga för oss om de lyckas ta sig in i kroppen. Lyckligvis har vi ett immunsystem som konstant är på sin vakt och fungerar som kroppens försvarssystem mot dessa mikroorganismer. Immunförsvarets viktigaste uppgift är nämligen att skydda oss mot att bakterier, virus och parasiter angriper kroppen och orsakar infektioner. Dessvärre kan dock immunförsvaret bli överaktivt och kan då angripa kroppens egen vävnad, vilket orsakar inflammation och vävnadsskada.

Personer med ett nedsatt immunförsvar tenderar till att lättare drabbas av infektioner av svårare karaktär. En sådan infektionssjukdom är sjukdomen septisk artrit, även känd som infektiös artrit. Septisk artrit är en ledsjukdom som främst orsakas av den gram-positiva bakterien Staphylococcus aureus. Septisk artrit betraktas som en av de farligaste ledsjukdomarna i dagsläget, då sjukdomen karaktäriseras av att den snabbt förvärerrar patientens hälsotillstånd. Trots optimal antibiotikabehandling ger septisk artrit upphov till permanenta skador i lederna hos uppemot 50 % av patienterna.


I denna avhandling har jag studerat möjliga orsaker till permanenta ledskador vid septisk artrit i en musmodell. Vidare har jag också studerat olika biologiska läkemedel och deras inverkan på stafylokock-inducerad septisk artrit och sepsis.

Sammantaget visar denna avhandling att ett alltför aktivt immunförsvar orsakat av antibiotika-avdödade stafylokocker kan ge upphov till bestående ledinflammation och skador. Jag har också kunnat påvisa att olika biologiska läkemedel, som används mot reumatoid artrit, kraftigt ökar risken för stafylokockinfektioner.
LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.


OTHER PUBLICATIONS

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<tbody>
<tr>
<td>AP1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>Coa</td>
<td>coagulase</td>
</tr>
<tr>
<td>Cna</td>
<td>collagen adhesin</td>
</tr>
<tr>
<td>ClfA</td>
<td>clumping factor A</td>
</tr>
<tr>
<td>ClfB</td>
<td>clumping factor B</td>
</tr>
<tr>
<td>CTLA4-Ig</td>
<td>cytotoxic T lymphocyte-associated antigen-4 immunoglobulin</td>
</tr>
<tr>
<td>DIC</td>
<td>disseminated intravascular coagulaton</td>
</tr>
<tr>
<td>FADD</td>
<td>FAS-associated death domain</td>
</tr>
<tr>
<td>FnBPA</td>
<td>fibronectin binding protein A</td>
</tr>
<tr>
<td>FnBPB</td>
<td>fibronectin binding protein B</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of NF-κB</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IL-1 Ra</td>
<td>interleukin-1 receptor antagonist</td>
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<tr>
<td>IRAK</td>
<td>interleukin-1 receptor-associated kinase</td>
</tr>
<tr>
<td>JUN</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function-associated antigen 1</td>
</tr>
<tr>
<td>LTA</td>
<td>lipoteichoic acid</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>MEKK</td>
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<tr>
<td>MKK88</td>
<td>MAPK kinase</td>
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<tr>
<td>MYD88</td>
<td>myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NK Cell</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>NKT Cell</td>
<td>natural killer T cell</td>
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<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>PVL</td>
<td>Paton-Valentine leukocidin</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RAGE</td>
<td>receptor for advanced glycation end products</td>
</tr>
<tr>
<td>RIP</td>
<td>receptor-interacting protein</td>
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<tr>
<td>SE</td>
<td>staphylococcal enterotoxin</td>
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<tr>
<td>SEI</td>
<td>staphylococcal enterotoxin-like toxin</td>
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<tr>
<td>Sak</td>
<td>staphylokinase</td>
</tr>
<tr>
<td>SpA</td>
<td>staphylococcal protein A</td>
</tr>
<tr>
<td>TAK</td>
<td>transforming-growth-factor-β-activated protein kinase</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
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<td>Abbreviation</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNFR</td>
<td>tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR-1-associated death domain protein</td>
</tr>
<tr>
<td>TRAF</td>
<td>tumor-necrosis factor receptor-associated factor</td>
</tr>
<tr>
<td>TSST</td>
<td>toxic shock syndrome toxin</td>
</tr>
<tr>
<td>vWbp</td>
<td>von Willebrand factor binding-protein</td>
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1. Introduction

Nearly half of the human population is at some-point colonized by *S. aureus*. Of these, 20% are persistently colonized while around 30% are intermittently colonized, mostly in the anterior nares and the skin [1]. However, one should not make the mistake of assuming that *S. aureus* is a harmless microbe that is only part of the normal flora. Rather, *S. aureus* is indeed a very virulent bacteria that causes a wide range of diseases, from simple wound infections and food poisoning to life-threatening conditions such as sepsis, meningitis and endocarditis [2]. Below I will briefly review two of the many infections caused by *S. aureus*, namely septic arthritis and sepsis.

1.1 Septic arthritis

Septic arthritis is rapidly progressing and devastating joint disease caused by pathogen infection. Prevalence of septic arthritis is around 6 cases per 100 000 in the general population and much higher in rheumatoid arthritis (RA) patients approaching about 70 cases per 100 000 [3]. *S. aureus* accounts for about 70% of the septic arthritis cases and has been shown to cause more severe infection than other microbes [3, 4]. The mortality rate is around 10-15% in non-RA patients with monoarticular arthritis, i.e. arthritis in a single joint. Polyarticular arthritis on the other hand is associated with a much worse prognosis, with the mortality rate ranging from 30-50% [3, 5]. Risk factors for septic arthritis include: increasing age, preexisting joint diseases (especially RA), intravenous drug abuse, prosthetic joints and diabetes mellitus [3, 5]. Treatment of septic arthritis consists primarily of antibiotics and joint aspiration to flush out the intra-articular pus containing both bacteria and infiltrating immune cells [6, 7]. One of the devastating aspects of septic arthritis is that despite optimal antibiotic treatment, almost half of the patients will develop irreversible joint destruction [5]. Definitive diagnosis of septic arthritis requires the isolation of the microbe from the synovial fluid, although due to the fast progressing nature of the disease, physicians do not and should not wait for culture results before initiating treatment with broad spectrum antibiotics [6].

Hematogenous spread of *S. aureus* to the synovial membrane of joints is the most common reported route of acquiring septic arthritis, although the bacteria can also be introduced directly into the joints by trauma (e.g. needle accident) or spread from neighboring tissues [7]. Once inside, the bacteria will employ different virulence factors to attach to the host factors and
proliferate while the host immune system will respond to the invading bacteria. It has been shown that the destruction of joints in *S. aureus* septic arthritis is not only caused by the invading microbe, but also by cells and molecules of the immune system, both the innate and adaptive [7].

The virulence factors involved, as well as the host response to the bacteria will be discussed in the coming chapters.

### 1.2 Sepsis

Sepsis is defined as the systemic inflammatory response due to an infection and is usually caused by bacteria such as *S. aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* [8]. *S. aureus* bacteremia is associated with higher mortalities than bacteremia caused by most other microbes and can develop to sepsis and severe sepsis [8]. Despite advances made in critical care and treatment, sepsis remains one of the foremost causes of death in critically ill patients. Mortality in sepsis is around 10-20% and increases significantly up to 80% if a septic shock develops [9, 10].

The pathogenesis of *S. aureus* sepsis is multifactorial and is mediated by components of the bacteria as well as the exaggerated immune response mounted by the host. Bacterial superantigens can cause non-specific activation of T-cells leading to massive polyclonal T cell activation resulting in vast release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) [11, 12]. Peptidoglycan and lipoteichoic acid, cell wall components of *S. aureus*, can also interact with CD14 molecules through toll like receptor 2 (TLR2) and stimulate the release of pro-inflammatory cytokines (TNF-α, IL-6) and chemokines (IL-8) further potentiating the systemic inflammation in sepsis [13-15]. This is followed by a massive release of anti-inflammatory cytokines in response to the inflammation whereby the immune regulation is rendered inactive, leading to a state of immunosuppression [16]. Without proper functioning immune system, the bacteria have free reign to proliferate and spread to different organs. Coagulation disorder, characterized by an excessive coagulation, is another attribute of sepsis. The coagulation cascade can be activated through the activity of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 leading to disseminated intravascular coagulation (DIC) [14, 17]. DIC is soon followed by thrombocytopenia, i.e. the lack of platelets in the blood resulting in massive bleeding from several sites and leading to organ failure [17]. Given together, the pathogenesis of sepsis includes systemic inflammation, loss of immune regulation and excessive coagulation that altogether will lead to multiple organ failure, shock and finally the demise of the host.
2 Virulence factors of S. aureus

*S. aureus* is a very resilient pathogen due to the various virulence factors it contains and produces, some of which are described below and illustrated in Figure 1.

2.1 Cell wall components

*S. aureus* expresses a capsular polysaccharide (CP) that functions as a virulent factor, enabling the bacteria to evade phagocytosis [18, 19]. Several serotypes of the CP have been identified and of those, CP5 and CP8 are the major ones. Most of the clinical isolates of *S. aureus* have the capability to produce either CP5 or CP8 [18]. *S. aureus* strains expressing the CP5 capsule significantly increase mortality as well as arthritis frequency and severity in *S. aureus* induced sepsis and septic arthritis, respectively, compared to the strains lacking the CP5 capsule [20]. This can be due to the downregulatory effects of CP5 on the uptake and intracellular killing ability of the phagocytes [20]. The CP8 serotype seems to be less virulent than the CP5 serotype as demonstrated by the ability of CP5 to cause higher bacteremia than the CP8 serotype in a mouse model of bacteremia. The CP5 producing strain also exhibited greater resistance to *in vitro* opsonophagocytic killing by neutrophils compared to the CP8 serotype [21].

The cell wall of *S. aureus* is made up of a 20-30 nm thick layer of peptidoglycan. Apart from being a protective barrier of the bacteria, peptidoglycan has other functions such as being a scaffold, whereby surface proteins that are fundamental for bacterial virulence can attach [22].

The major structural features of peptidoglycan consist of linear glycan strands made up of alternating N-acetylglucosamine and N-acetylmuramic acid residues that are linked by β-1-4 bonds [23]. The glycan strands are cross-linked by short peptides made up of D-alanine, L-lysine, D-glutamic acid and L-alanine [24]. The ε-amino groups L-lysine of nearby peptides are cross-linked to D-alanine of other peptides through pentaglycine bridges, thus giving rise to the 3-dimensional structure of the peptidoglycan [25].

