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Study of inflammatory responses in experimental staphylococcal septic arthritis model induced by Staphylococcus aureus and extracellular vesicles

Estudo sobre as respostas inflamatórias em modelo experimental de artrite séptica induzida por Staphylococcus aureus e suas vesículas extracelulares

Versao Corrigida

Farah Fatima

Ribeirão Preto

2018

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A thesis submitted to Ribeirão Preto Medical School, University of São Paulo for the requirements to obtain doctorate of philosophy (PhD).

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Farah Fatima

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Dedication

I dedicate this thesis to my loving parents who always been a continuous source of unconditional love, prayers and great support at every step of my life.

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List of Abbreviations

µCT: Micro Computed Tomography

BSA: Bovine Serum Album

CFU: Colony-Forming Unit

ClfA: Clumping Factor A

ClfB: Clumping Factor B

ELISA: Enzyme-Linked Immunosorbent Assay

EVs: Extracellular Vesicles

FnBPA: Fibronectin-Binding Proteins A

FnBPB: Fibronectin-Binding Protein B

ICTP: Cross- linked C-Terminal Telopeptides of Type 1 Collagen

IL-6: Interleukin 6

ISEV: International Society for Extracellular Vesicles

MVBs: Multivesicular Bodies

MVs: Microvesicles

NTA: Nanotracking Analysis

OA: Osteoarthritis

PBMCs: Peripheral Blood Mononuclear Cells

PMNs: Polymorphonuclear Granulocytes

RA: Rheumatoid Arthritis

S. aureus: Staphylococcus Aureus

SA: Septic Arthritis

SEA: Staphylococcal Enterotoxins-A

SFMCs: Synovial Fluid Mononuclear Cells

TLR: Toll like receptors

TNF-a: Tumour Necrosis Factor Alpha

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CHAPTER I

Radiological features of experimental staphylococcal septic arthritis by micro computed tomography scan

ABSTRACT

ABSTRACT

FATIMA, F. Study of inflammatory responses in experimental staphylococcal septic

Septic arthritis (SA), also called infectious arthritis, is an inflammatory disease of the joints that is started by an infectious agent. The most common causative infectious agent in SA is *Staphylococcus aureus (S. aureus).* The pathogenesis of SA includes a complex inflammatory response involving both innate and adaptive immune system. Cytokines released from macrophages such as TNF-α, IL-1β and IL-6 have been classically indicated as the major players of the severe inflammation that precedes cartilage and bone destruction and permanent joint dysfunction during SA. Radiological evidence is often present but does not differentiate mechanical from septic loosening of the joints. Therefore, if there is any index of suspicion of infection, must be aspirated for microbiological evaluation. Recently, imaging technologies such as micro computed tomography (μCT) scan have been widely used for preclinical models of autoimmune joint disorders. However, the radiological features of SA in mice are still largely unknown. In current study, NMRI mice were intravenously or intra-articularly inoculated with *S. aureus* Newman or LS-1 strain. The radiological and clinical signs of septic arthritis were followed for 10 days using μCT. We assessed the correlations between joint radiological changes and clinical signs, histological changes, and serum levels of cytokines. On days 5-7 after intravenous infection, bone destruction verified by μCT became evident in most of the infected joints. Radiological signs of bone destruction were dependent on the bacterial dose. The most commonly site affected by septic arthritis was the distal femur in knees. The bone destruction detected by μCT was positively correlated with histological changes in both local and hematogenous septic arthritis. The serum levels of IL-6 were significantly correlated with the severity of joint destruction. Collectively, our data shows that μCT is a sensitive method for monitoring disease progression and determining the severity of bone destruction in a mouse model of septic arthritis; whereas, IL-6 is a potential biomarker for bone destruction in septic arthritis.

Keywords:

Staphylococcus Aureus, Septic arthritis, CT-scan, Animal model, TNF-alpha, Cytokines, IL-6

RESUMO

FATIMA, F. Estudo sobre as respostas inflamatórias em modelo experimental de artrite séptica induzida por Staphylococcus aureus e suas vesículas

A artrite séptica (AS), também chamada de artrite infecciosa, é uma doença inflamatória das articulações iniciada por um agente infeccioso. O agente causal mais comum da SA é Staphylococcus aureus (*S. aureus*). A patogênese da SA inclui uma resposta inflamatória complexa envolvendo sistema imune inato e adaptativo. As citocinas liberadas a partir de macrófagos, tais como TNF-α, IL-1β e IL-6, foram classicamente apontadas como os principais mediadores da inflamação grave que precede a destruição da cartilagem e osso e a disfunção articular permanente mediante a AS. A evidência radiológica está frequentemente presente, mas não diferencia o afrouxamento mecânico do septo das articulações. Portanto, se houver algum indício de suspeita de infecção, deve ser aspirado para avaliação microbiológica. Recentemente, as tecnologias de imagem como a micro tomografia computadorizada (μCT) foram amplamente utilizadas para modelos pré-clínicos de distúrbios articulares auto-imunes. No entanto, as características radiológicas da AS em camundongos ainda são amplamente desconhecidas. No estudo atual, os camundongos NMRI foram inoculados intravenosamente ou intra-articularmente com a cepas de *S. aureus* Newman ou LS-1. Os sinais radiológicos e clínicos da artrite séptica foram acompanhados durante 10 dias usando μCT. Avaliamos as correlações entre alterações radiológicas conjuntas e sinais clínicos, alterações histológicas e níveis séricos de citocinas. Nos dias 5-7 após a infecção intravenosa, a destruição óssea verificada por μCT tornou-se evidente na maioria das articulações infectadas. Os sinais radiológicos de destruição óssea eram dependentes da dose bacteriana. O local mais comumente afetado pela artrite séptica foi o fêmur distal nos joelhos. A destruição óssea detectada pelo μCT foi correlacionada positivamente com alterações histológicas na artrite séptica local e hematogênica. Os níveis séricos de IL-6 foram significativamente correlacionados com a gravidade da destruição das articulações. Coletivamente, nossos dados mostram que o μCT é um método sensível para monitorar a progressão da doença e determinar a gravidade da destruição óssea em um modelo de artrite séptica do mouse; enquanto que a IL-6 é um potencial biomarcador de destruição óssea na artrite séptica.

Palavras-Chave: Staphylococcus aureus, Artrite séptica, Modelo animais, Micro tomografia computadorizada, Histoquímica, ELISA, Citocinas, TNF-alpha, IL-6

_____ *INTRODUCTION*

1 INTRODUCTION

1.1 Septic arthritis

Septic arthritis (SA), also called infectious arthritis, is an inflammatory disease of the joints that is started by an infectious agent. It is the most rapidly progressive joint disease due to its destructive character including severe joint inflammation followed by irreversible cartilage/bone destruction and later permanent joint dysfunction [1, 2]. The general estimated incidence of SA in industrialized countries is about 4-10 cases per 100,000 persons per year, with the highest rates being found in those under 15 and over 55 years old [2]. The most important risk factor for SA is preexisting joint pathologies, especially rheumatoid arthritis (RA) or prosthetic joint surgery. In these patients, SA incidence increases to 70 per 100,000 persons.

1.1.2 Etiology

Staphylococcus aureus (*S. aureus*) has been reported to be the most common etiological agent in both children and adult SA [1, 3, 4]. Streptococci from groups A, B, C and G are also commonly isolated from SA in immunocompromised hosts or in patients with severe gastrointestinal or genitourinary infections [5]. *Streptococcus pneumoniae*, *Escherichia coli*, *Proteus sp.*, *Salmonella sp.*, *Serratia marcescens*, and *Neisseria sp*. have also been reported as causal agents of SA [6].

S. aureus is the primary cause of bacterial arthritis in 40% of cases from England and Wales, 56% of cases from France and 37% of cases from tropical Australia [7-9]. Of particular note, the isolation of *S. aureus* from arthritis lesions increases to 80% in joint infections in patients with concurrent rheumatoid arthritis (RA) and in those with diabetes.

1.2 Virulent factors of Staphylococcus aureus

A variety of virulence factors are associated with the ability of *S. aureus* to trigger SA, some of which are directly related to the ability of *S. aureus* to colonize the joint whereas others are related to the effect of *S. aureus* on host immunity. The virulence elements which allow *S. aureus* to adhere to certain tissues to initiate infection are classified as adhesins. The important adhesin types which facilitate the initial anchoring of *S. aureus* in the joints are: the clumping factors (ClfA and ClfB) and fibronectin-binding proteins (FnBPA and FnBPB).

ClfA is a surface protein (adhesion) that has been reported to bind with fibrinogen and fibrin [10], which facilitates *S. aureus* to cause disease, initially established in a rat model of

endocarditis [11, 12]. In fact, ClfA is able to clump bacterial cells and to promote their adherence to plasma-conditioned biomaterials, blood clots, and to catheter-damaged heart valves [10-13]. The contribution of ClfA to the pathogenesis of *S. aureus* was evaluated in a murine model of SA. The mice infected intravenously with ClfA mutant bacteria devoid of ClfA exhibited strikingly reduced arthritis severity in comparison to mice infected with the wild-type bacteria that expressed ClfA [14]. Moreover, previous passive immunization with anti-ClfA antibodies or active immunization with ClfA showed less severe arthritis. Collectively, this indicates the role of ClfA in SA.

Collagen-binding protein is another adhesin that was originally isolated from the cell surface of *S. aureus*, and is known to mediate the attachment of *S. aureus* cells to cartilage [15]. The arthritogenic properties of this protein were studied with two classes of *S. aureus* mutants, which confirmed the contribution of collagen adhesin gene of *S. aureus* strain. The animals injected with the strain containing this gene developed arthritis whereas the animals injected with mutant *S. aureus* strain lacking this gene did not develop symptoms with few exceptions [16].

It has been demonstrated that the fibronectin-binding proteins (FnBPA and FnBPB) expressed by *S. aureus* are associated with the recognition of fibronectin, fibrinogen and elastin [17-19]. These FnBPs not only facilitate *S. aureus* adherence but also stimulate further invasion of different cell types, such as osteoblasts, fibroblasts and, epithelial and endothelial cells and trigger bacterial invasion [20-22]. It has been suggested that FnBPs might provide a mechanism by which the staphylococci could evade host defenses and escape being killed by antibiotics.

In addition to the clumping factors and fibronectin-binding proteins, the biofilm-forming capacity of staphylococci has been described as major virulence determinant in mediated *S. aureus* infection [23, 24]. Biofilms are communities of bacterial cells, present on a surface, that are held together by a matrix of extracellular substances from both the bacteria and the host. It has been suggested that articular structures and implanted medical devices serve as potential support for the growth of bacterial communities [25, 26].

In addition, it has been shown that *S. aureus* secretes a large number of enzymes and toxins which are implicated as potential virulence factors in infectious SA [27]. Subsets of these molecules hold unique ability to activate a large number of T lymphocytes expressing certain Vβ sequences and therefore, are able to display superantigenic properties.

These superantigens (SAg) containing TCR Vβ elements can activate large number of T cell pool [28]. In fact, Vβ recognition can bind to antigen-presenting cells via MHC class II molecules simultaneously, and such interaction allows T cell proliferation and elevated level of cytokine release [29, 30]. The contribution of SAg in the development of SA has been evidently observed in experimental model of arthritis [31-33]. The toxic shock syndrome toxin-1 (TSST-1) SAg has been more frequently detected (47%) in the synovial fluid of SA patients [34].

1.3 Pathogenesis of septic arthritis

One of the major hallmarks of septic arthritis is the persistence of substantial inflammation that precedes cartilage and bone destruction. The pathogenesis of septic arthritis is linked to elevated virulence of *S. aureus* compared to other infectious agents is primarily attributed to diverse immune evasion strategies presented by *S. aureus* [35]. This includes complex inflammatory response involves both innate and adaptive immune system, and the participation of pro-inflammatory cytokines causing bone damage in the affected joints [1]. Of particular note, the immune-evasion strategies by *S. aureus* such as intracellular survival in osteoblasts [36], neutrophils [37], and endothelial cells [38] may cause the persistence of joint infection. Some of the evasion mechanisms such as the expression of an extracellular capsule, the release of formylated peptides and the production of molecules accompanied by superantigenic properties have been correlated with higher arthritogenicity. Formylated peptides could mediate the recruitment of neutrophils into the synovial tissue, which aggravate the joint destruction [39-42].