Due to the critical role it plays in maintaining bacterial structure, growth and viability, peptidoglycan is a target for antibiotics as well as the immune system. Toll-like receptors (TLRs), Nod-like receptors (NLRs), mannose-binding lectin and lysozyme are some of the components of the immune system that can recognize peptidoglycan [26].
Peptidoglycan is a very strong inducer of inflammation and stimulates the release of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6. Furthermore, studies have shown that peptidoglycan alone can induce arthritis in mice [27] and repetitive inhalation of components of peptidoglycan can lead to bone loss [28].

Another component of the S. aureus cell wall is teichoic acid. Teichoic acid attached to the peptidoglycan layer is known as wall teichoic acid while that attached to the lipid is known as lipoteichoic acid (LTA). LTA is made up of a hydrophilic 1,3-linked polyglycerolphosphate backbone that is linked to a glycolipid that anchors LTA in the bacterial membrane [29, 30].

LTA, just like peptidoglycan, is a strong inducer of inflammation that binds to TLR2 and its co-receptors CD14 and CD36, activating macrophages and inducing the release of several pro-inflammatory cytokines such as TNF-α and IL-6 [31, 32].

The release of LTA and peptidoglycan from S. aureus results in systemic inflammatory response, largely due to their ability to stimulate greater adherence of granulocytes to endothelial cells and secretion of large amounts of IL-8 and monocyte chemotactic protein-1 (MCP-1) [33]. The systemic activation of endothelial cells will lead to excessive leukocytes aggregation and can subsequently lead to multiple organ failure due to accumulation of leukocytes in several organs [33]. Indeed, it has been shown that LTA and peptidoglycan from S. aureus act in synergy and cause shock and multiple organ failure, which coincides with the expression of iNOS in several organs as well as with a massive release of pro-inflammatory cytokines such as interferon gamma (IFN-γ) and TNF-α [34, 35].

Several enzymes such as lysostaphin are able to digest S. aureus peptidoglycan. Lysostaphin is a metalloendopeptidase capable of cleaving the crosslinking pentaglycine bridges in the staphylococcal cell wall [36]. However, the enzymatic digestion of antibiotic-killed S. aureus by lysostaphin had no effect on the severity of arthritis caused by antibiotic-killed S. aureus (Paper I) [37].
2.2 Bacterial DNA

*S. aureus* DNA can induce the production of pro-inflammatory cytokines such as TNF-α, IL-6 and IFN-γ [13]. Indeed, the injection of *S. aureus* DNA in mice led to rapid activation of macrophages followed by massive release of TNF-α that triggered lethal shock in mice [38]. Furthermore, previous results from our lab showed that *S. aureus* DNA containing CpG motifs induce arthritis [39]. However, DNA from antibiotic-killed *S. aureus* plays a minor role in mediating arthritis caused by antibiotic-killed *S. aureus* (Paper I) [37].

2.3 Surface proteins

*S. aureus* expresses several surface proteins that play a crucial role in enabling the bacteria to adhere to the host cells, aid in invasion of the bacteria and evade the immune response mounted by the host [40]. Adherence of bacterial products to host tissues is one of the important steps in initiation of colonization and infections [41]. *S. aureus* surface proteins usually recognize and adhere to several components of the extra cellular matrix (ECM) such as fibronectin, fibrin and collagen [41].
**Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)** are surface proteins expressed by *S. aureus* that are anchored on the cell wall peptidoglycan. Most MSCRAMMs contain a carboxyl-terminal sorting signal containing LPXTG motif that is cleaved by *S. aureus* sortase enzymes before being covalently anchored to the cell wall peptidoglycan [42]. Notable members of this group include fibronectin binding protein A and B (FnBP A and FnBP B), Collagen adhesin (Cna), Protein A, Clumping factor A and B (CfA and CfIB) [40].

**Clumping factor A** (CfA) binds to soluble fibrinogen and has been shown to inhibit complement-mediated phagocytosis [40, 43]. In *S. aureus* septic arthritis and sepsis, CfA is an important virulence factor that promotes the pathogenesis of the diseases and can be a target for generation of vaccine against *S. aureus* infections. Passive immunization of mice with rat and rabbit anti-CfA antibodies gave protection against *S. aureus* induced septic arthritis and sepsis [44].

Furthermore, CfA mediates binding of *S. aureus* to human platelets, an important virulence mechanism in the pathogenesis of *S. aureus* caused endocarditis [45].

**Staphylococcal protein A** (SpA) not only evades the innate immunity by inhibiting opsonophagocytosis, but also has the ability to alter the response of the adaptive immunity by binding to both Fc region of IgG and Fab regions of the B-cell receptor, thereby inducing apoptosis by functioning as a B-cell superantigen [46, 47]. SpA can also diminish the pro-inflammatory signaling of TNF-α by binding to its receptor TNFR1 [48, 49].

**Fibronectin binding proteins** are important in helping the bacteria to adhere to and invade cells of the host and together with CfB and SpA play a role, although not yet fully understood, in forming *S. aureus* biofilms [40]. Fnbps are also involved in the pathogenesis of *S. aureus* induced sepsis [50].

**Collagen adhesin** (Cna) binds to collagen and helps to mediate the binding of *S. aureus* to cartilage [51] and was shown by Patti et al to be involved in the pathogenesis of *S. aureus* septic arthritis [52]. Mice injected with *S. aureus* lacking Cna developed significantly less frequent signs of clinical arthritis (27%) compared with mice infected with wild type *S. aureus* (70%) [52].

*S. aureus* also secretes other proteins that, although not covalently attached to the cell wall peptidoglycan, are still surface-associated proteins and act as
adhesins - they are commonly known as **secreted expanded repertoire adhesive molecules (SERAMS)**. Coagulase (Coa), von Willebrand factor binding protein (vWbp), extracellular fibrinogen binding protein and extracellular adherence protein (Eap) are several examples of SERAMs [53]. The ability of *S. aureus* to clot human blood is mediated by the direct binding of Coa and vWbp with the hosts’ prothrombin. The resulting staphylothrombin complex will eventually convert fibrinogen to fibrin, thus forming fibrin clots.[54]. Eap plays multiple roles in *S. aureus* infections such as acting as an adhesin, inhibits wound healing and involved in biofilm formation [49, 55].

### 2.4 Secreted proteins

*S. aureus* **superantigen like proteins** (SSLs) is another group of proteins secreted by *S. aureus* that have similar structures as superantigens but lack superantigenic activities. Several SSLs have been identified that have been shown to be able to interfere with the innate immune response [49, 56]. Of these, SSL3 can bind to TLR2 and inhibit the production of TNF-α by macrophages stimulated by heat-killed *S. aureus* or peptidoglycan [49, 56].

*S. aureus* also secretes numerous other proteins such as chemotaxis inhibitory protein of *S. aureus*, staphylococcal complement inhibitor and formyl peptide receptor-like-1 inhibitory protein that aid the bacteria to evade opsonization and phagocytosis [2].

**Enzymes** secreted by *S. aureus* include catalase, proteases, hyaluronidase, lipases, nuclease and staphylokinase. Apart from exploiting host tissues and converting them into nutrients for the bacteria, *S. aureus* enzymes also facilitate invasion and evasion of the immune system [2]. Hyaluronidase breaks down hyaluronic acid that holds the cells of the body together, thus facilitating the invasion of *S. aureus* further into tissues [57]. Staphylokinase, which mediates the digestion of fibrin clots via activation of plasminogen to plasmin, has been shown to promote the establishment of *S. aureus* skin infections, but at the same time decrease the severity of the disease [58]. Intriguingly, fibrinolysis activated by staphylokinase prevents biofilm formation and promote detachment of biofilms [59].

### 2.5 Toxins

The secretion of toxins is another virulence weapon of *S. aureus* that bacteria use to manipulate and gain the upper hand against the immune system. *S.
*Staphylococcus aureus* secretes large amounts of toxins with several different virulence factors.

Several toxins secreted by *S. aureus* have superantigenic properties that have the ability to cause non-specific activation of T-cells, leading to massive polyclonal T cell activation followed by vast release of cytokines with subsequent fever, shock and multiple organ failure [11, 12]. It was long assumed that superantigens bind only to the TCR on the T-cells and MHC class II molecules on antigen presenting cells (APCs) [60]. However, it has since emerged that superantigens can also bind to CD28, thus forming a more stable complex than previously thought [61].

Toxic shock syndrome toxin 1 (TSST-1), staphylococcal enterotoxins (SE) (AE, G-1, R and T) as well as staphylococcal enterotoxin like toxins (SEls) (J-Q, S, U, V and X) are all superantigen toxins produced by *S. aureus* [62].

Of the staphylococcal enterotoxins, **SEB** and **SEC** are known to cause non-menstrual toxic shock syndrome (TSS) [63]. Furthermore, SEs have long been known to cause food poisoning whereas SEls were thought not to have emetic properties. However, recent studies have found that some newly discovered SEls (I-Q) have emetic properties and may play some role in staphylococcal food poisoning [64].

**TSST-1** accounts for almost half of all non-menstrual TSS in the general population and almost all cases of menstruation associated TSS [65]. In addition, clonal expansion of CD4+ Vβ11+ T cells induced by *S. aureus* producing TSST-1 toxin has been shown to be involved in the pathogenesis of *S. aureus* septic arthritis [66].

Another set of toxins secreted by *S. aureus* includes the hemolysins (also known as alpha (α), beta (β), and gamma (γ) toxins), cytolytic peptides (phenol soluble modulins) and bi-component leukocidins (including Panton-Valentine leukocidin (PVL)). All are characterized by their ability to cause cell lysis by forming pores in the cell membrane [67].

The first to be discovered and most studied of the **hemolysins** is the α-toxin, with an ability to form pores and lysis of a broad range of cell types such as peripheral blood monocytes, platelets and keratinocytes, as well as cells of the endothelium [68]. In addition to α toxins, γ toxins produced by *S. aureus* are also a critical virulence factor in *S. aureus* induced septic arthritis since mice injected with a mutant lacking both α and γ toxins showed significantly
less frequent and less severe arthritis compared to wild-type strains producing both α and γ toxins [69].

As mentioned, PVL has adhesion properties, damages leukocytes and has also been implicated in the pathogenesis of necrotizing pneumonia [70, 71].

**Phenol soluble modulins** are largely produced by community associated methicillin-resistant *S. aureus* (CA-MRSA) and trigger lysis of both red and white blood cells. Furthermore, they are involved in biofilm formation and are known to cause aggressive *S. aureus* infections. [72].