Histopathological analysis of swollen joints shows pannus formation, hypertrophy and proliferation of the synovial tissue, as well as cartilage and bone destruction [27]. Clinically, experimental arthritis has been described to occur in both fore and hind paws and is characterized by visible erythema and edema.

In addition to bacterial virulence factors, it is now well established that the joint-damaging process is associated with host immune response. It has been considered that the local bacterial proliferation is favored by a rapid recruitment of activated macrophages and polymorphonuclear granulocytes (PMNs) subsequently followed by T cells [42, 43]. Bacteria could start protein synthesis with a formyl methionine residue, and the resultant formylated peptides are considered potent chemoattractants for PMNs [44]. The mice infected with a mutant strain lacking the

ability to produce formylated peptides demonstrate much more discrete recruitment of PMNs in the synovial tissue.

Cytokines such as TNF-α, IL-1β and IL-6 released from macrophages have been characteristically described as major players of the severe inflammation that precedes cartilage and bone destruction during SA. Importantly, cytokines have been shown to stimulate osteoclast differentiation and bone resorption in a synergistic fashion [45]. TNF-α, considered the most osteoclastogenic cytokine, which activates NF-kB resulting in the survival of osteoclasts [43]. Recently Ali *et al.* [46], has shown that via TNF receptor 1, antibiotic-killed *S. aureus* causes long-lasting joint inflammation that might lead to post-infectious complications of septic arthritis. However, it is also important to highlight the ability of cytokines to protect the host against the infectious agent [47]. The protective role is demonstrated by the explanation that the mice lacking IL-1R type 1 can develop a much more severe SA compared with intact controls, in response to infection with *S. aureus* [48].

T cells and their cytokines have been clearly demonstrated in S. aureus-induced SA, but not the B cells [49]. In the affected joints from experimentally infected mice, the contribution of T cells was indicated by predominant presence of CD4+ phenotype [32]. Assays developed for targeting of T cell-derived cytokines confirmed the participation of CD4+ subset. IL-17 is a recently described cytokine, produced by T cell subsets and many innate-like T cells [50], and is considered an important mediator of RA in both, humans and mice[51]. It has been suggested that IL-17A is implicated in local rather than systemic host defense against *S. aureus*-induced SA. Augmenting body of evidence suggests that SAg can stimulate a great fraction of T cells, which is followed by their proliferation and subsequent secretion of cytokines and chemokines. The possible contribution of SAg such as TSST-1 to SA development has been demonstrated in various reports [31-33].

Collectively, host–bacterium interactions and the associated inflammatory responses during pathogenesis of *S. aureus* arthritis are summarized in **figure 1**.

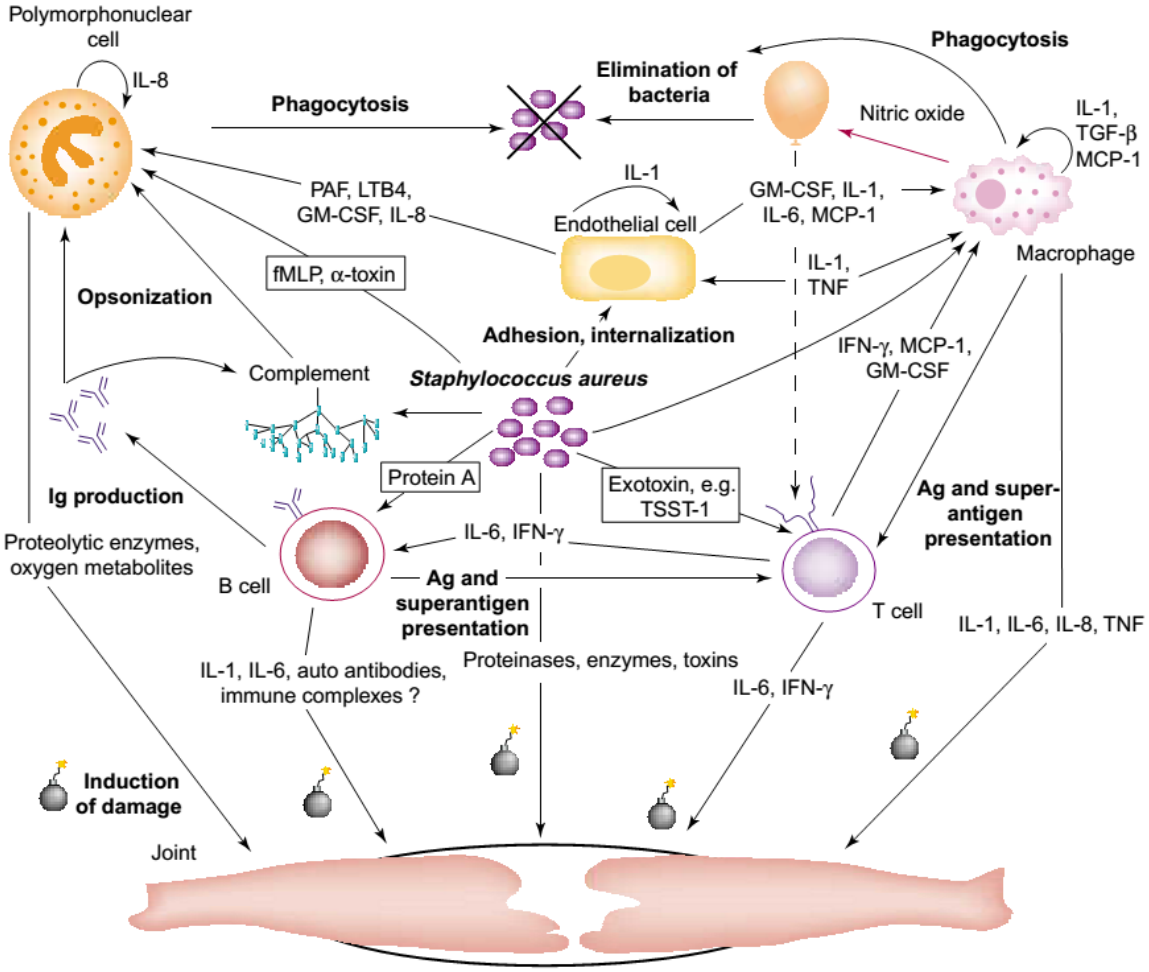

Figure 1. Hypothetical and simplified scheme of host–bacterium interactions during *S. aureus* induced arthritis and related immune responses (Figure adopted from Tarkowski *et al* [52].

1.4 Diagnosis

Diagnosis includes histopathological examination, fluid examination for microbial detection. Usual case-definition relies on criteria that requires one of four points to be met: (i) isolation of a pathogenic agent from an affected area; (ii) isolation of a pathogenic agent from another source (e.g. blood) in the context of a suspicious infection; (iii) typical clinical features and turbid fluids in the presence of previous antibiotic treatment; and (iv) postmortem or pathological features suspicious of disease onset.

Radiological evidence is often present but does not differentiate mechanical from septic loosening. Therefore, if there is any index of suspicion of infection, must be aspirated for microbiological evaluation [5].

1.5 Treatments and therapy

In past few decades, challenge is posed by increasing antibiotic resistance of *S. aureus* and spread of highly virulent resistant strains. *S. aureus* possess a plethora of elements and components that provoke host response and contribute to the pathogenesis of *S. aureus*-mediated septic arthritis. Host responsiveness protects the host against bacteria, but sometimes potentiates the infection severity when staphylococcal danger signals trigger exaggerated host responses. The imbalanced immune responses result in either deteriorated bacterial clearance resulting in chronic osteomyelitis or excessive inflammation leading to tissue damage.

Septic arthritis management is growing in complexity with the advent of novel and antibiotic resistant causative microorganisms and within the current climate of increased immunosuppression. Findings from animal models and patients are shedding light on disease pathogenesis and the possibility of novel adjunctive treatments, including systemic corticosteroids, cytokines and anti-cytokines, and bisphosphonates.

It has been shown that administration of sulfatide, a myelin self-glycosphingolipid that activates a type II NKT cell subset, significantly improved the survival rate of mice with *S. aureus* sepsis. The protective effect of sulfatide treatment depended on CD1d, but not on type I NKT cells [53]. Administration of IFN-γ before or after *S. aureus* inoculation is sown to decrease mortality rate but also could increase arthritis development [54]. Moreover, anti–tumour necrosis factor (anti-TNF) treatment attenuates joint inflammation and bone destruction **Figure 2**. Importantly,

combination therapy of antibiotics and a TNF inhibitor resulted in a quicker relief of septic arthritis in mice compared to the antibiotic monotherapy [55].

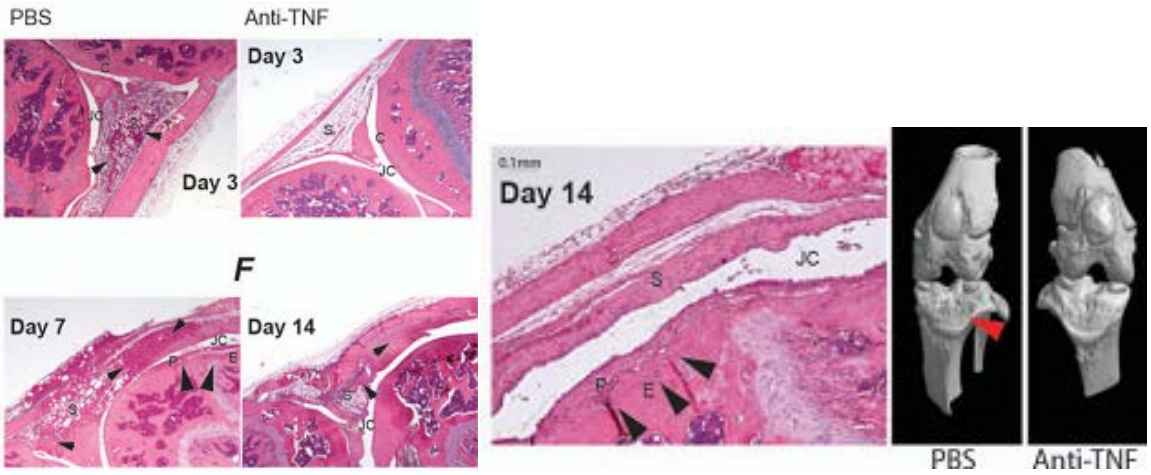

Figure 2. Anti–tumour necrosis factor (anti-TNF) treatment attenuates joint inflammation and bone destruction. Figure adopted from Ali *et al.*, 2015 [46].

1.6 Experimental models of septic arthritis

Undoubtedly, animal models are invaluable source for understanding the pathogenesis of many human diseases. Unfortunately, the investigation of septic arthritis in humans is hampered by difficulty in establishing the infection onset time and also difficulty in obtaining tissue samples from different regions of the joint such as the cartilage, the subchondral bone and the synovial membrane [56]. Due to such hindrances mice infected with *S. aureus* is used as an experimental model of arthritis.

For a long time (4 decades ago) the rabbits intra-articularly injected with bacteria have been used to study septic arthritis [57]. However, as in many other diseases where immune system plays a central role, mice have been adopted for such experimental studies. This is mainly because the characteristics of the murine model are known to closely mirror the changes detected in human septic arthritis, such as the severity of periarticular bone erosivity [43]. The immune system of mice is considered similar to the human counterpart in many aspects and is particularly well characterized. There is also a plethora of mice strains lacking (knockout mice) or overexpressing certain genes (transgenic), which enables a deeper investigation of their implications to pathologies under investigation. The general application of our own experimental models will be discussed in material methods and results section.