**Exfoliative toxins** (ETs) (A and B) are also secreted by *S. aureus* and are responsible for causing staphylococcal scalded skin syndrome (SSSS) [73].
3 The immune response during *S. aureus* infections

Hitherto we have seen that *S. aureus* possess multifactorial virulence factors with an ability to cause a range of diseases, some of which are life threatening. However, the immune system is also well equipped to defend and fend off the invading intruder.

In the coming chapter, the immune responses, both the innate and adaptive immunity mounted during *S. aureus* infections, will be discussed briefly.

3.1 Innate immunity

3.1.1 Neutrophils

Neutrophils are the most abundant type of white blood cells (WBCs) in the body, constituting around 50-70% of all WBCs and play a very important role in the innate immunity. During *S. aureus* infections, neutrophils are quickly recruited from the blood and migrate to the infection site via a process known as chemotaxis [74].

Breach of the epidermal barrier by pathogens such as *S. aureus* results in the internalization of the bacteria by resident macrophages and dendritic cells which produce pro-inflammatory cytokines such as IL-1 (α and β), IL-6, and TNF-α [74-76]. The cytokines will activate the endothelial cells to produce chemokines such as CXCL1, 2 and 5 as well as selectins (P and E selectins), integrin ligands and intercellular adhesion molecule 1 (ICAM-1). These are needed for the rolling, adhesion and migration of the neutrophils through the endothelium to the infection site as shown in Figure 2 [74-76].

Neutrophils have several pattern recognition receptors (PRRs), such as TLRs that can recognize different conserved molecules from microbes, so-called pathogen-associated molecular patterns (PAMPs). At the infection site, PRRs on the neutrophils will recognize PAMPs from bacteria, which is subsequently internalized. Around the internalized bacteria, a cellular compartment known as phagosome will be formed which in turn fuses with lysosomes to form phagolysosomes. The rapid release of reactive oxygen species through oxidative burst, antibacterial peptides that have microbicidal effects, proteinases that degrade bacterial components and proteins that sequester essential bacterial nutrients are some of the mechanisms employed
by the neutrophils in the phagolysosomes to neutralize the internalized bacteria [74-76].

Neutrophils also possess the ability to kill bacteria extracellularly by releasing its DNA, known as neutrophil extracellular traps (NETs). This process involves forming a web-like structure interconnected with histones and containing anti-microbial agents such as defensins and myeloperoxidase that trap the bacteria and eliminate it [77, 78]. Neutrophils are killing machines that do an excellent job phagocytizing bacteria and thus have a short life span (1-2 days) as a regulatory precaution to avoid tissue damage [79].

![Figure 2](image_url)

Figure 2. Transmigration of neutrophils through the endothelium: At the site of an infection, resident macrophages and dendritic cells will internalize the invading bacteria releasing pro-inflammatory cytokines such as TNF-α and IL-1β. The release of cytokines will stimulate neighboring endothelial cells to produce selectins, ligands for integrins as well as chemokines. This will mediate the rolling, adhesion and transmigration of neutrophils from the endothelium to the site of infection. ICAM-1 = Intercellular Adhesion Molecule 1, LFA-1 = Lymphocyte function-associated antigen 1, TNF-α = Tumor necrosis factor alpha, IL-1β = Interleukin 1 beta. Neutrophils are absolutely essential in protecting the host against live S. aureus infections, as clearly exhibited by the significantly higher mortality as well as arthritis caused by S. aureus in neutrophils depleted mice compared to
wild type controls [80]. On the other hand, the depletion of neutrophils did not have any impact in arthritis caused by antibiotic-killed S. aureus, whereas double depletion of both neutrophils and monocytes attenuated the arthritis induced by antibiotic killed S. aureus (Paper I) [37]. The same phenomenon was observed in arthritis caused by intra-articular injection of high mobility group box chromosomal protein 1 (HMGB-1) [81].

3.1.2 Macrophages

Macrophages are outstanding phagocytes that not only eliminate S. aureus but also function as antigen presenting cells and are involved in activating the adaptive immunity in case of serious breaches. Activated macrophages are also potent secretors of the pro-inflammatory cytokine TNF-α, whose role in S. aureus infections will be described later.

Two distinct subtypes of macrophages have been described with opposing activities: M1 macrophages and M2 macrophages. M1 macrophages, also known as “classically activated” macrophages, are pro-inflammatory. The enzyme nitric oxide synthase (iNOS) is expressed by M1 macrophages and helps convert arginine into nitric oxide (NO), which inhibits proliferation of infected cells [82, 83]. Microbial products, such as LPS or the pro-inflammatory cytokine IFN-γ, stimulate the M1 macrophages phenotype that will result in a Th1 immune response [84]. This will lead to the production of more pro-inflammatory cytokines such as IL-12, TNF-α and IFN-γ in a positive feedback loop, thus maintaining the M1 macrophage phenotype [85].

M2 macrophages, or “alternatively activated macrophages”, are anti-inflammatory and give rise to a Th2 immune response and thus promote cell proliferation and wound repair. The anti-inflammatory cytokine IL-4 promotes the differentiation of macrophages into M2 macrophages and stimulates the production of IL-10, which further enhances the phenotype of M2 macrophages [85].

Macrophages have specific names depending on the tissue on which they reside. For example, Kupffer cells are macrophages that are found in the liver, whereas microglia, adipose tissue macrophages and osteoclasts are found in the central nervous system, adipose tissue and bones, respectively [86]. Osteoclasts and osteoblasts play an important role in maintaining bone homeostasis by degrading and synthesizing bones, respectively [87]. In S. aureus septic arthritis, mice lacking IL-15 were found to have a reduced number of osteoclasts in their joints, which also coincided with reduced severity and less joint destruction compared to wild-type mice [88].
Activation of osteoclasts requires the receptor activator of nuclear factor kappa-B-ligand (RANKL), a member of the TNF superfamily that is found on the surface of osteoblast, to bind to receptor activator of nuclear factor kappa-B (RANK) on the surface of osteoclasts. RANKL has been implicated in \textit{S. aureus} infections, and its inhibition reduces bone loss in \textit{S. aureus} septic arthritis [89].

Macrophages have been shown to play dual roles in \textit{S. aureus} infections. On the one hand, Verdrengh \textit{et al} showed that macrophages are involved in aggravating \textit{S. aureus} arthritis and the deficiency of macrophages attenuated the disease [90]. On the other hand, the ability of the host to clear invading bacteria in the kidneys is impeded, thus leading to higher mortality [90]. Further studies also showed that macrophages are involved in arthritis triggered by bacterial DNA containing CpG motifs [91].

As was highlighted by the results from our study, macrophages do not seem to play a major role in arthritis induced by antibiotic-killed bacteria (\textit{Paper I}) [37]. However, as mentioned before, double depletions of both monocytes and neutrophils significantly attenuated the disease (\textit{Paper I}) [37].

\textit{S. aureus} is generally considered to be an extracellular pathogen. However, there is strong emerging evidence indicating that phagocytized \textit{S. aureus} can indeed survive inside macrophages. Thus, instead of getting rid of the bacteria, macrophages can be used by surviving \textit{S. aureus} as a protection pad against antibiotics as well as a means to disseminate to other tissues [92, 93].

### 3.1.3 Natural Killer (NK) cells

NK cells are a type of white blood cells that play an important role in the innate immune system. NK cells respond to and eliminate virus-infected as well as tumor cells and do not require antibodies or MHC to respond to these cells. NK cells play a protective role during \textit{S. aureus} infections [94, 95]. The depletion of NK cells in mice before inoculation with a toxic shock syndrome toxin-1 (TSST-1) secreting strain of \textit{S. aureus} is associated with higher susceptibility to develop \textit{S. aureus} septic arthritis as compared to wild-type control mice [94]. Further studies have also shown that NK cells depleted mice are significantly more susceptible to pulmonary \textit{S. aureus} infections compared to wild-type mice [95], underscoring the protective role of NK cells against \textit{S. aureus} infections.
3.1.4 The complement system

The complement system serves as the first line of defence and is a crucial part of the innate immune system that helps the host defend against many pathogens. It is made up of several plasma proteins and can be activated through 3 different pathways: the classical, the alternative and the lectin pathway (Figure 3).

Whenever bacteria are successful in breaching the physical barriers, the complement system, regardless of the activation pathway, will recognize this and form enzyme complexes known as C3 convertases whose task is to cleave the complement component 3 (C3) into two different proteins. The C3a, also known as anaphylatoxin, is pro-inflammatory and helps with the recruitment of the phagocytes to the infection site, whereas the C3b opsonizes the invading S. aureus, thus making it easier to be phagocytized [96, 97].

Apart from opsonizing the bacteria, the complement system can also form a lytic complex known as membrane attack complex (MAC) on the surface of invading bacterial cells that will lead to the lysis and eventual death of the microbe. However, the MAC recognizes only gram-negative bacteria and thus S. aureus is spared from the potent killing ability of MAC mechanism [98].

The complement system is imperative to the host defence during S. aureus infection as its deficiency renders the host defenseless and significantly increases the susceptibility to S. aureus infections [99]. Recent data from our lab show that mice lacking the complement component 3 (C3-) are highly susceptible to S. aureus septic arthritis. Kidney abscesses formation as well as bacterial burden in the kidneys are also negatively affected in the C3- mice compared to the wild type controls [100]. The results underscore the importance of the complement system in fending off S. aureus infections.
Figure 3. The complement system. The complement system can be activated via three different pathways: the classical pathway, the alternative pathway and the lectin pathway. All the pathways will converge at the formation of a C3 convertase complex. The complex will cleave the C3 protein into C3a and C3b, ultimately leading to pathogen opsonization, release of inflammatory mediators, and formation of MAC that results in the lysis of target cells.
3.2 Adaptive immunity

3.2.1 T-cells

T-cells or T-lymphocytes are an integral part of adaptive immunity. They originate in the bone marrow but mature in the thymus, hence the name T-cells. T-cells are recognized from other lymphocytes due to their unique T-cell receptor displayed on the cell surface. Several subsets of T-cells have been identified, of which three are well studied: T helper cells (T_h cells), Cytotoxic T-cells (CTLs) and Regulatory T-cells (Tregs).