1.6.1 NMRI, C57BL/6, 1295 V and BALB/c models

Induction of experimental arthritis by *S. aureus* infections has been successful with certain mice strains such as NMRI, C57BL/6, 1295 V and BALB/c [58-60]. This important to consider that even though *S. aureus* is the leading cause of infectious arthritis, not all the strains are arthritogenic. Tarkowski group (University of Gothenburg, Sweden) has greatly contributed to establishing the basis of SA models [52]. This group usually has been employing a bacterial strain denominated LS-1, originally isolated from a spontaneous outbreak of *S. aureus*-mediated arthritis in a mouse colony [61]. Importantly, the production of the exotoxin TSST-1 contributes to arthritogenicity. It is largely due the reason that mice injected with TSST-1-producing *S. aureus* strain was able to develop more frequent and severe disease as compared to strains that do not produce this SAg [32, 33]. However, *S. aureus* strains are also able to produce other Sag such as enterotoxin C and enterotoxin A that have capacity to trigger septic arthritis [62].

Additionally, the bacterial dose and route of administration are relevant parameters in these models, which typically ranges from 7.106 to 107 *S. aureus* colony-forming units (cfu) per mouse, and to be injected via intravenous route [52]. There is a consensus that the best way to trigger SA is by the intravenous route. In particular, such procedure could better mimic a large majority of bacterial joint infections in humans that are believed to originate from the blood [63]. However, it has been shown that the bacterial injection via retro-orbital route is fast, highly effective and importantly could generate a pathology that is very homogeneous. This model offers the analysis of disease development having daily joint inspection. To evaluate the arthritis intensity, a clinical scoring (i.e. arthritic index) is recorded using a system where macroscopic inspection yields a score of 0-3 points for each limb (1 point = mild swelling and/or erythema; 2 points = moderate swelling and erythema; 3 points = marked swelling and erythema). Finally, the arthritis index is built by dividing the total score (i.e. number of arthritic limbs by the number of animals used in each experimental group) [32, 33, 62]. Such model has been extensively used to assess bacterial virulence factors, host defense mechanisms and the immunopathogenetic mechanisms that cause the severity in joint destruction [14, 27, 52].

The study of septic arthritis in humans is hindered by the fact that it is challenging to establish the infection onset time and also the difficulty in obtaining the tissue samples from the different parts of the joint [56]. An optimal animal model resembling the human disease is necessary to interrogate the distinct mechanisms of disease pathology in order to identify potential biological targets in pursuit of novel therapeutics. Mouse model for *S. aureus* hematogenous septic arthritis is a well-established animal model [64], which has clarified the involvement of several bacterial virulence factors in relation to host immune cell types and cytokines in the pathogenesis of this disease. This model exhibits similar features to human septic arthritis that provides a straightforward and rapid means of producing this pathology [64].

The establishment of animal model for *S. aureus* mediated septic arthritis has demonstrated the involvement of several bacterial virulence factors, as well as some host immune cell types and cytokines, in the pathogenesis of the disease [52]. This model parallels the pathogenesis of human disease closely in that the pathogen is introduced haematogenously by intravenous injection. More than 90% of mice develop septic arthritis within 24 hours of inoculation and their joints have a severe degree of bone erosion similar to changes seen in the human septic joint. This model initially developed at Gothenburg Sweden, has been widely used worldwide to study

both host and bacterial virulence factors implicated in the establishment of infection and immune responses.

1.7 Imaging Technologies

Over the last two decades, imaging approaches such as ultrasonography, magnetic resonance imaging (MRI) and computed tomography (CT) have made major advances in the early diagnosis and therapeutic monitoring of autoimmune joint disorders in patients[65] and in various experimental models for autoimmune joint diseases to gain a deeper understanding of disease pathophysiology [66]. Among those methods, micro CT (μCT) has been extensively used in rodent models for osteoporosis [67, 68], osteoarthritis [69], and rheumatoid arthritis[70, 71], due to the short acquisition time and instantaneous identification and quantification of disease progression. μCT was used as a supplementary method in our previous studies to determine the extent of bone destruction in mice with septic arthritis [46, 72, 73]. However, systematic descriptions of radiological changes of joints in mouse models for septic arthritis are still largely lacking.

1.7.1 Micro computed Tomography Scan (μCT)

Micro-CT is an x-ray imaging in 3D on a small scale with massively increased resolution. This technique provides 3D microscopy of very fine scale internal structure of objects non-destructively. It has several advantages over other imaging techniques such as short acquisition time, good spatial resolution (50 μm) and moreover all three planes (transverse, coronal, and sagittal) are acquired simultaneously.

In clinical studies it provides instantaneous identification and quantification of disease progression. It has been widely used now for small animal models to detect morphological changes such as rodent models for osteoporosis, osteoarthritis and rheumatoid arthritis.

AIM AND OBJECTIVES

2. AIM AND OBJECTIVES

Permanent joint dysfunction due to bone destruction remains a major challenge in patients with septic arthritis. Recently, imaging technologies such as micro computed tomography (μCT) scan have been widely used for preclinical models of autoimmune joint disorders. However, the radiological features of septic arthritis in mice are still largely unknown.

The aim of the current study was to determine the radiological features of experimental *S. aureus* septic arthritis in mice. The specific objectives are;

1. To determine the onset time point of bone destruction by μCT scan after infection.
2. To determine which joint is mostly effected post infection and the specific bone having maximum destruction.
3. Correlation between bone destruction verified by μCT scan and several parameters of septic arthritis.
4. To study the local infection model of septic arthritis and comparing its radiological and histological signs.
5. To identify potential biomarkers of bone destruction.

METHODOLOGY

3. MATERIAL METHODS

3.1 Mice

Female NMRI mice, 6–8 weeks old, were purchased from Charles River Laboratories (Sulzfeld, Germany). In total, 150 mice were used in this study. They were bred and housed in the animal facility of the Department of Rheumatology and Inflammation Research, University of Gothenburg. The mice were kept under standard conditions of temperature and light, and were fed laboratory chow and water ad libitum. The Ethical Committee of Animal Research of Gothenburg approved the study.

3.2 Bacterial strains and reagents

S. aureus laboratory strain Newman and clinical isolate LS-1 [61], were cultured separately on blood agar plates for 24 h, harvested, and kept frozen at -20°C in phosphate-buffered saline (PBS) containing 5% bovine serum album (BSA) and 10% dimethyl sulfoxide (DMSO). Before experiments, the bacterial suspension was thawed, washed in PBS, and adjusted to the required concentration [55].

3.3 Kinetic study of bone destructions in mice with septic arthritis

10 NMRI mice were intravenously (i.v) injected with *S. aureus* newman ($8x10^6$ cfu/mouse). All 4 limbs were inspected for clinical signs of arthritis at day 3, day 5, day 7 and day 10. The mice were anesthetized with an intraperitoneal injection of 200µl of ketamine/xylazine mixture. The affected joints with signs of septic arthritis (redness and swelling) along with both knees were then scanned *in vivo* on respective days with Skyscan 1176 micro CT (Bruker, Antwerp, Belgium). After scanning, the mice were allowed to wake up by receiving 150-200 µl of antisedan as the antidote and resumed the experiment until day 10.

3.4 Dose dependent inoculation of staphylococcal septic arthritis

To study whether bone destructions in septic arthritis are bacterial dose-dependent, two experiments were performed using both *S. aureus* Newman and LS-1. In the first experiment, two groups of NMRI mice (n=8-10 mice/group) were inoculated with *S. aureus* LS-1 intravenously (i.v.) into the tail vein with 0.2 ml of staphylococcal suspension in two doses ($1x10^6$ and $1x10^7$ cfu/mouse). In the second experiment another two groups of NMRI mice (n=8-9) were injected (i.v) with *S. aureus* Newman in two doses (2×10^6 and 1.2×10^7 cfu /mouse). In

the next three experiments, the correlation between bone destructions and different parameters of staphylococcal arthritis were assessed. The mice (n = 48) were inoculated with a suboptimal arthritogenic dose of 1.1–1.7x10^6 cfu of *S. aureus* Newman to induce variant degree of septic arthritis.

3.5 Clinical examination

All the mice from these 5 experiments were regularly weighed and clinically examined for arthritis incidence and severity by two observers blinded to the dose given to the groups. After sacrificing the mice at day 10, the kidneys were obtained for the assessment of bacterial persistence, serum samples were collected to assess the levels of cytokines and the 4 limbs were obtained for radiological examination of bone erosions. Thereafter, the limbs from *S. aureus* Newman infected mice were further microscopically evaluated for the expression of synovitis and destruction of cartilage and bone.

To understand which particular joints are most often affected by septic arthritis in mice, five independent experiments were performed. 42 NMRI mice were i.v. injected with the optimal septic arthritis dose (5-8 x 10^6 cfu/mouse) of *S. aureus* Newman strain. All 4 limbs were collected after sacrificing the mice on day 10. Micro computed tomographic scan was carried out to determine the extent of bone destruction.

3.6 Mouse model for local *S. aureus* arthritis

NMRI mice (n=10) were inoculated intra-articularly in the knee joints with *S. aureus* Newman strain (1x10^3 cfu/ knee). The mice were sacrificed 10 days later. Knee joints were collected for µCT scan and histological examination.

3.7 Clinical evaluation of septic arthritis

Observers blinded to the groups visually inspected all 4 limbs of each mouse. Arthritis was defined as erythema and/or swelling of the joints. To evaluate the severity of arthritis, a clinical scoring system ranging from 0 to 3 was used for each limb (0, no inflammation; 1, mild visible swelling and/or erythema; 2, moderate swelling and/or erythema; 3, marked swelling and/or erythema). The arthritis index was constructed by adding the scores from all 4 limbs for each animal as described previously [55]. Arthritis that involved 5 or more joints simultaneously was defined as polyarthritis.

3.8 Bacteriologic examination

Kidneys were aseptically removed and blindly assessed by two investigators (M.N and F.F) for abscesses. A scoring system ranging from 0 to 3 was used (0, healthy kidneys; 1, 1 or 2 small abscesses on kidneys without structural changes; 2, more than 2 abscesses but <75% of kidney tissue involved; and 3, large amounts of abscesses with >75% of kidney tissue involved). Afterward, the kidneys were homogenized, diluted serially in PBS, and transferred to agar plates containing 5% horse blood. Bacteria were grown for 24 h and quantified as cfu.

3.9 Micro computed tomography

For *in vivo* kinetics study of bone destruction, the scanning was done at 55 kV and 434 mA, with a 1-mm aluminum filter. The exposure time was 47 ms. The X-ray projections were obtained at 0.7° intervals with a scanning angular rotation of 180° (procedure published [74]).

For all other experiments, joints were fixed in 4% formaldehyde for 3 days and then transferred to PBS for 24 h. Afterwards, all 4 limbs were scanned and reconstructed into a three-dimensional (3D) structure with a Skyscan1176 micro-CT (Bruker, Antwerp, Belgium) with a voxel size of 35 _m. The scanning was done at 55 kV and 455 mA, with a 0.2-mm aluminum filter. The exposure time was 47 ms. The X-ray projections were obtained at 0.7° intervals with a scanning angular rotation of 180°. The projection images were reconstructed into three-dimensional images using NRECON software (version 1.5.1; Bruker). After reconstruction, the 3D structures of each joint were blindly assessed by 2 observers (T.J. and F.F) using a scoring system from 0 to 3(0, healthy joint; 1, mild bone destruction; 2, moderate bone destruction; 3, marked bone destruction).

3.10 Histopathological examination of joints

After the scanning, the joints were decalcified, embedded in paraffin, and sectioned with a microtome. Tissue sections were stained with hematoxylin and eosin. All the slides were coded and assessed in a blinded manner by two observers with regard to the degree of synovitis and cartilage/bone destruction. The extent of synovitis and cartilage/bone destruction was judged as previously described [46].

3.11 Measurement of cytokine levels

The cytokine levels of TNF-α, IFN-γ IL-10, IL-6, IL-4 and IL-17 in serum were determined using a Cytometric Bead Array (CBA) mouse inflammation cytokine kit (BD Biosciences) and analyzed using a FacsCanto2 flow cytometer (BD Biosciences). The data was analyzed using FCAP array software (BD Biosciences).

3.12 Measurement of bone destruction marker

The serum levels of cross- linked C- terminal telopeptides of Type 1 collagen (ICTP) were determined by mouse ICTP ELISA kit according to the protocols recommended by the manufacturer (My Biosource.com) and analyzed by SoftMAx pro ELISA reader.

3.13 Statistical analysis

The statistical significance between groups was assessed using the Mann–Whitney U test and the $\chi 2$ test. Spearman's correlation was used to calculate correlation coefficients. The GraphPad Prism (version 6) software was used for calculations. The results are reported as the mean ± the standard error of the mean (SEM) or median. A two-tailed p value of <0.05 was considered statistically significant.

RESULTS

4. RESULTS

4.1 Examination of bone destruction by µCT scan after infection

To study the development of bone destructions in mice with septic arthritis, ten NMRI mice were intravenously infected with *S. aureus* Newman and followed by *in vivo* CT scan on different days (day 3, 5, 7 and 10) after infection. The 3-dimensional joint structures were semi-quantitatively assessed by 3 observers in a blind manner, using a scoring system from 0 to 3 (**Figure 3A**). The mice showing clinical signs of arthritis at day 10 also had highest detectable bone erosion by µCT scan. Interestingly, on day 3 only 20 % of bone destructions were possible to be detected by µCT-scan (**Figure 3B**), whereas on day 5 we were able to detect 8 out of 10 bone erosions. Importantly, on day 7 bone destructions became evident in all infected joints, suggesting that µCT-scan is sensitive to detect most of the joint infections on day 7 after intravenous infection. The representative pictures (**Figure 3C**) demonstrate the progression of bone destructions with the passage of time. On day 0 the knee joint was completely intact and healthy. Signs of bone destructions were starting to appear on day 3 at fibula whereas structural changes also appeared on femur. On day 5 relatively mild bone destructions became evident on both distal femur and tibia. On day 7 distal femur and tibia were moderately infected, and heavily eroded with large bone erosions on day 10 (**Figure 3C**).

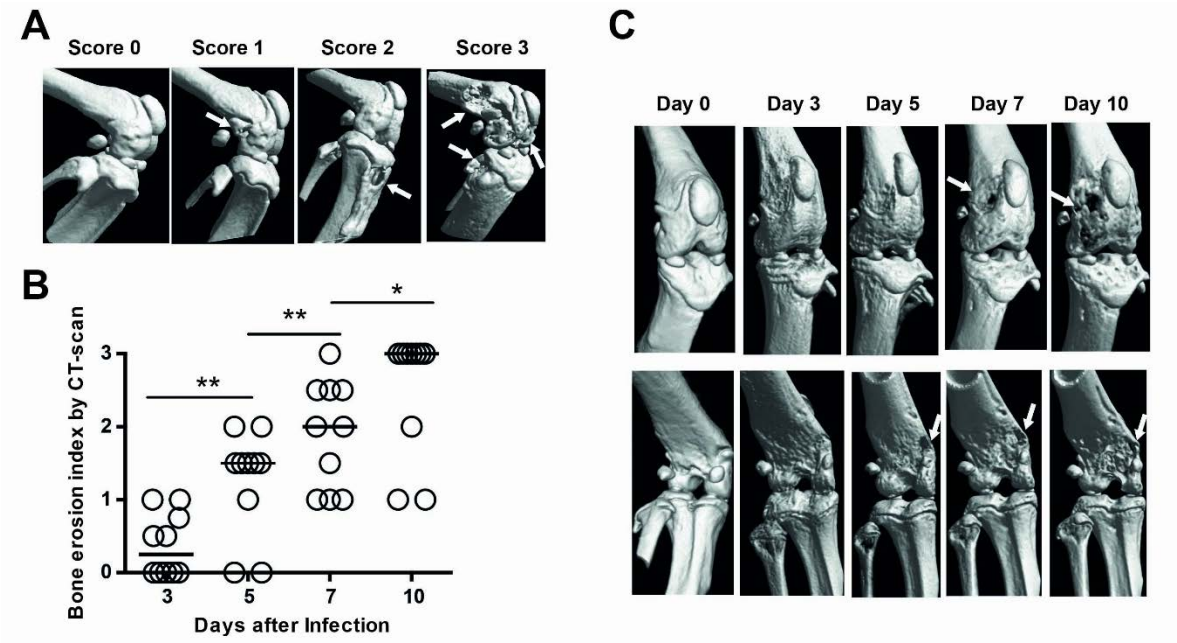

Figure 3. Micro-CT scan is useful to monitoring the progression of bone destruction in mice with *S. aureus* septic arthritis. Ten NMRI mice injected intravenously with *S. aureus* Newman (8 x106 cfu/mouse) were serially scanned to identify bone destruction by μCT on days 0, 3, 5, 7, and 10. (**A)** Representative CT images demonstrating the scoring system for determining the extent of bone destruction in affected joints: intact knee joint (score 0), a knee joint with mild bone destruction on the distal femur (score 1; arrow), a knee joint with moderate bone erosion on the proximal tibia (score 2; arrow), and a heavily destroyed knee joint on the distal femur and proximal tibia (score 3; arrows). (**B)** Changes in bone destruction severity by μCT scan with time in affected joints. **(C)** Representative CT images in both anterior (upper panel) and posterior (lower panel) views of a knee joint showing progression of bone destruction caused by septic arthritis on different days after infection: Intact knee joint on day 0; Suspected but no clear sign of bone damages on day 3; clear signs of bone damage at the distal femur on a posterior view on day 5 (arrow); moderate bone erosion at the distal femur on both anterior and posterior views on day 7 (arrows); heavily destroyed knee joint at the distal femur and fibula on both anterior and posterior views on day 10 (arrows) [74].

4.2 Bacterial dose-dependent radiological signs of bone destructions

Higher dose ($1x10^7$ cfu/mouse) of *S. aurues* strains, LS-1, significantly increased the severity of clinical arthritis in mice compared to the lower dose ($1x\ 10^6$ cfu/mouse). The difference was clear already on day 3 (p=0.03) after bacterial inoculation and increased overtime until day 7 (p= 0.01) and stabilizing at the end of the experiment on day 10 (p= 0.06) (**Figure 4A**). A very similar trend was also observed when the *S. aureus* Newman strain was used, although no statistical significance was reached (**Figure 4B**).

To confirm the clinical arthritis data, µCT was applied to determine whether bone destruction in joints is bacterial dose-dependent. We found that both severity and frequency of bone erosions were significantly higher in NMRI mice infected with the high dose of both *S. aureus* strains (LS-1 and Newman) compared to the lower doses. In mice infected with higher dose of *S. aureus* LS-1, both the severity (p=0.03) and frequency (18% vs 5%, p=0.008) were significantly higher compared to the lower dose of bacteria (**Figure 4C**). Despite no significance was reached regarding the clinical arthritis, significant dose-dependent pattern in both severity and frequency was observed when *S. aureus* Newman was used (**Figure 4D**).

In line with results from radiological signs of septic arthritis, the histopathological changes of septic arthritis were also more severe in mice receiving higher dose of *S. aureus* Newman compared to the lower dose. The extent of joint destruction (p=0.04) as well as the histologically verified synovitis (p=0.05) were more severe in NMRI mice receiving higher dose of *S aureus* Newman (**Figure 4E**).

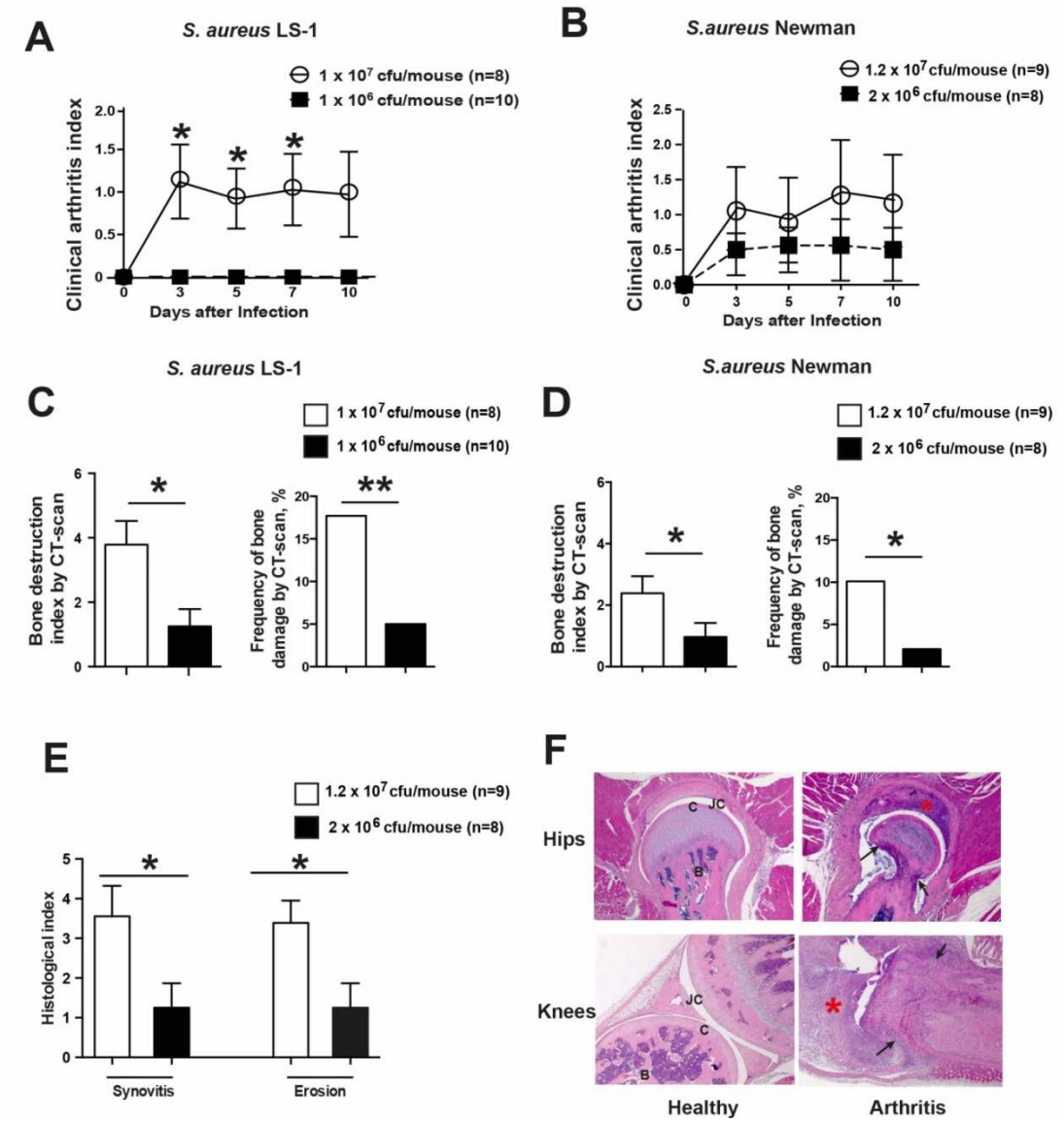

Figure 4. Bone destruction verified by µCT is dependent on bacterial dose in mice with septic arthritis. NMRI mice (n = 8-10/group) were inoculated intravenously with two doses of S. aureus LS-1 (1 x 106±1 x 107 cfu/mouse) and Newman (2 x 106±1.2 x 107 cfu/mouse). The severity of clinical arthritis in the mice infected with (A) S. aureus LS-1 and (B) S. aureus Newman was observed until the animals were euthanized on day 10. The bone destruction scores and frequency of bone damage from all 4 limbs of mice infected with the (C) LS-1 or (D) Newman strains were analyzed by µCT scan. (E) Histological evaluation of the joints from all 4 limbs 10 days after infection. (F) Representative micrograph of intact hip (upper left) and knee (lower left) joints as well as the heavily inflamed hip (upper right) and destroyed knee (lower right) joints from mice inoculated with S. aureus strain Newman. Original magnification ×10. The asterisks indicate heavily inflamed synovium. Arrows indicate the bone erosion. Statistical evaluations were performed using the Mann—Whitney U test. Data are presented as the mean values ± standard errors of the mean. Abbreviations: B, bone; C, cartilage; JC, joint cavity; S, synovial tissue [74].