T_h cells (CD4^+ T-cells) express CD4 glycoprotein on their surface, recognize antigens presented by major histocompatibility complex class II (MHCII) and secrete cytokines that are necessary for both the cell-mediated and humoral immune response [101]. CTLs (CD8^+ T-cells) express CD8 glycoproteins, recognize antigens presented by MHCII and eliminate virus infected and tumor cells [101]. Regulatory T-cells play an important role in maintaining balance by preventing immune response to self-antigens and suppressing excessive immune response that can cause autoimmune diseases [102].

CD4^+ T-cells differentiate into two major subgroups: Th1 and Th2 cells. Th1 cells mainly secrete the cytokines IFN-γ and IL-2, respond to intracellular microbes and stimulate phagocyte mediated uptake and elimination of microbes [103, 104]. Th2 cells usually respond to extracellular pathogens such as gastrointestinal parasites, secrete mainly IL-4 and IL-5 cytokines and promote eosinophil activation and phagocyte-independent immune response [103, 104].

Two signals are necessary for a T-cell to be fully activated and respond to an antigen. The first signal is provided when the T-cell receptor (TCR) binds to an antigen presented by antigen-presenting cell (APC) via MHC II. However, this signal is not enough to activate the T-cell; rather a second co-stimulatory signal between CD80/86 on the APC and CD28 on the T-cell is essential [105]. Cytotoxic T-lymphocyte-associated protein 4 (CTLA4), a naturally occurring protein receptor expressed on the surface of the T-cells, has the ability to inhibit the activation of the T-cell by competitively binding to CD80/86.

CTLA4-Ig, a biologic that inhibits the full activation of T-cells, (discussed more in coming chapters) down regulates the Th2 response and has little effect on Th1 response [106]. The same phenomenon is seen in S. aureus infections where septic arthritis mice pre-treated with CTLA4-Ig exhibited
lower levels of IL-4, a Th2 cytokine compared to control mice (Paper II) [107].

Although both CD4+ and CD8+ T cells are found in the inflamed synovium, CD4+ T cells make up the overwhelming part. Furthermore, depletion of CD4+ cells significantly ameliorates the course of septic arthritis in mice, whereas depletion of CD8+ T cells does not alter the course of arthritis compared to control mice [108]. Thus, it appears that CD4+ T cells are pathogenic during *S. aureus* septic arthritis due to their ability to produce pro-inflammatory cytokines such as TNF-α and IFN-γ via activated macrophages [108]. Recent results also found that CD4+ T cells promote the pathogenesis of *S. aureus* pneumonia [109]. In line with previous results, depletion of CD4+ T cells improved the pathology of the lungs, which also correlated with decreased levels of pro-inflammatory cytokines such as TNF-α, IL-β and IL-6 in the CD4-/− mice [109].

3.2.2 Natural Killer T (NKT) cells

NKT cells are unique subset of T cells that can have features of both T cells as well as NK cells. While other subsets of T cells recognize protein antigens, NKT cells are unique in that they recognize lipids and glycolipids and make up a tiny percentage of blood T cells. Studies from *S. aureus* triggered sepsis indicate that NKT cells do not play any significant role in the course of the disease [110].

3.2.3 B-cells

Unlike T-cells, B-cells do not seem to be the driving force behind the pathogenesis of *S. aureus* infections. Studies from murine *S. aureus* septic arthritis model show that B-cell deficient mice do not differ from the wild-type controls with regards to arthritis, mortality as well as clearance of the bacteria [111].

There have been several studies regarding the generation of antibodies against specific virulence factors of *S. aureus* with varying success. Vaccination with recombinant fragments of collagen adhesin gave protection against *S. aureus* induced sepsis and death in mice [112]. Antibodies generated from vaccination with fibronectin binding protein from *S. aureus* gave also protection against *S. aureus* induced endocarditis in rats [113]. Furthermore, both active and passive immunization of mice with rat and rabbit anti-ClfA antibodies protected against *S. aureus* induced septic arthritis and sepsis [44]. Targeting the toxins secreted by *S. aureus* could also be a viable approach of vaccination in *S. aureus* infections. Collins et al showed
that the intranasal administration of SEA in mice protected mice against SEA induced toxic shock [114]. However, the virulence factors of *S. aureus* are numerous, ranging from the virulent structure of the bacteria to the toxins, enzymes and surface proteins. Generating a single vaccine that gives protection against all virulence factors expressed by *S. aureus* would be at least very challenging if not impossible [3].

3.3 Receptors involved in the immune response in *S. aureus* infections

3.3.1 Receptor for advanced glycation endproducts (RAGE)

RAGE is a damage-associated molecular pattern (DAMP) multiligand receptor that has the ability to recognize several pro-inflammatory ligands that are generated during inflammation and infection. RAGE was discovered as a receptor for advanced glycation endproducts (AGE); highly oxidant substances that have been implicated in several diseases such as diabetes and atherosclerosis [115]. RAGE is localised and expressed on a wide variety of cells such as monocytes/macrophages, T-cells, endothelial cells, vascular smooth muscle cells, glomerular epithelial cells and neurons [116, 117].

The calcium-binding S100 calgranulins (S100A8, S100A9 and S100A12) are some of the ligands RAGE interacts with. They are mainly produced by neutrophils and activated macrophages, and play an important role in the inflammatory diseases [118]. Furthermore, leukocytes recruitment is enhanced in some inflammatory disorders associated with elevated RAGE expression through the interaction of RAGE with β2-integrin Mac-1, another ligand of RAGE that is mostly found on the surface of neutrophils and macrophages [119].

HMGB1 is another ligand of RAGE and has also been implicated in a number of inflammatory diseases [120]. Extracellular HMGB1 can be secreted from activated macrophages [121] and other immune cells [122, 123] after their activation by cytokines or toll-like receptor (TLR) ligands. It can also be released passively from damaged and necrotic cells [124], and acts therefore as an endogenous danger signal / damage-associated molecular pattern (DAMP) mediating subsequent inflammation.

RAGE seems to play different roles in local and systemic *S. aureus* infections. In local infections, RAGE and HMGB1 have been shown to
contribute to lung injury during *S. aureus* pneumonia [125]. Also, RAGE is involved in the induction of arthritis caused by antibiotic-killed *S. aureus* as RAGE-/- exhibited less severe and less frequent arthritis compared to wild-type controls (Paper I) [37].

In experimental models of severe sepsis and systemic infection, the inhibition of RAGE is associated with better survival rates. RAGE-/- had significantly higher survival rates (80%) compared to wild type mice (37%) in cecal ligation and puncture (CLP) model of polymicrobial sepsis as well as a model of systemic listeriosis [126]. The injection of anti-murine RAGE antibody significantly improved the survival rate of mice, highlighting the involvement of RAGE in the pathogenesis of sepsis [126]. On the other hand, preliminary results from our lab show that RAGE is not involved in the pathogenesis of *S. aureus* septic arthritis since RAGE-/- mice did not differ with their wild-type counterparts regarding arthritis, mortality as well as weight loss (unpublished data).

### 3.3.2 Toll like receptors (TLRs)

TLRs are pattern recognition receptors (PRRs) and are an integral part of the innate immune system. More than 10 different TLRs have been discovered thus far and most of them recognize different PAMPs. For example, TLR4 recognizes LPS from gram-negative bacteria as well as LTA from gram-positive bacteria [32], while TLR5 mostly recognizes bacterial flagellin [127].

Peptidoglycan from *S. aureus* is recognized by TLR2, which initiates an immune reaction immediately through nuclear factor κB (NF-κB) [128]. TLR2 has been found to play a pro-inflammatory and a catabolic role in septic arthritis [129]. We showed that in arthritis caused by antibiotic-killed *S. aureus*, the absence of TLR2 was associated with less severe arthritis compared to wild-type controls, although no difference regarding the frequency of arthritis was observed (Paper I) [37]. It is known that TLR2 deficient mice infected with *S. aureus* are still capable of producing significant pro-inflammatory cytokines, probably due to other TLRs recognizing and reacting to *S. aureus* [130]. Moreover, TLR2 can also form heterodimer with TLR6 to recognize other components of the gram-positive bacteria [131]. Since we could not see a difference in frequency (although in arthritis), it is possible that other TLRs could initiate an inflammatory response.
In *S. aureus* infections, deficiency of TLR2 in mice is associated with impaired bacterial clearance and significantly higher mortalities compared to wild type mice, underscoring the protective role of TLR2 in *S. aureus* infections [132].

### 3.4 Cytokines

Several cytokines are secreted by cells of both the innate and the adaptive immunity and have different roles in *S. aureus* infections. Some of them will briefly be discussed below and are exhibited in Table 1.

*Table 1: The role of cytokines in *S. aureus* infections.*

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cell source</th>
<th>Role in <em>S. aureus</em> infections</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Macrophages</td>
<td>Aggravate <em>S. aureus</em> induced septic arthritis but protective in sepsis [133].</td>
</tr>
<tr>
<td></td>
<td>T-cells</td>
<td></td>
</tr>
<tr>
<td>IL-1</td>
<td>Macrophages</td>
<td>Protective in <em>S. aureus</em> induced septic arthritis and sepsis [134].</td>
</tr>
<tr>
<td></td>
<td>Dendritic cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endothelial cells</td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td>Monocytes</td>
<td>Protective in <em>S. aureus</em> induced sepsis but not septic arthritis [135].</td>
</tr>
<tr>
<td></td>
<td>Macrophages</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dendritic cells</td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>Th2 cells</td>
<td>Dual role in <em>S. aureus</em> induced septic arthritis and sepsis depending on the genetic background of the host [136, 137].</td>
</tr>
<tr>
<td>IL-10</td>
<td>Monocytes</td>
<td>Protective in <em>S. aureus</em> induced septic arthritis [138].</td>
</tr>
<tr>
<td></td>
<td>Dendritic cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T-cells</td>
<td></td>
</tr>
<tr>
<td>IL-17</td>
<td>Th17 cells</td>
<td>Protective in local but not systemic <em>S. aureus</em> infection [139].</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>NK cells</td>
<td>Protective in <em>S. aureus</em> induced sepsis but aggravates septic arthritis [158].</td>
</tr>
<tr>
<td></td>
<td>T-cells</td>
<td></td>
</tr>
</tbody>
</table>
Although **TNF-α** was only discovered during the last half-century, it has become one of the most studied proteins in the body due to its role in inflammation and many diseases. TNF-α is one of the cytokines involved in the acute-phase reaction and is mainly secreted by activated macrophages as well as CD4+ cells, neutrophils, mast cells and NK cells [141].

With a molecular weight of 25.6 kDa, the human TNF-α is made up of 233 amino acids and is primarily expressed on the cell surface as membrane-bound TNF-α. The soluble form of TNF-α is secreted through the activity of tumor necrosis factor-α-converting enzyme (TACE), a metalloprotease enzyme, which cleaves the N-terminal 76 amino acids of the membrane-bound TNF-α [142]. There are two known receptors through which TNF exerts its biological activity: TNF receptor type 1 (TNFR1) and TNF receptor type 2 (TNFR2).

TNFR1, also known as p55, is expressed on most cells of the body and can be activated by both membrane-bound and soluble TNF-α, whereas TNFR2 is mostly expressed on the immune cells and usually activated by membrane-bound TNF-α [142]. Most studies on TNF receptors have focused on TNFR1 and not much is known regarding TNFR2. However, some functional differences between the two receptors have been reported. TNFR1 is mostly pro-apoptotic and its activation will ultimately lead to a cell death in most cases, whereas TNFR2 activation during infections or trauma will have the opposite effect, i.e. stimulate cell survival of target cells e.g. osteoclasts [143, 144]. On the other hand, some pro-survival function of TNFR1 and pro-apoptotic function for TNFR2 have been reported due to cross talk between the two different receptor types [143].

As mentioned above, the signaling pathways of TNF are quite complex and may result in cell survival/proliferation or apoptosis. The pathways are briefly discussed below (Figure 4).

In the **NF-κB activation pathway**, TNFR1 binds to Tumor necrosis factor receptor type 1-associated death domain protein (TRADD) which recruits TNFR-associated factor 2 (TRAF2), the receptor-interacting protein (RIP) and inhibitor of nuclear factor-κB (NF-κB) kinase (IKK), which will eventually lead to the activation of NF-κB [145].

Another pathway is the **death receptor pathway** whereby TNFR1 binds to TRADD and FAS-associated death domain (FADD) is recruited. This will in turn recruit caspase-8 followed by the activation of caspase-3 and eventually cell death [145].
The mitogen-activated protein kinases (MAPK) pathway involves the recruitment of TRAF2 after binding to the adaptor protein TRADD. This leads to the subsequent recruitment of MAP/ERK kinase kinase 1 (MEKK1) and MAPK kinase 7 (MKK7). This will eventually activate c-Jun N-terminal kinase [145].

**Figure 4.** An overview of the signaling pathways mediated by TNF-α. TNF-α can activate different signaling pathways that result in the induction of apoptosis or inflammation via production of several pro-inflammatory cytokines. (Modified from Aggarwal BB, 2003). TNFR (1 & 2) = Tumour-necrosis factor receptor, TRADD = Tumor necrosis factor receptor type 1-associated death domain protein, TRAF2 = TNFR-associated factor 2, MAPK = mitogen-activated protein kinase, MEKK1 = MAP kinase kinase 1, MKK (3&7) = MAPK kinase, JUN = c-Jun N-terminal kinase, NF-κB = nuclear factor κB, RIP = receptor-interacting protein, IKK = inhibitor of NF-κB, AP1 = activator protein 1 FADD = FAS-associated death domain.

TNF-α has a contrasting role during S. aureus infections. In S. aureus arthritis, the levels of TNF-α have been shown to be highly elevated in the
synovial fluid isolated from patients. Furthermore, it has been suggested that the levels of the cytokine could function as a predictor in determining the prognosis of the disease, with higher levels associated with worse prognosis [146].

Studies have also shown that TNF/lymphotoxin (LT)-α double knockout mice have significantly less severe *S. aureus* arthritis compared to the wild type mice [133]. Indeed, we could also show that tumor necrosis factor receptor type I (TNFRI) knockout mice exhibited less arthritis compared to wild type mice in antibiotic-killed *S. aureus* arthritis (Paper I) [37]. Anti-TNF treatment (Enbrel®) was also able to abrogate arthritis induced by antibiotic-killed *S. aureus* (Paper I) [37]. Additionally, in the *S. aureus* skin infection model, mice pre-treated with anti-TNF agent (Enbrel®) exhibited smaller lesion (abscess) sizes compared to the control PBS-treated mice according to preliminary results from our lab.

On the other hand, the lack of TNF-α is associated with impaired ability of the host to successfully clear invading *S. aureus* in the kidneys (Paper II) [107, 133], thus leading to also higher mortalities [133].

**IL-1** cytokine family is a group of 11 cytokines that play an important role in the inflammatory response. Of these, most is known regarding IL-1α, IL-β, and IL-1 Ra. IL-1α plays a central role in the induction of fever, sepsis and inflammation and is produced by activated macrophages, neutrophils as well as endothelial and epithelial cells. IL-β is predominantly produced by activated macrophages as a pro-protein and is cleaved by caspase 1 into its active mature form [147]. It plays an important role in pain, inflammation and cartilage degradation in several inflammatory diseases [148, 149].

There are three IL-1 receptors, IL-1 receptor type 1 (IL-1RI), IL-1RII as well as IL-1 receptor accessory protein (IL-1RAcP), all of which can bind to IL-α, IL-β and IL-1Ra [147]. Due to its lack of cytosolic part that is signaling-competent, IL-1RII functions as a decoy receptor [150] while IL-1RAcP serves as a co-receptor and is required for signal transduction. IL-1Ra is a natural occurring antagonist of both IL-α and IL-β by binding to the same receptors, thus blocking their pro-inflammatory biological effects. IL-1Ra is clinically significant and has been produced to combat several auto-inflammatory syndromes [151].

IL-1 can activate two different signaling pathways by binding to its receptors, as shown in Figure 5. IL-1 binds to its receptors namely, IL-1R1 and IL-1RAcp forming a trimeric complex. This results in the recruitment of adaptor
protein myeloid differentiation primary response gene 88 (MYD88), IL-1 receptor activating kinases (IRAK) as well as TRAF6 [147, 152]. From this stage, TRAF6 can activate JNK signaling pathway via the MAPK6 or NF-κB signaling pathway via TGFβ activated protein kinase 1 (TAK1)[147, 152].

IL-1, through the signaling adaptor molecule MYD88 has been shown to be vital for neutrophils recruitment and host defence against S. aureus cutaneous infection [153]. Of note, TLR2 also share the same signaling pathway as IL-1R, i.e. through MYD88, but did not have the same impact on S. aureus cutaneous infection as IL-1R-MYD88 signaling [153].

Furthermore, both IL-1α and IL-1β play an important role in protecting the host against S. aureus wound infection [154]. However, it was shown that IL-1β played a more prominent role in deeper intradermal S. aureus skin infections compared to IL-1α [154] whereas the latter was more crucial in S. aureus superficial skin infections [76, 155]. Although both IL-1α and IL-1β signal through the IL-1R, there are some key differences regarding the cellular source as mentioned above. IL-1α is mainly produced by epithelial cells and is continuously released from prestores expressed in keratinocytes during infection [156]. Furthermore, the stimulation of keratinocytes by components of S. aureus cell wall such as Peptidoglycan and LTA trigger an autocrine IL-1α signaling loop resulting in continuous production of neutrophil chemokines [154, 157]. On the other hand, the recruitment of neutrophils in S. aureus skin infections depends on the expression of IL-1β on bone marrow derived cells and not resident skin cells [155].

In S. aureus systemic infections, IL-1R signaling is also essential to the host protection against the bacteria as shown by Hultgren et al. IL-1R−/− mice inoculated with S. aureus developed significantly higher S. aureus septic arthritis and sepsis compared to wild-type IL-1R+/+ mice [134].
Figure 5. IL-1 signaling pathways: The activation of IL-1 signaling pathway leads to secretion of pro-inflammatory cytokines and chemokines that will ultimately lead to inflammation. (Modified from Medzhitov R, 2001). IL-1RAcP = IL-1 receptor accessory protein, MYD88 = Myeloid differentiation primary response gene 88, IRAK = Interleukin-1 receptor-associated kinase, TRAF6 = TNF receptor-associated factor 6, TAK1 = transforming-growth-factor-β-activated protein kinase 1, MKK6 = MAPK kinase 6, NFκB = nuclear factor κB, JUN = c-Jun N-terminal kinase.

IFN-γ is a potent pro-inflammatory cytokine that plays an important role in both the innate and adaptive immunity and is mainly produced by NK cells as well as T-cells. Apart from inhibiting viral and even bacterial infections, IFN-γ activates and stimulates the macrophages to better phagocytize intracellular invaders.
Different roles of IFN-γ in *S. aureus* triggered sepsis and septic arthritis have been described. Mice deficient of IFN-γ receptor develop significantly more severe and frequent arthritis [140]. The mortality levels due to sepsis are also significantly increased during the early stages of the infection in the mice lacking IFN-γ receptor, whereas in later stages the reverse is true with higher mortality levels in the wild-type mice [140].

Likewise, *in vivo* administration of IFN-γ before and after inoculation of *S. aureus* improved the survival of the mice while at the same time increased the severity and frequency of arthritis [158]. The positive effects on mortality due to *in vivo* administration of IFN-γ correlated with improved phagocytosis and better clearance of the bacteria in both, the liver and the kidneys. On the other hand, treatment of the mice with anti-IFN-γ monoclonal antibodies attenuated the severity and frequency of arthritis due to lower levels of serum TNF-α, IL-6 and IL-1β [158].

**IL-4** is an anti-inflammatory cytokine that play several roles in the immune system such as differentiation of naïve T-cells into Th2 cells as well as the differentiation of B-cells into plasma cells. In *S. aureus* infections, dual role of IL-4 has been described depending on the genetic background of the host. In inbred C57BL/6 mice, IL-4 was shown to be a driving force of septic arthritis and sepsis by significantly impairing the capability of the host to clear the bacteria [136]. However, in another inbred strain, 129SV mice, the opposite was true, i.e. IL-4 protected the mice from *S. aureus* induced sepsis [137].

Although **IL-6** has been shown to have some anti-inflammatory features, it is usually regarded as a pro-inflammatory cytokine [159]. Macrophages and T cells mainly produce IL-6 during infections or trauma. In *S. aureus* infections, IL-6 is usually elevated together with other pro-inflammatory cytokines such IL-β and TNF-α [146].

Synovial IL-6 together with synovial lactate and synovial fluid white blood cells count have been touted as a good parameters for diagnosing septic arthritis [160].