4.5 Weight development

Both *S. aureus* LS-1 and Newman infected mice had negative weight development during the course of infection. Only mice receiving *S. aureus* LS-1 had significantly more weight loss (**Figure 5A and 5B**). In accordance with this, the significant difference was only found in mice receiving LS-1 strain regarding the bacterial load in kidneys between high and low bacterial doses strain (**Figure 5C and 5D**).

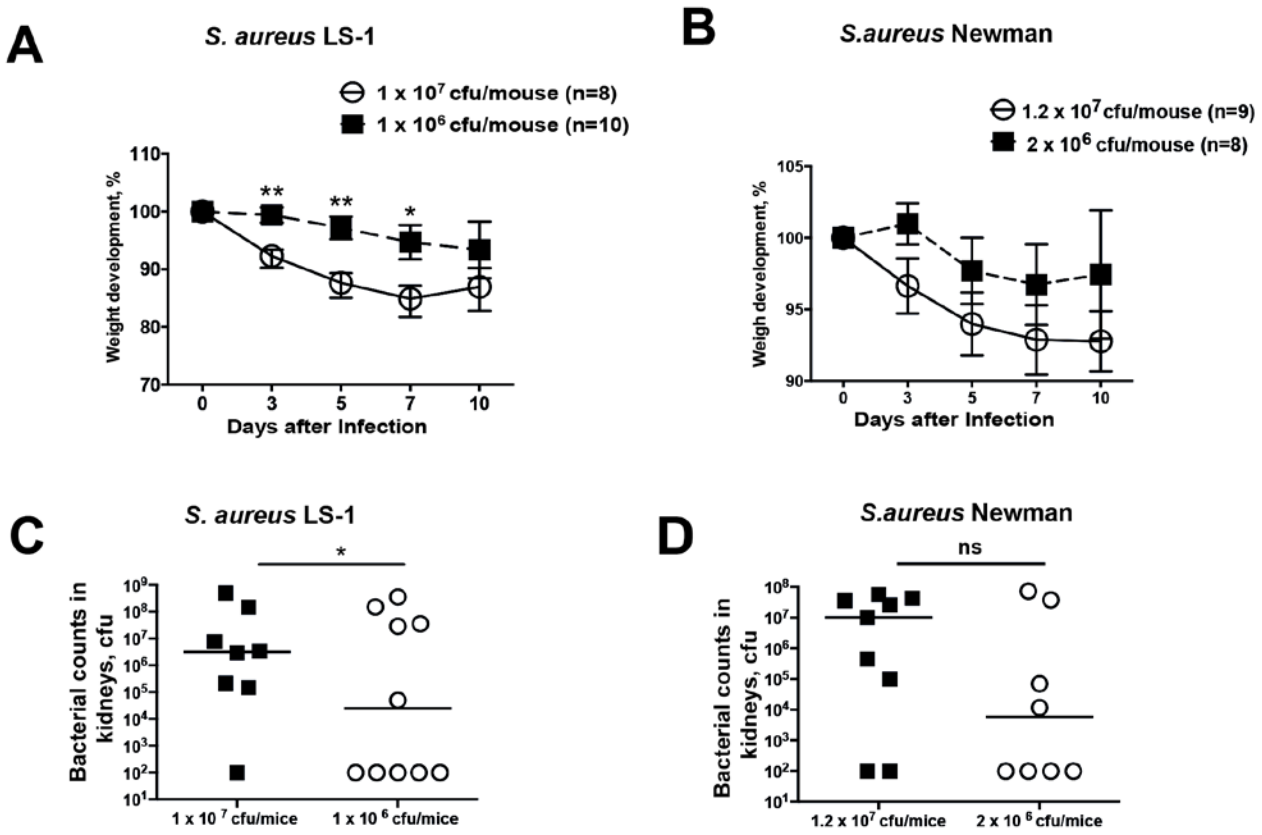

Figure 5. The weight development and bacterial load in kidneys in mice with *S. aureus* septic arthritis. NMRI mice (n = 8–10) inoculated intravenously with two doses of *S. aurues* LS-1 (1x 10^6–1x 10^7 cfu/mouse) and *S. aurues* Newman (2x 10^6–1.2 x 10^7 cfu/mouse) were sacrificed on day 10 after infection. **(A-B)** Percentage changes in body weight registered from day 0 in mice infected with **(A)** *S. aureus* LS-1 and **(B)** *S. aureus* Newman. **(C-D)** Persistence of bacterial strains **(C)** *S. aureus* LS-1 and **(D)** *S. aureus* Newman in kidneys of NMRI mice. Mean±SEM. * $p<0.05$. ** $p<0.01$. Mann-Whitney U test.

4.6 Most commonly affected joints in septic arthritis were knees

42 NMRI mice from 5 independent experiments were evaluated for a more detailed sub group analysis to investigate which particular joints were affected by septic arthritis in mice receiving optimal septic arthritis dose (5-8x10^6 cfu/ mouse) of *S. aureus* Newman. More than half of the knee joints displayed signs of bone destruction. This was followed by 28.5 % of shoulder joints, 27.2 % of feet (including ankles and toes), and 20.2 % of hip joints. In this analysis, the least frequently affected joints were hands (including wrists and fingers) and elbows, which only represent 11.9 % and 5.9%, respectively. We further analyzed both the severity and frequency of bone destruction in respective joints from upper and lower limbs (**Figure 6A and 6B**). Interestingly, knee joints had strikingly more frequent and severe bone damages compared to the elbow joints (p<0.0001), whereas shoulder and hips, as well as hands and feet showed no significant difference (**Figure 6B**). Description about **Figure 6C** through **6H** is provided in legends.

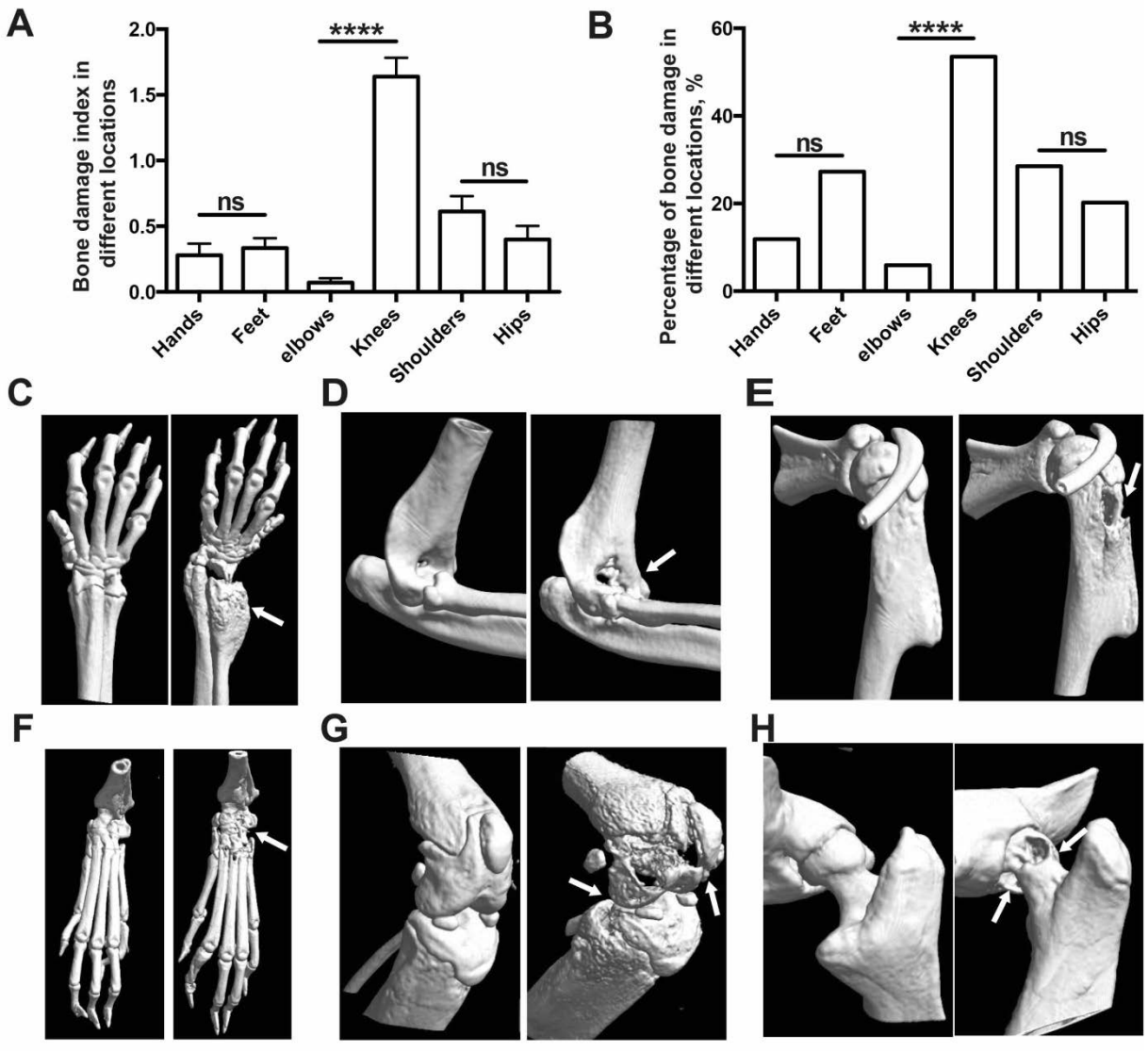

Figure 6. Knee joint is most often affected in *S. aureus* septic arthritis. NMRI mice (n = 42) were intravenously injected with *S. aureus* strain Newman (5–8 x 10^6cfu/mice) and all joints from 4 limbs were examined by μCT scan on day 10 after infection. Severity **(A)** and frequency **(B)** of bone destruction in different locations were compared. **(C-H)** Representative computed tomographic images showing both intact (Left) and heavily eroded (Right) joints, **(C)** wrist, **(D)** elbow, **(E)** shoulder, **(F)** ankle, **(G)** knees and **(H)** hips. Arrows indicate the bone erosion. Statistical evaluations were performed using the Mann-Whitney U test and Fisher exact test. Data are presented as the mean values ± standard errors of the mean.

Very similar pattern was observed when mice were infected with *S. aureus* clinical strain LS-1 (**Figure 7A and 7B**); suggesting this finding is highly reproducible.

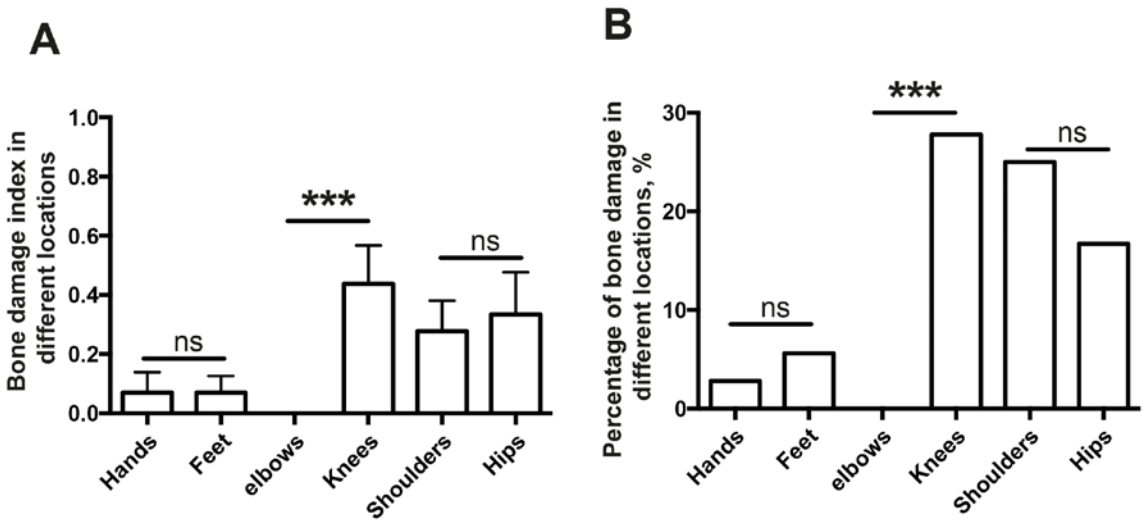

Figure 7. Knee joint is the most often affected in septic arthritis induced by *S. aureus* LS-1. NMRI mice (n = 8–10) were intravenously injected with *S. aureus* LS-1 ($1x10^6$–$1x\ 10^7$cfu/mouse) and all joints from 4 limbs were examined by μCT scan on day 10 after infection. Severity **(A)** and frequency **(B)** of bone destruction in different locations were compared. ns = not significant; Mann-Whitney test *U* test or Fisher's exact test.

4.7 Distal femur was the most often affected part by septic arthritis in knees

To further evaluate which part of the knee joints are the most engaged during the septic arthritis, we evaluated all knees from those 42 NMRI mice by µCT scan. Most frequently affected site in knee joints is distal femur (47.6 %), followed by proximal tibia (32.1 %) and proximal fibula (16.7 %) (**Figure 8A**). Moreover, 23.8 % of knees had two or more than two bones affected. Computed tomography scans showing different sites of the knee affected by septic arthritis. **(Figure 8B**) Heavily destroyed femur, (**Figure 8C**) evident destruction on the tibia, (**Figure 8D**) infected fibula, (**Figure 8E**) damaged knee joints with involvement of several bones including femur, tibia and fibula.

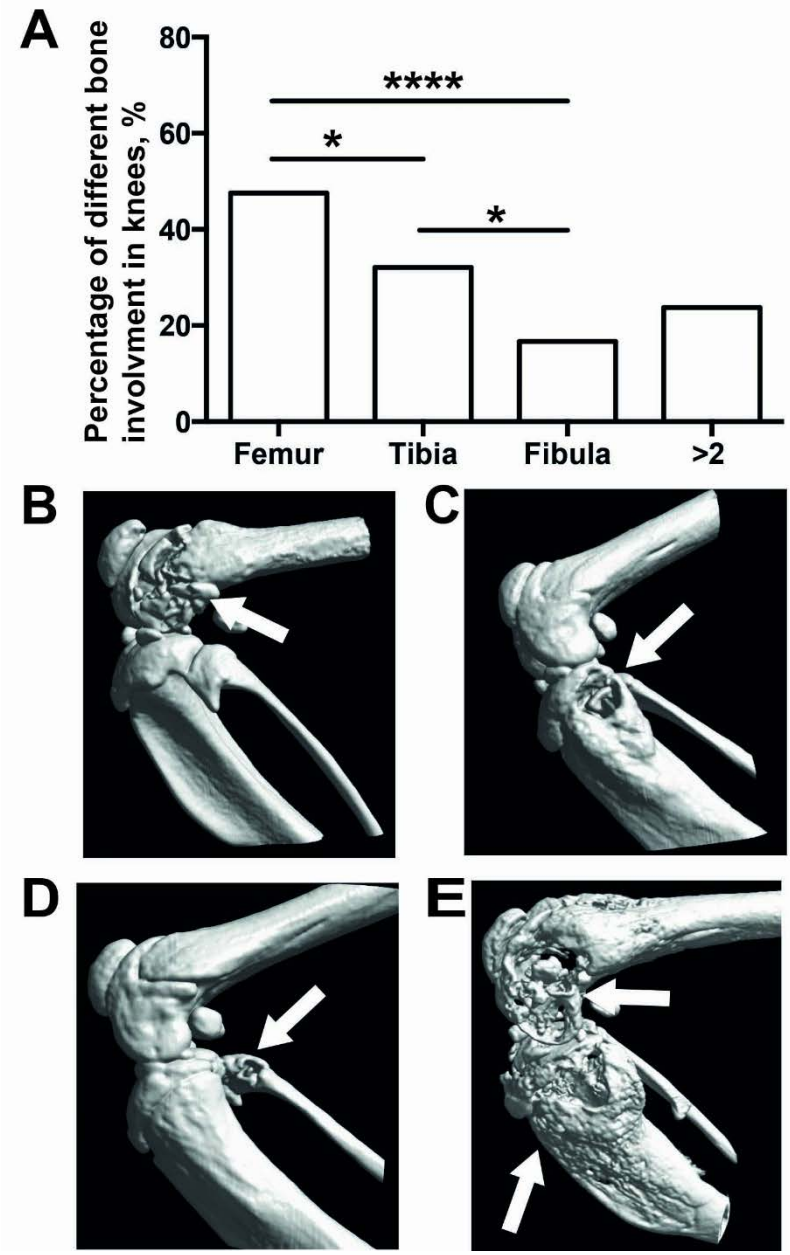

Figure 8. Most commonly affected site of the knee joints is the distal femur. (A) Percentage of different sites within the knee joints of NMRI mice infected with *S. aureus* Newman. **(B-E)** Computed tomography scans showing different sites of the knee affected by septic arthritis. **(B)** Heavily destroyed femur, **(C)** evident destruction on the tibia, **(D)** infected fibula, **(E)** damaged knee joints with involvement of several bones including femur, tibia and fibula.

4.8 ICTP cannot be used as the biomarker for bone destruction in septic arthritis

Cross-linked carboxyterminal telopeptide of type I collagen (ICTP), a bone turnover marker, is considered to be useful in the early assessment of skeletal metastases in cancer patients [75, 76]. We hypothesized that ICTP might be used as a relevant bone destruction biomarker in septic arthritis. The serum levels of ICTP were compared in mice infected with different doses of *S. aureus* LS-1 and Newman strains. No significant difference was found between the higher and lower doses of *S. aureus* in both strains with regard to the bone destruction marker ICTP **(Figure 9A** and **9B)**, suggesting that ICTP is probably not the useful biomarker for bone destructions in septic arthritis.

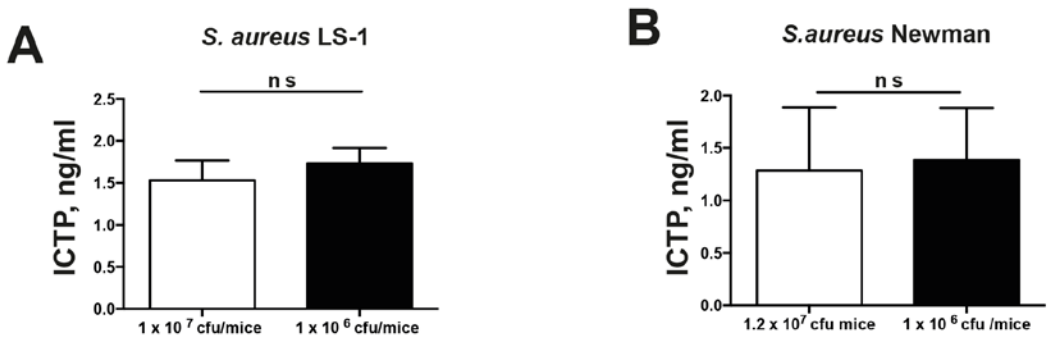

Figure 9. Serum levels of cross-linked carboxyterminal telopeptide of type I collagen (ICTP) are not associated with extent of bone destruction in septic arthritis. NMRI mice (n = 8–10) were inoculated intravenously with two doses of *S. aurues* LS-1 (1x 10^6 -1x 10^7 cfu/mouse) and *S. aurues* Newman (2x 10^6–1.2 x 10^7 cfu/mouse) were sacrificed on day 10 after infection. Blood was collected and serum levels of ICTP were determined. Mean±SEM; ns = not significant; Mann-Whitney U test.

4.9 Polyarthritis is not the predictor for worse outcome of *S. aureus* systemic infection

Polyarthritis, defined as the mice having 5 or more infected joints, was reported to be indicative of poor prognosis of the disease in septic arthritis patients [77].To study whether polyarthritis can be used as the predictor for worse outcome of *S. aureus* infection, 42 mice infected with *S. aureus* Newman were divided into 3 different groups according to the number of joints affected by septic arthritis (Group 1 with 5 or more infected joints; group 2 with 2-4 infected joints; and group 3 with 1 or no infected joint). Nine Out of 42 mice (21.4 %) developed polyarthritis on day 10, whereas only 4.7 % of infected mice showed no sign of clinical arthritis until the end of experiment. No significant differences were found regarding both weight loss % (**Figure 10A**) and kidney bacterial load (**Figure 10B**) among groups, indicating inadequate correlation between polyarthritis and weight loss as well as bacterial clearance in kidneys.

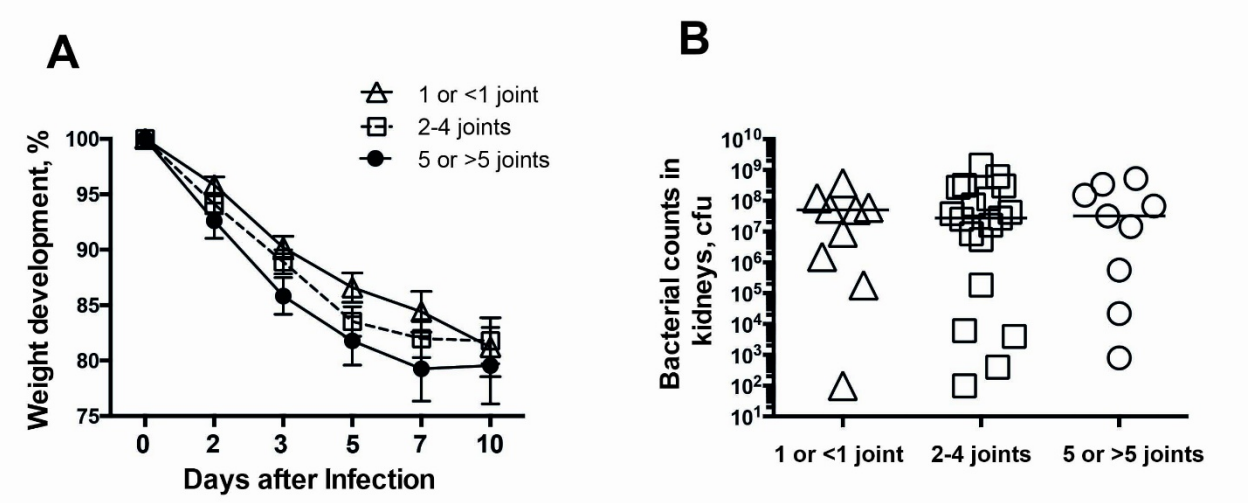

Figure 10. Polyarthritis is not associated with weight development and bacterial clearance in mice with *S. aureus* septic arthritis. Forty-two NMRI mice inoculated with the *S. aureus* strain Newman (5–8 x 10^6 cfu/mice) were sacrificed 10 days after infection. Bone destruction was analyzed in all joints from 4 limbs by μCT scan. The animals were divided into 3 groups: 1) mice with 5 or more infected joints; 2) mice with 2–4 infected joints; and 3) mice with 1 or no infected joint.**(A)** Percentage changes in body weight and **(B)** persistence of *S. aureus* in the kidneys was recorded and compared among groups. Statistical evaluations were performed using the Mann—Whitney U test and Fisher exact test. Data are presented as the mean values ± standard errors of the mean.

4.10 Correlations between bone destruction verified by µCT and several parameters of septic arthritis

Permanent reductions in joint function due to joint destruction and deleterious contractures occur in up to 50% of patients with septic arthritis [78]. There is still no biomarker available for bone destructions in septic arthritis. To this purpose, we statistically determined correlations between radiological signs of bone destructions and several cytokines as well as some parameters of septic arthritis in 48 NMRI mice infected with *S. aureus* Newman strain.

Bone destructions by µCT analysis were positively correlated with histological changes ($p=<0.0001$, $r=0.54$), suggesting high accuracy and sensitivity of µCT scan in detecting septic arthritis. It also showed relatively good correlation with weight loss % ($p=0.0028$, $r= -0.42$) and serum level of inflammatory cytokine IL-6 ($p=0.0042$, $r=0.41$). This suggests that IL-6 might be used as the biomarker for bone erosions in septic arthritis. A significant correlation was also found between clinical arthritis score and bone destruction determined by µ CT scan ($p=0.05$, $r =0.28$).

In case of kidney bacterial counts, the best correlation was found with serum levels of pro inflammatory cytokine TNF-α ($p=<0.0001$, $r= 0.67$). A significant correlation was also found with weight loss % ($p=0.0115$, $r= -0.36$) and serum levels of IL-6 ($p=0.0154$, $r= 0.35$).