**IL-10** is popularly known to be an anti-inflammatory cytokine produced mainly by monocytes and to a smaller extent the lymphocytes. IL-10 promotes Th2 response while downregulating Th1 cytokine secretion by macrophages and monocytes. IL-10 plays a crucial role protecting the host against *S. aureus* septic arthritis by promoting bacterial clearance [138].
**IL-12** is also another immune mediating cytokine primarily produced by monocytes, macrophages and dendritic cells. Apart from stimulating the differentiation of naive T-cells to Th1 cells, IL-12 is also involved in the production of IFN-γ and TNF-α via T-cells and NK-cells. In *S. aureus* infections, IL-12 is crucial for the survival of the host and deficiency of IL-12 is associated with significant accumulation of *S. aureus* in many organs leading ultimately to the demise of the host [135].

**IL-17A** is a pro-inflammatory cytokine and one of the six members of IL-17 cytokine family. IL-17A is produced by activated Th17 subset of T-cells. IL-17A plays a significant role in host defense against local *S. aureus* infections due to its ability to produce chemokines that attract and recruit neutrophils [161]. Thus, in local *S. aureus* infection, IL-17A-/- mice developed more synovitis and erosions as well as more weight loss compared to the wild-type mice [139]. On the other hand, IL-17A-/- mice did not differ from wild-type mice regarding the severity and the frequency of arthritis induced by antibiotic-killed *S. aureus* (Paper I) [37].
4 Biologics against rheumatoid arthritis

The last few decades have seen the emergence of new type of drugs against RA and other autoimmune diseases known as biologics. Biologics, as the name suggests, are medicine derived from biological sources that target specific cells and molecules of the immune system. The development of biologics has revolutionized how RA patients are treated and have significantly improved the quality of life of many patients who otherwise were not responding to traditional disease modifying anti rheumatic drugs (DMARDs) [162].

There are a broad range of biologics against RA currently in use (Table 2) and are administered as a monotherapy or in conjunction with non-biological DMARDs.

Table 2. Biologics currently used in RA. (Modified from Ghia et al 2013)

<table>
<thead>
<tr>
<th>Biologics</th>
<th>Brand name</th>
<th>Structure</th>
<th>Type</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etanercept</td>
<td>Enbrel</td>
<td>Human dimeric fusion protein</td>
<td>Fusion protein</td>
<td>TNF-α and LT-α</td>
</tr>
<tr>
<td>Infliximab</td>
<td>Remicade</td>
<td>Chimeric (murine-human) mAb</td>
<td>IgG1</td>
<td>TNF-α</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>Humira</td>
<td>Human mAb</td>
<td>IgG1</td>
<td>TNF-α</td>
</tr>
<tr>
<td>Certolizumab</td>
<td>Cimzia, Pegol</td>
<td>PEGylated humanised mAb</td>
<td>Fab' fragment</td>
<td>TNF-α</td>
</tr>
<tr>
<td>Golimumab</td>
<td>Simponi</td>
<td>Human mAb</td>
<td>IgG1</td>
<td>TNF-α</td>
</tr>
<tr>
<td>Anakinra</td>
<td>Kineret</td>
<td>Human IL-1 Ra Receptor antagonist</td>
<td>IL-1R</td>
<td></td>
</tr>
<tr>
<td>Tocilizumab</td>
<td>Actemra</td>
<td>Humanised mAb</td>
<td>IgG1</td>
<td>IL-6R</td>
</tr>
<tr>
<td>Abatacept</td>
<td>Orencia</td>
<td>Human dimeric fusion protein</td>
<td>Fusion protein</td>
<td>CD80/86</td>
</tr>
<tr>
<td>Rituximab</td>
<td>MabThera, Rituxan</td>
<td>Chimeric (murine-human) mAb</td>
<td>IgG1</td>
<td>CD20</td>
</tr>
</tbody>
</table>
4.1 TNF inhibitors

TNF inhibitors are one of the main types of biologics currently in use and there are five different TNF inhibitors that have been approved for the treatment of RA and other arthritis diseases (Figure 6) [163, 164].

*Etanercept (Enbrel®)*

Etanercept is a synthetic fusion protein made of two identical soluble 75-kilodalton human TNF-receptors bound to the Fc region of human type 1 immunoglobulin G (IgG1) [165, 166]. Naturally occurring soluble TNF-receptors inhibit TNF-α and thus block its pro-inflammatory response and etanercept functions similarly albeit more intensely. Etanercept successfully binds to both, TNF-α and LT-α (previously known as TNF-β).

The dimeric makeup of etanercept gives it an advantage over naturally occurring soluble TNF-receptors by greatly increasing its binding affinity to TNF whereas the Fc region significantly increases the half-life of the drug [165, 166].

*Infliximab (Remicade®)*

Infliximab was approved shortly after etanercept for the treatment of RA in patients who had inadequate response to methotrexate (MTX). Infliximab, unlike etanercept, is not a fusion protein but rather a chimeric monoclonal antibody that is 75% human and 25% murine [167, 168]. The variable regions of the antibody are murine while the constant region is of human origin. Infliximab binds to TNF-α with high affinity and specificity, preventing TNF-α binding to its receptor and thus rendering it inactive [167, 168].

Unlike etanercept, infliximab does not bind to LT-α but has the ability to bind to both monomeric and trimeric forms of TNF-α, thus increasing the efficacy of the drug [169].

*Adalimumab (Humira®)*

Adalimumab was the third anti-TNF drug to be approved after etanercept and infliximab. Unlike its predecessors, adalimumab is a fully human monoclonal antibody that bind to TNF-α and blocks its pro-inflammatory effect [170]. Although adalimumab as a monotherapy leads to a significant rapid improvement in RA patients [171], its therapeutic effect is even greater when
given together with MTX [170]. Within a decade after its launch, adalimumab has become the best-selling drug in the world [172].

**Certolizumab pegol (Cimzia®)**

Certolizumab pegol is the first and currently the only PEGylated Fab' fragment of a humanized IgG1 anti-TNF monoclonal antibody approved for the treatment of RA [173]. Unlike the rest of anti-TNF inhibitors, it lacks the Fc region thus making it less likely to illicit complement-dependent cytotoxicity (CDC) [174, 175]. Certolizumab pegol in combination with MTX is a very effective therapeutic agent against RA [176, 177].

**Golimumab (Simponi®)**

Golimumab is the latest TNF-inhibitor to be approved for the treatment of RA. Golimumab, just like adalimumab, is a fully human IgG1 monoclonal antibody against TNF-α [178]. Golimumab is very stable and binds to TNF-α with high affinity and neutralizes its pro-inflammatory response [178]. Similar to its four anti-TNF predecessors, golimumab together with MTX leads to significant, rapid disease improvement in RA patients [179, 180].

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**Figure 6.** Structures of anti-TNF agents: Etanercept is a fusion protein of the extracellular domain of TNFR2 and the Fc region of IgG1. Infliximab is a chimeric monoclonal antibody that is 75% human and 25% murine. The variable regions of the antibody are murine while the constant region is of human origin. Unlike infliximab that contains murine parts, adalimumab and golimumab are fully human IgG1 antibodies. Certolizumab pegol consists of a PEGylated Fab' fragment of a humanized IgG1.
4.2 CTLA4-IG

*Abatacept (Orencia®)*

CTLA4-Ig (abatacept) is a fusion protein consisting of the extracellular domain of human CTLA4 bound to a fragment of human Fc region of IgG1 [105]. It is the only T-cell modulating drug approved for the treatment of RA. It has a high affinity for CD80/86 and competitively binds to it. The binding of CTLA4-Ig to CD80/86 prevents it from engaging CD28 on the T-cell and blocks the second co-stimulatory signal need for the activation of the T-cell, thus rendering the cell inactive (Figure 7) [105, 181]. Of note, T-cells can be activated via other co-stimulatory pathways other than the CD80/86-CD28 pathway, although CTLA4-Ig also manages to limit this type of activation [105, 182].

![Activation of T-cell](image1)

**Inhibition of T-cell**

![Inhibition of T-cell](image2)

**Figure 7.** Mechanism of action of CTLA4-Ig. For a naïve T-cell to be activated, two signals must be present. The first is achieved when an antigen is presented by APCs via MHC molecule to a TCR on the T-cell. However this signal on its own is not enough to fully activate the T-cell. Rather, a second co-stimulatory signal between the CD80/86 on the APC and CD28 on the T-cell must be present. CTLA4-Ig competitively binds to CD80/CD86 on the APC, thus preventing it’s binding to CD28 on the T-cell. Without the second co-stimulatory signal, the T-cell will remain inactive. APC = Antigen presenting cell, TCR = T-cell receptor, MHC = Major histocompatibility complex.
4.3 IL-1 receptor antagonist

Anakinra (Kineret®)

Anakinra, an IL-1 Ra, was the third biologic to be approved for the treatment of RA few years after infliximab and etanercept. Anakinra competitively binds to IL-1R1 and thus blocks the activity of IL-1α and IL-1β and their subsequent pro-inflammatory effects [183]. Anakinra in combination with MTX has led to significant improvement in RA patients compared to patients only on MTX [184, 185].

Although there were great expectations with anakinra, it never succeeded as the TNF-inhibitors and nowadays due to better efficacy exhibited by other biologics, IL-1 Ra is falling out of favor and is mostly prescribed to patients who do not respond well to other biologics. On the other hand, anakinra is the main therapeutic agent in many auto-inflammatory syndromes [183].

4.4 IL-6 inhibitor

Tocilizumab (Actemra®)

Another anti-cytokine biologic that has been approved for the treatment of RA is tocilizumab. Tocilizumab blocks the damaging pro-inflammatory effects of IL-6 by competitively binding to both membrane-bound and soluble IL-6 receptors, thus preventing the cytokine from exerting its biological pro-inflammatory function [186]. Tocilizumab is a humanized receptor monoclonal antibody (mAb) that is made up of mouse mAb grafted into human IgG1 [186].

In RA patients with inadequate response to traditional DMARDs or other biologics, tocilizumab administered together with MTX or alone significantly improves the disease activity [187, 188].

4.5 B-cell depletion

Rituximab (MabThera®)

Rituximab is the only biologic that targets the B-cells currently approved for the treatment of RA. Rituximab is a chimeric (murine-human) mAb with constant human and variable mouse regions that binds to CD20 expressed primarily on B-cells. Lysis of B-cells by Rituximab occurs through direct
induction of apoptosis, antibody-dependent cellular killing as well as complement-mediated lysis [189]. Apart from being expressed on the surface of almost all normal pre-B and mature B-cells, CD20 is also found on more than 90% of B-cell lymphomas [190]. Originally, rituximab was used mainly for B-cell malignancies, however it was also approved for the treatment of RA due to its efficacy in patients who had inadequate response to other DMARDs or biologics [191, 192].

4.6 Janus-kinase inhibitor

*Tofacitinib (Xeljanz®)*

Tofacitinib is the one of the latest drugs to be approved for the treatment of RA in USA. Tofacitinib is not a biologic but rather small molecules inhibitor and can be taken orally unlike biologics, which are usually injected. Tofacitinib targets and inhibits the Janus kinase (JAK) 1, JAK3 and to a lesser degree also JAK2 of the tyrosine kinases family, thus disrupting the JAK-STAT signaling pathway that is linked to inflammation associated with RA [193, 194]. Tofacitinib alone or in combination with MTX has been shown to lead to a rapid reduction in signs and symptoms of RA in patients with inadequate response to traditional DMARDs or anti-TNF therapy [193, 195-197].
5 Infection risks associated with biologics in RA

Even before biologic treatment of RA became standard routine, RA patients were at an increased risk of developing serious infections. RA patients had greater frequency of skin and soft tissue infections as well as septic arthritis and sepsis compared to the general population [198, 199].

The emergence of biologics and the fact that they are immunosuppressing agents might further increase the risks of infections in patients receiving these agents.

Infections reported among RA patients’ receiving biologics alone or in combination with other DMARDs will be discussed below and are summarized in Table 3.

Table 3. Infections risk associated with biologics.

<table>
<thead>
<tr>
<th>Biologics</th>
<th>Risk of infections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-TNF Agents</td>
<td>Tuberculosis [200], candidiasis [200], coccidioidomycosis [200], histoplasmosis [200], aspergillosis [201], listeriosis [200], nocardiosis [200], pneumonia [201], chickenpox [202], herpes zoster [202], septic arthritis [203]</td>
</tr>
<tr>
<td>CTLA4-Ig</td>
<td>Septic arthritis [107], herpes simplex [204]</td>
</tr>
<tr>
<td>IL-1 Ra</td>
<td>Tuberculosis [205], pneumonia, [206], septic arthritis [207], sepsis [207]</td>
</tr>
<tr>
<td>Tocilizumab</td>
<td>Candidiasis [208], listeria monocytogenes [209]</td>
</tr>
<tr>
<td>Rituximab</td>
<td>Hepatitis B reactivation [210], pneumocystis pneumonia [210]</td>
</tr>
<tr>
<td>Tofacitinib</td>
<td>Tuberculosis reactivation [211], herpes zoster [212]</td>
</tr>
</tbody>
</table>
5.1 Infection risk associated with TNF-inhibitors in RA

As mentioned before, TNF-α is an essential part of the immune system and its inhibition could increase the likelihood of pathogens gaining the upper hand against the immune system.

Etanercept and infliximab were approved for RA several years before adalimumab. Therefore, most studies on patients and even on animals are from these two TNF-inhibitors. Golimumab and certolizumab pegol were approved just few years back and not so many studies are available yet regarding the risk of infections in patients receiving these agents.

During the trial period of etanercept and infliximab, there were no significant increased risk of infections reported for these agents. However, successive studies have shown increased risk of serious infections in RA patients on these agents [213].

In general, the risk of serious infections that require hospitalization, irrespective of the pathogen responsible, is increased in RA patients treated with TNF-inhibitors [214-217].

Interestingly, although RA patients treated with etanercept, infliximab and adalimumab have an increased risk of tuberculosis, infliximab stands out with being associated with highest risk of TB infection [167, 200, 218, 219]. Furthermore, studies on mice have also shown similar pattern of extra increased risk of TB in mice treated with infliximab compared to other TNF-inhibitors [220].

The risk of listeriosis infection is also increased in RA patients treated with anti-TNF agents, especially infliximab [200, 221]. Furthermore, RA patients who are administered anti-TNF treatments are also at an increased risk of nocardiosis and pneumonia infections [200, 222, 223].

Anti-TNF agents administered to RA patients in general, and infliximab in particular are also associated with increased risk of other granulomatous fungal infections, such as candidiasis, coccidiodomycosis, histoplasmosis and aspergillosis [200, 201, 224].

TNF-α has been shown to be important in the protection against Mycobacterium Tuberculosis due to its ability to stimulate the formation and maintenance of protective granulomas. Thus, its inhibition will likely impair...
the ability of the host to successfully fend of the invading pathogens [219, 225-227]. However, it’s worth asking why infliximab is associated with higher risk of granulomatous infections in both mice models and in RA patients compared to etanercept, although both are very effective biologics that inhibit TNF-α successfully. To find the answer to that question, one has to look at both the structure and the mode of action of infliximab and how it inhibits TNF-α.

Infliximab binds and blocks both soluble and membrane bound TNF with high avidity whereas etanercept binds mostly to soluble TNF. Furthermore, infliximab has been shown to quickly and irreversibly bind to TNF and has a longer half-life in the serum, thus almost completely inhibiting all TNF compared to etanercept which has a shorter half-life, whose binding is reversible and not as complete as with infliximab [219, 227]. Therefore, there could be some TNF, although minimal amounts, still present to recruit necessary cells and cytokines to contain the infiltrating pathogen in patients receiving etanercept compared to the patients treated with infliximab [219, 227].

Serious skin and soft tissues infections as well as chickenpox and herpes zoster infections are increased in RA patients undergoing anti-TNF therapy [202, 228-230]. Furthermore, RA patients with resolved hepatitis B infection; the risk of virus reactivation is increased with anti-TNF treatment, especially with infliximab [231, 232].

Apart from infections, malignancies, especially hematological cancers, are reportedly increased in RA patients administered anti-TNF therapy [217, 233].

The risk of infections in RA patients receiving anti-TNF therapy is higher during the first few months of treatment initiation. Afterwards, the risk starts to decrease or remains stable the longer duration the treatment takes [214, 234]. Several potential answers regarding this phenomenon have been suggested. With regard to higher risk of infections early on, it could be just the truth, i.e. anti-TNF therapy increases the risk of infection in susceptible patients early on [234]. Another explanation could be that maybe doctors are biased and are more prone to treat infections early on during the treatment with anti-TNF [234]. As for the decrease of risk of infections later on, this could be due to the fact that patients on anti-TNF who develop infections stop treatment and thus will not be included in longer follow-up periods [214, 234].
5.2 Infection risk associated with CTLA4-Ig in RA

CTLA4-Ig, the only T-cell co-stimulator approved against RA, is prescribed mainly for RA patients who have inadequate response to anti-TNF therapy. However, due to its safety profile, CTLA4-Ig is gaining attraction [235, 236].

CTLA4-Ig is associated with slightly increased risk of serious infection in RA patients [237]. There are no extensive studies regarding the risk of specific infections in RA patients receiving CTLA4-Ig.

In animal studies, CTLA4-Ig has been shown to significantly increase mortality in a murine model of herpes simplex virus infection [204]. This could depend on almost complete ablation of the anti-HSV CD4+ and CD8+ T-cell responses by CTLA4-Ig due to anergy and reduced cell numbers respectively [204].

5.3 Infection risk associated with IL-1 Ra in RA

Meta-analyses have shown that IL-1 Ra increases the risk of serious infections in RA patients, especially in higher doses [235, 238]. Just like anti-TNF therapy in RA patients, the risk of serious infections was highest during the first six months and is further increased when patients have comorbidities factors [235, 238].

Only a few studies are available regarding the risk of tuberculosis in patients prescribed IL-1 Ra and the results from those studies give conflicting conclusion. Some conclude that IL-1 Ra does not elevate the risk of tuberculosis [239] while others studies paint the opposite picture [205]. Furthermore, the risk of pneumonia is slightly elevated in RA patients treated with IL-1 Ra [206]. In a rabbit model of pneumonia, IL-1 Ra treatment increased the bacterial burden in the lungs compared to the untreated animals [240].

5.4 Infection risk associated with Tocilizumab in RA

Tocilizumab significantly increases the risk of serious infections in RA patients, especially in higher doses [241, 242]. Tocilizumab is specifically associated with higher risk of serious respiratory infections in RA patients [243, 244]. Thus far, no increased risks of TB reactivation have been reported.
in RA patients treated with tocilizumab [241, 244]. There is also no increased risk of hepatitis or malignancies reported with tocilizumab treatment in RA patients [241].

Of note, patients with dental infections receiving tocilizumab are at increased risk of developing systemic streptococcal infections [245]. In mice, IL-6 deficiency is associated with increased risk of candidiasis [208] and listeria monocytogenes [209, 246].

5.5 Infection risk associated with Rituximab in RA

Rituximab has been in use for treatment of hematological malignancies longer than in RA treatment and thus most studies available are from patients from the former group. In RA patients, rituximab is associated with slightly increased risk of serious infections [247, 248]. Rituximab treatment increases the risk of hepatitis B reactivation in lymphoma patients with previous resolved hepatitis B. [210, 249]. The risk of pneumocystis pneumonia is also slightly increased in patients receiving rituximab [250]. In mice, the depletion of B cells has been shown to impair the ability of the host to successfully fend off disseminating virus infection [251].

5.6 Infection risk associated with Tofacitinib in RA

Tofacitinib is associated with a small increased risk of serious infections [211, 252]. Studies have also shown that the risk of tuberculosis reactivation is elevated in RA patients receiving tofacitinib [211, 252]. Similar results from mice have also shown that, indeed, tofacitinib reduces the host’s containment of the bacteria and further promotes its replication in the lungs [253]. RA patients treated with tofacitinib also face an increased risk of herpes zoster, although no conclusive explanation behind the underlying mechanism has been identified [212, 254]. Nevertheless, type 1 (IFN-α and IFN-β) and type II (IFN-γ) responses are crucial for the antiviral defence in humans [255] and both of them signal through the JAK-1 receptor which is inhibited by tofacitinib [212].
6 S. aureus in the era of biologics in RA

Thus far we have seen that biologics in RA increase the risk of other infections. A pattern that can be seen in these infections is that they are mostly granulomatous infections and viral infections, such as herpes zoster and reactivation of hepatitis. However, studies regarding the risk of S. aureus infections in RA patients treated with biologics are not very comprehensive. Below, the risk of infection associated with S. aureus in RA patients who were administered biologics will be discussed.

Previous studies have shown that colonization with S. aureus significantly increased the risk of infections in the patients in intensive care units [256, 257], while active surveillance together with subsequent decolonization decreased colonization by S. aureus and hospital-acquired infections [258].