4.11 Radiological and histological signs correlated well in local infection model of septic arthritis

NMR1 mice infected with *S. aureus* Newman strain (1×10^3 cfu /knee) on the knee joints were evaluated for bone destruction by µCT scans after sacrificing on day 10. Thereafter, histological analysis was also carried out. A significant correlation was found between the radiologically determined bone destructions by µCT scan and histologically determined signs of erosion ($p= 0.0088$, $r=0.56$) as well as signs of synovitis ($p=0.0089$, $r= 0.56$).

_____ *DISCUSSIONS*

5. DISCUSSIONS

One of the reasons why S. aureus-induced SA is considered a medical emergency is because this disease rapidly progresses to joint destruction. µCT offers the unique opportunity to visualise three dimensional micro bone architectural changes occurring in the various types of arthritis models. Extremely aggressive and rapid cartilage and bone destruction is one of the major hallmarks of septic arthritis [1, 2] and therefore, we hypothesize that µCT is an excellent tool for the assessment of bone damage in our murine model of septic arthritis. In this study, we systematically studied the radiological features of *S. aureus* septic arthritis (both hematogenous and local) in mice by µCT. Our data suggest that µCT scan is a useful supplementary method to clinical assessment and bacteriological analysis in our model for septic arthritis [74].

Various *S. aureus* strains differ in several virulence determinants and therefore, the radiological pattern of bone destructions caused by different strains may have some variations. In present study, both clinical strain (LS-1) and laboratory strain (Newman) were used and they differ in expression of several virulence factors. For instance, Newman is known to express staphylococcal enterotoxins-A (SEA) but lack the TST gene, which produces toxic shock syndrome toxin-1 (TSST-1) [79], whereas the LS-1 strain has been shown to produce large amounts of TSST-1 [80]. Despite those differences between two strains, the clinical features and radiological changes in mice with septic arthritis induced by these two strains were very similar, suggesting radiological evaluation of bone damage by µCT scans is stable and consistent in septic arthritis elicited by different *S. aureu*s strains.

To determine whether appearance of radiological signs was bacterial dose-dependent, bone erosion severity and frequency were compared using µCT following the administration of a standard low-dose of *S. aureus* inoculum (10^6 CFU/mouse) versus a 1-log-higher dose (10^7 CFU/mouse). Despite the fact that no significant difference was observed in the clinical evaluation of arthritis between different doses, significant differences were observed in both the severity and frequency of bone damage by µCT scan indicating good sensitivity of this method for the detection of bone damage. In addition, the µCT data was also correlated well with histological evaluations that are the gold standard for determination of disease onset and progression in our model. This suggests that µCT can be used as an alternative non-invasive

technique for diseases index assessment and to quantify bone damages in septic arthritis mouse model.

With more than 20 years of experience on our unique septic arthritis model, we noticed a major limitation of clinical assessment of septic arthritis in mice- clinical arthritis scoring is usually impossible to assess for deep joints, including the knees, elbows, hips, and shoulders, since the redness and swelling is undetectable in those joints by clinical observation. Our data demonstrated that μCT scan could easily detect these deeper joints that are extensively involved during septic arthritis, hence overcoming the limitation of the clinical arthritis assessment.

The most frequently involved joints in patients with septic arthritis are the knees,, followed by hip, shoulders, wrists, and ankles [78] , which is in full agreement with current data in our animal model. This is another strong evidence supporting that our mouse model for septic arthritis closely resembles the human disease. It has been repeatedly observed that the larger joints in the weight bearing lower limbs are mostly involved in septic arthritis as compared to the upper extremities [81]. More than 50 % of knees affected by septic arthritis compared to the 5.9 % of elbow joints in our study demonstrate the similar manner. Strikingly higher involvement of knees than elbows was also observed in our previous studies [72]. Previously proposed explanations for this was that generally it is easier to aspirate synovial fluid from the large joints of the lower limbs such as the knee, compared with joints of the upper extremities during routine examination of septic arthritis patients. Our data suggest that above mentioned explanation might be imprecise. Anatomic difference or difference in bacterial adhesion molecules between joints might be the cause of such difference. However, future studies are largely needed to elucidate the underlying mechanism.

Knees were most often involved in *S. aureus* induced septic arthritis. However, it was largely unknown which part of knees was most susceptible to bacterial infection. In present study, distal femur was demonstrated to be most frequently eroded compared to tibia and fibula, suggesting that distal femur is the most vulnerable region during the course of septic arthritis. Osteosarcoma, a primary tumor disease, is also most commonly founded in the distal femur that is the anatomic site associated with maximum growth velocity with abundant blood supply[82]. We speculate that those two distinct joint diseases may have some similarities in their early stages of disease development.

Early diagnosis and prompt treatment with appropriate antibiotics are crucial determinants for better prognosis in septic arthritis. To find the new diagnostic biomarkers for septic arthritis would enable early intervention and intensive treatment of the disease which otherwise delayed, results in permanent joint and bone damage. So far there is no evidence of reliable bone marker to detect bone destruction in septic arthritis patients. Serum ICTP has been shown to be a valuable index of bone turnover in several pathological situations, including bone metastases of breast [83], prostate [76], lung [84] cancer cells and multiple myeloma [75]. However in the present study no significant association was observed between serum ICTP levels and bone destructions, proving it to be an inadequate marker in septic arthritis.

Increased levels of cytokines such as IL- l, IL-6, and TNF have been interpreted as an indicator of the inflammatory state. It is unlikely that these cytokines could serve as “biomarkers” in inflammatory disease, as they are linked to the general disease biological processes, hence not specifically associated with a particular disease. Additionally, lack of correlation is often observed between cytokine levels (in serum/plasma) and clinical endpoints. Surprisingly, in our study, IL-6 positively correlated with the severity of bone destructions verified by µCT scan, suggesting that IL-6 might be used as the marker to determine the extent of bone damage in septic arthritis. Indeed, IL-6, despite not being disease specific, was shown to be more sensitive than other serum cytokines for the prediction of therapeutic response of rheumatoid arthritis patients [85]. Bacterial load in kidneys, one of most important parameters for *S. aureus* systemic infections, reflects the strength of host immune system against invaders. The excellent positive correlation between bacterial counts in kidneys and TNF levels in serum strongly indicates the potent role of TNF in *S. aureus* systemic infections, which is in agreement with our previous studies [46, 55].

Polyarthritis was known to be indicative of poor prognosis of the disease in septic arthritis and associated with higher mortality [77]. Surprisingly, our data demonstrate that mice with polyarthrtis had similar weight development and almost identical kidney CFU counts compared with the mice with arthritis in 1 or less than 1 joint, suggesting that although bacterimia initiates hematogenous septic arthritis, severity of joint infections is independent of the disease parameters of sepsis, such as weight loss and kidney bacterial load.

There are many advantages of using μCT scan in our mouse model for septic arthritis. Firstly, as mentioned before, it can easily detect deep joints including knees, hips, shoulders, which cannot be analyzed by clinical analysis. Also, this non-invasive imaging technology enables *in vivo* scanning to follow up the joint morphological changes occurring in the same animals at different intervals instead of sacrificing groups of animals at given time points. Thirdly, the technique permits the visualization of the external and inner part of the same joints without altering the specimen, making it useful for later histological analyses [86]. Importantly, the excellent correlation between μCT scan analysis and histological evaluation of septic arthritis demonstrate the accuracy and sensitivity of μCT scan to identify the joints with septic arthritis at least in the late phase of the disease.

Apparently, μCT also has some limitation. Despite the fact that major radiological changes in bone destruction became visible in some joints (20%) already on day 3 after infection, the earliest time-point to definitely identify all bone erosions caused by septic arthritis was fairly late (7 days post infection or later), suggesting μCT imaging is not so useful for early diagnostic of septic arthritis in clinical praxis. The reason might be that μCT scan is not sensitive method to detect joint effusion, soft tissue swelling, and para-articular abscesses, which usually occur several days before bone damage. For early diagnostic, magnetic resonance imaging is known to be more sensitive with high accuracy [87]. A standard scan of all joints of one mouse generates an image-volume that is several GB in size [88]. Computers require multiple, high-end graphic cards as well as large amounts of memory to reconstruct the generated images into 3D volumes. Management and analyses of such large data can sometimes be problematic [88]. Also, since the image analysis is subjective, the reliability of results is largely dependent on the method standardization and well-trained and experienced technicians.

6. CONCLUSIONS

For the first time we reported [74], that the radiological signs of bone destructions in mice with septic arthritis are bacterial dose-dependent. Just like human disease, the most commonly affected joints were knees, and the distal femur was the most susceptible site. The bone destruction detected by μCT scan analysis was positively correlated to histological changes and clinical signs of septic arthritis [74]. Interestingly, the serum levels of IL-6 were significantly correlated to the severity of bone destruction in septic arthritis. Our data strongly suggest that

μCT scan is useful tool to evaluate the extent of joint destructions in septic arthritis mouse model.

CHAPTER II

Studying the inflammatory responses in septic arthritis induced by Staphylococcus aureus-derived vesicles

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ABSTRACT

Extracellular vesicles (EVs) are heterogeneous population of nano- and micro-sized vesicles secreted from almost every cell type. The process of EV secretion seems to be evolutionary conserved across the species kingdoms, ranging from simple prokaryotes to higher eukaryotes including bacteria, viruses, and parasitic protozoa such as leishmania and malarial parasites, fungi, plants and animals. Recent data suggests that *Staphylococcus aureus* (*S. aureus*) bacteria secretes EVs that could mediate host-pathogen interactions. EVs have been investigated in various bacterial species which modulate the secretion of immunoregulatory molecules such as cytokines and may have key role in infection. However, their role in *S. aureus* septic arthritis has not been explored yet. In current study, we postulate novel perspectives for the implementation of *S. aureus*-derived EVs *in vitro* as well as *in vivo* model of septic arthritis. EVs derived from *S. aureus* were applied to stimulate mice splenocytes *in vitro* as well as intra-articularly and the cytokine levels were measured.

Our results showed that *S. aureus* derived EVs potentially provoke the production of pro-inflammatory cytokines. TNF-α, and IL-6 were significantly elevated in splenocytes *in vitro* after EV-based stimulation. Moreover, NMRI mice were injected with variable doses of EVs intra-articularly and mice were observed for 10 days to examine inflammation and development of septic arthritis. Bone and cartilage destruction was assessed by histochemistry analysis to score the joint erosion. Altogether, our results demonstrate the putative role of *S. aureus*-derived EVs in provoking inflammation and immunological responses suggesting that these vesicles could induce and disseminate systemic immune response during the development of septic arthritis.

Keywords: Extracellular vesicles, exosomes, microvesicles, *Staphylococcus aureus*, TNF-α, and IL-6, inflammation, septic arthritis

RESUMO

As vesículas extracelulares (VEs) são populações heterogêneas de vesículas de nano e microesferas, segregadas de quase todos os tipos celulares. O processo de secreção de VEs parece ser evolutivo e conservado em todos os reinos das espécies, que vão desde procariotas simples até eucariotas superiores, incluindo bactérias, vírus e protozoários parasitários, como leishmania e malária, fungos, plantas e animais. Dados recentes sugerem que a bactéria Staphylococcus aureus (*S. aureus*) secreta VEs que poderiam mediar as interações hospedeiro-patógeno. As VEs foram investigadas em várias espécies bacterianas que modulam a secreção de moléculas imuno regulatórias, como citocinas e podem ter um papel fundamental na infecção. No entanto, seu papel na artrite séptica de *S. aureus* ainda não foi explorado. No presente estudo, postulamos novas perspectivas para a implementação de VEs derivados de *S. aureus in vitro*, bem como modelo *in vivo* de artrite séptica. VEs derivadas de *S. aureus* foram aplicadas para estimular esplenócitos de camundongos *in vitro*, bem como foram inoculadas intra-articularmente e os níveis de citocinas foram medidos. Nossos resultados mostraram que VEs derivadas de *S. aureus* provocam potencialmente a produção de citocinas pró-inflamatórias. TNF-α e IL-6 foram significativamente elevados em esplenócitos *in vitro* após estimulação com VEs. Além disso, doses variáveis de VEs foram injetadas por via intravenosa em camundongos NMRI os quais foram observados durante 10 dias para avaliar a inflamação e o desenvolvimento de artrite séptica. A destruição de osso e cartilagem, bem como a erosão articular foram avaliadas por histoquímica. Em geral, nossos resultados demonstram o papel putativo das VEs derivadas de *S. aureus* na indução da inflamação e respostas imunológicas, sugerindo que essas vesículas poderiam promover e disseminar a resposta imune sistêmica durante o desenvolvimento da artrite séptica.