Additionally, in a multicenter study of bacteremia caused by S. aureus, the majority of cases of bacteria isolated in the bloodstream originated from colonies in the nasal mucosa [259]. A meta-analysis of published studies showed that colonization with methicillin-resistant S. aureus (MRSA) is associated with an increased risk of MRSA infection [260].

Treatment with anti-TNF agents plus MTX in RA patients may predispose patients to S. aureus colonization [261]. Furthermore, patients who are already colonized by S. aureus are more likely to remain colonized after anti-TNF therapy than patients who are not on biologics [262].

It is known that the risk of septic arthritis increases several folds if the patient has RA [198, 199]. There is evidence pointing towards that RA patients exposed to anti-TNF therapy are even more susceptible to septic arthritis than RA patients on non-biologic DMARDs. In a prospective observational study from the British Society for Rheumatology Biologics Register, Galloway and colleagues found that anti-TNF therapy (etanercept, infliximab and adalimumab) in RA patients doubled the risk of septic arthritis [203]. However, the anti-TNF treated patient cohort had more severe disease at baseline, which is an important risk factor for the development of septic arthritis [203].

The risk of septic arthritis relapse can also be increased in patients receiving anti-TNF therapy despite adequate longtime antibiotics treatment [263]. This in particular is very worrisome, and physicians should immediately discontinue any anti-TNF therapy if patients acquire other infections.
Fei et al showed that mice treated with etanercept had more bacteria accumulating in their kidneys compared to the PBS-treated control mice [264]. The same pattern has been seen in our study where mice pre-treated with etanercept developed significantly more severe kidney abscesses as well as a higher bacterial burden in the kidneys (Paper II) [107]. Further studies also showed that TNF deficiency was associated with significantly higher intracerebral bacterial loads as well as more severe brain abscess formation compared to wild type mice infected with S. aureus [265]. Collectively, these studies highlight the crucial role of TNF in controlling S. aureus induced abscess formation.

Different role of anti-TNF therapy in S. aureus sepsis has been reported. On the one hand, several studies indicate that in patients with sepsis, anti-TNF therapy might reduce the overall mortality [266, 267]. However, anti-TNF therapy itself is associated with an increased risk of acquiring sepsis in the first place in RA patients [268, 269].

Pre-treatment with CTLA4-Ig (abatacept) significantly increases the susceptibility of mice to develop S. aureus septic arthritis in a mouse model (Paper II) [107]. Furthermore, the frequency of polyarthritis, which has far worse prognosis regarding mortality in patients [270], is significantly higher in CTLA4-Ig treated mice (Paper II) [107]. However, pre-treatment with CTLA4-Ig did not lead to increased abscess formation in the kidneys of mice infected with S. aureus (Paper II). This is not quite unique and it has previously been shown that blockade of T-cell activation by CTLA4-Ig prevented abscess formation in mice infected with different bacterial pathogens including S. aureus [271]. Recent results show that T-cell signaling through CD28 contributed to MRSA pneumonia and preventing T-cell co-stimulation significantly ameliorated the course of the disease in mice [109].

As mentioned previously, IL-1R is essential in the protection of the host against S. aureus arthritis and sepsis [134]. Thus, one could assume that IL-1 Ra (anakinra) treatment might increase the susceptibility of S. aureus septic arthritis and sepsis in RA patients. Indeed, in our mouse model, the mice pre-treated with IL-1 Ra and then infected with a S. aureus strain to mimic the clinical situation as in RA patients, developed significantly more septic arthritis and sepsis compared to the control untreated animals (Paper III) [207]. Kanangat et al showed that IL-1Ra enhances the growth of S. aureus in a concentration-dependent manner, i.e. the addition of IL-Ra and IL-1β significantly enhanced the growth of S. aureus in cell culture medium [272]. However, in our study, although IL-1Ra increased the susceptibility of septic
arthritis and sepsis in mice, it did not lead to an increase of bacterial burden in the kidneys or joints (Paper III) [207].

C-reactive protein (CRP) is an acute-phase protein that rises during inflammation and infection and is used as a key marker clinically for inflammation [273]. Of note, IL-6 has been shown to stimulate synthesis of CRP [273, 274] and inhibition of IL-6 by tocilizumab leads to lower levels of CRP. Tocilizumab seems to mask severe S. aureus [275, 276] and pneumonia [277, 278] infections in RA patients by keeping the CRP levels normal or only slightly elevated.
Figure 8. Summary of the main findings of the thesis. (Right panel) Intra-articular injection of antibiotic-killed *S. aureus* is capable of inducing and maintaining joint inflammation. This is likely mediated through TLR2, TNFR1 and RAGE receptors. The cross talk of neutrophils and monocytes seems to be responsible for this type of arthritis. T-cells are of minor importance in the pathogenesis of this type arthritis. Among bacterial factors, insoluble cell debris plays an important role in inducing joint inflammation. (Left panel) Anti-TNF, CTLA4-Ig and IL-1 Ra aggravate *S. aureus* systemic infections with different clinical manifestations. IL-1 Ra increased the susceptibility of mice to *S. aureus* induced septic arthritis and sepsis. CTLA4-Ig therapy significantly increased the susceptibility to *S. aureus* septic arthritis in mice. Anti-TNF therapy, on the other hand, deteriorated host bacterial clearance, resulting in more-severe weight loss and kidney abscesses.
7 Combination therapy in *S. aureus* arthritis

Septic arthritis is a dangerous, fast progressing erosive disease with high morbidity and mortality. It is no doubt that neutralizing the bacteria is imperative in controlling *S. aureus* septic arthritis. However, it is also clear that eliminating the bacteria alone is not enough to completely improve the prognosis of the disease. Up to 50% of patients receiving adequate antibiotic treatment will not regain full joint function, mostly due to joint and cartilage damage associated with the immune response mounted by the host [3]. We have shown that antibiotic-killed *S. aureus* can induce and maintain destructive arthritis (Paper I), thus removing any doubts that the immune system is involved in the pathogenesis of *S. aureus* septic arthritis. Therefore, the need to find an alternative therapy to *S. aureus* septic arthritis is indeed of great importance.

Several studies testing the combination therapies of antibiotics and anti-inflammatory drugs to control the exaggerated immune response have had varied results. Combining antibiotics and antioxidants and free radical scavengers ameliorated the course of *S. aureus* septic arthritis in mice [279-281]. Bisphosphonates, normally used in the treatment of osteoporosis in order to inhibit bone loss, in combination with antibiotics also led to significantly less bone loss in *S. aureus* septic arthritis compared to treatment with antibiotics alone [282]. The further addition of glucocorticoids to the combination of bisphosphonates and antibiotics decreased the osteoclastic activity seen in *S. aureus* septic arthritis, thus significantly reducing the potential of skeletal damage [282].

Furthermore, animal studies have shown that combination therapy of glucocorticoids with antibiotics ameliorated the course of *S. aureus* septic arthritis in mice [283, 284]. These animal studies were followed by a double-blind, randomized, placebo-controlled study of dexamethasone, a steroid, in combination with antibiotics in children with septic arthritis. The results showed significant reduction in residual joint dysfunction as well as shortened duration of symptoms in children receiving the combination therapy [285]. However, it is known that long-term use of glucocorticoids is associated with several adverse side effects such as inducing secondary osteoporosis [286]. In addition, since there is a rapid bone resorption during *S. aureus* induced septic arthritis, the likelihood is increasing that
glucocorticoid treatment can aggravate the development of osteoporosis [287].

Osteoprotegerin (OPG), a decoy receptor of RANK-L produced by osteoblasts, efficiently inhibits RANK-L and prevents osteoclast formation and activation. Combination therapy of OPG and antibiotics prevented bone loss in *S. aureus* induced septic arthritis suggesting potential therapeutic role of RANK-L inhibitors in combination with antibiotics in the treatment of *S. aureus* septic arthritis [89].

Biologics are superior to other anti-inflammatory agents in a sense that they can target specific molecules or cells of the immune system. TNF-α is involved in the pathogenesis of *S. aureus* septic arthritis and can be a good target for combination therapy [133, 264]. Indeed, the combination of antibiotics and anti-TNF agent significantly improved severity and frequency of septic arthritis compared to antibiotic monotherapy in a mouse model [264]. However, as with all immunosuppressors, it is critical to test the side effects of any new potential therapy before moving in the direction of any clinical trials.
8 General conclusions and future perspectives

As mentioned, the inflammatory response mounted by the host immune system to antibiotic-killed S. aureus could be one of the explanations of such a great percentage of septic arthritis patients never regaining full joint function (Paper 1) [37]. This is in line with Feis study [264], which showed that anti-TNF therapy in combination with antibiotics has been shown to have greater effect than antibiotics alone. However as mentioned above, potential dangers associated with biologics should be carefully studied. Thus, we tested the effects of several biologics in the absence of antibiotics in S. aureus induced septic arthritis.

In Paper II [107] we showed that both anti-TNF and CLTA4-Ig pre-treatment aggravate S. aureus infections in the absence of antibiotics, but will different clinical manifestations. Although anti-TNF does not increase the severity or frequency of septic arthritis compared to the control mice, it impairs the ability of the mice to efficiently eliminate bacteria in the kidneys. CTLA4-Ig on the other hand does not impair the ability of the mice to clear the bacteria compared to the controls but rather increases the severity and frequency of arthritis.

We also studied the role of IL-1Ra, a biologic that is used against several auto-inflammatory syndromes as well as RA in the same setting as above. Pre-treatment with IL-1Ra significantly increased the susceptibility to S. aureus arthritis and sepsis in mice but did not have any detrimental impact in the ability of the mice to clear bacteria as demonstrated in Paper III [207].

The combination of anti-TNF therapy and antibiotics proved to have the potential to be a novel therapeutic approach to treat S. aureus induced septic arthritis.

Our future plans are to study combination therapy of antibiotics with CTLA4-Ig or IL-1Ra, since both drugs do not hamper the ability of mice to eliminate bacteria. Our hypothesis is that a combination of these biologics with relevant antibiotics to control the infection would give a better outcome than antibiotics alone.

Our findings demonstrate that anti-TNF therapy deteriorates the clearance of S. aureus in mice while CTLA4-Ig and IL-1Ra treatment significantly increases the risk of destructive septic arthritis. Thus, if our findings are also
valid in human setting, patients at high risk of \textit{S. aureus} bacteremia such as those undergoing hemodialysis or peritoneal dialysis [288] should not be prescribed these types of biologics. Patients already undergoing anti-TNF, CTLA4-Ig or IL-1Ra treatment should be monitored carefully and the drugs immediately discontinued if the patients exhibit apparent signs of systemic infection.
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