Palavras-Chaves:

Vesículas extracelulares, Staphylococcus aureus, Artrite séptica, Esplenócitos, Citocinas pró-inflamatórias, ELISA, TNF-α, IL-6, Histoquímica

INTRODUCTION

1. INTRODUCTION

1.1 Extracellular vesicles (EVs)

Extracellular vesicles (EVs) are nano- and micro-sized vesicles secreted by almost every cell types. EVs were initially reported from reticulocytes [89-91], and antigen presenting cells (APCs) such as B lymphocytes, dendritic cells and mast cells [92-94]. The process of EV secretion seems to be ubiquitous and evolutionary conserved across the species kingdoms [95], ranging from simple prokaryotes to higher eukaryotes – including bacteria [96-98], viruses [99-102], parasitic protozoa such as leishmania and malarial parasites [103-107], plants [108-110], fungi [111-113] and animals such as those secreted from microglia and neurons [114-116], muscle cells [117], adipocytes [118], malignant effusions and tumour cells [119-121], and stem cells [122, 123].

EVs transport a repertoire of biomolecules including nucleic acids such as DNA including genomic DNA and mitochondrial DNA [124-130], mRNAs [131, 132], small and long non-coding RNAs (ncRNAs, lncRNA) [133]. The International Society for Extracellular Vesicles (ISEV) has designated a generalized term Extracellular Vesicles (EVs) to represent heterogeneous population of vesicles which can be classified into exosomes (40-200nm), microvesicles (100-1000nm), apoptotic bodies and large oncosomes with variable sizes above 1000nm [134].

1.1.1 Biogenesis and secretion of extracellular vesicles

The process of EV secretion seems to be evolutionary conserved. However, the mechanisms of their formation and secretion are not fully understood. Among all classes of EVs, the formation of exosomes is comparatively well characterized and seems to be highly conserved and tightly regulated process taking place at endosomal compartments (early endosomes), and matured within multivesicular bodies (MVBs) of late endosomes (**Figure 11**). Upon maturation in MVBs, exosomes are then released to the extracellular milieu upon fusion with the plasma membrane. Exosome trafficking is thought to be carried out by Rab GTPases. Collectively, several factors participate in exosome biosynthesis, sorting, maturation and secretion into extracellular milieu (extensively reviewed by Nawaz *et al.*, 2014 [120]).

In addition to exosomes, cells also release other type of vesicles; some are produced through outward budding of plasma membrane (not from endosomes) and direct release from plasma membrane (see [120], and **Figure 11b**). As compared to exosomes, these vesicles are larger in size and are called microvesicles (MVs). Sometimes apoptotic bodies are also considered as a part of vesicles. Usually cells undergoing apoptosis produces apoptotic blebs and are known as apoptotic vesicles (**Figure 11c**). Moreover, in recent years there have been also reported even larger membrane derived vesicles, called large oncosomes [135]. Parasites and microbes usually secrete MVs and are commonly known as outer membrane vesicles.

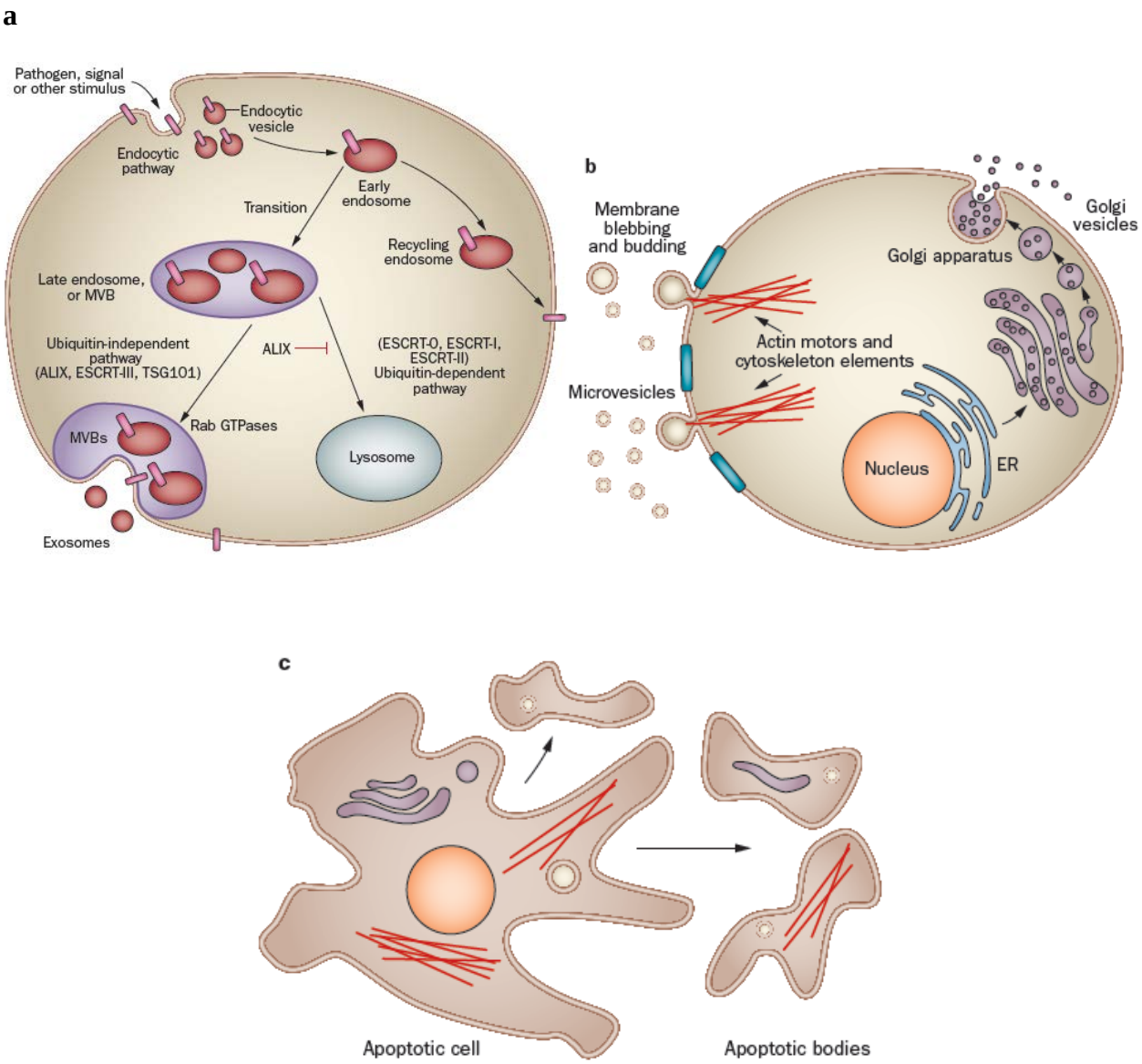


Figure 11: Biogenesis of extracellular vesicles (EVs): (a) Exosomes are formed by endocytosis. These endocytic vesicles mature to early endosomes, and then into late endosomes also known as multivesicular bodies (MVBs). MVBs can be sorted for lysosomal degradation or they can fuse with the plasma membrane and be released outside in the form of exosomes. Rab GTPases regulate MVB fusion with the plasma membrane and release of exosomes. **(b)** Microvesicles are directly secreted from plasma membrane, whereas **(c)** apoptotic vesicles are generated through apoptotic protrusion of cells (Adopted from Nawaz *et al*., 2014, Nature Reviews Urology [120].

1.2 Isolation and purification of extracellular vesicles

There are a variety of methods available for the isolation of EVs, and more are being developed, some of them are poorly standardized. These include ultrafiltration, density gradient centrifugation, size exclusion chromatography and affinity isolation, polymeric precipitation and the microfluidic devices [134, 136]. Each method has different isolation efficiencies when applied to different samples, such as blood plasma, milk, urine, and cell culture media.

A comparison of several conventional as well as high throughput technologies for the isolation and characterization of different samples has been recently undertaken with a limelight of their pros and cons [120, 136]. Recently, ISEV has made a critical analysis of various techniques implemented for the isolation of EVs and have made very important recommendations [134, 137]. Differential ultracentrifugation remains the most widely used primary isolation method comparable to several other techniques and is suitable for isolation from large-volume of samples. However, for the isolation of EVs from low volume samples the size exclusion chromatography is now a more widely used technique which allows separation of EVs from the bulk mix of soluble proteins. In this method the separation is purely based on particle size, therefore contaminating particles in the EV size range such as lipoprotein complexes may be co-isolated [134, 138]. When intended to capture a selective population of vesicles, the immuno-affinity capture offers and alternative method for higher degree of selection. The method can yield pure vesicle subpopulations (i.e. exosomes, microvesicles), but this method is highly influenced by both the choice of affinity reagent and the ligand density on different populations of vesicles [134].

Other methods include microfluidic devices, filtration and various commercially available kits. The commercial kits could make the use of volume-excluding polymers such as polyethylene glycol (PEG) which enable rapid isolation of EVs from culture media or body fluids. However, such polymers may also co-precipitate protein complexes that contaminate our isolates. Therefore, the ISEV has recommended different isolation techniques based on different principles each will enrich for different subpopulations of vesicles (separate for exosomes, and microvesicles). Since each method potentially co-isolates the contaminants such as protein complexes and lipoproteins to different degrees, the ISEV proposes the utilization of

combinations of techniques, such as density gradient centrifugation followed by size exclusion or immuno-affinity capture [134].

The method of choice should be taken into account based on sample type, volume and the yield, integrity, purity of EVs required for specific downstream analysis as well as the available instrumentation and processing time [120]. This implies whether the sample is derived from cell-culture media or body-fluids, and whether intended for proteomic analysis or nucleic acid profiling. Therefore, the factors of different isolation methods for EVs have impact on amount, type and purity of EVs recovered from various sources, as well as may have effect on downstream analysis of vesicles.

1.2.1 Characterization of extracellular vesicles

After the isolation procedures, one need to characterize heterogeneous population of vesicles for their size determination, detection of common markers on the surface of vesicles, morphology and concentration (quantification) and downstream analysis, for which there is a variety of techniques used [120, 136, 139, 140]. Characterization of EVs currently present various challenges, mainly due to their small size, complexity of their cargo (contents) and the physical parameters of available instruments for measuring nanosized vesicles.

The size as well as size distribution, and the concentration of individual subpopulations of vesicles (exosomes and microvesicles) is commonly determined by Nanotracking Analysis (NTA), and Zetaview [141-148], and tunable resistive pulse sensing **(**qNANO) [149]. The electron microscopy is used to assess the submicron phenotype of vesicles [139, 147, 150-152], whereas the flow cytometry is implemented for enumerating, phenotyping and sorting of vesicles based on their size distribution [153, 154]. Western blotting is implemented when aim is to detect EVs markers such as CD63, CD81, and CD9. A recent survey conducted by ISEV has stated that the most widely used techniques for EVs characterization are western blotting (74%), single-particle tracking (SPT, 72%) and electron microscopy (60%) [155].

Albeit, the flow cytometry remains a popular tool for measuring vesicle populations [153, 154], however fundamental principles and limitations of the instrument needs to be considered [156]. EVs isolated by ultracentrifugation may cause aggregation of EVs thus rendering difficulties in flow cytometric analysis or single particle tracking analysis [155]; whereas, those isolated

through kits, might represent difficulty for vesicle analysis with western blotting. Recently, qNANO based measurements of EV concentration have demonstrated a standardized method with a feasibility to facilitate comparable and reproducible results that further needs to be validated or reproduced [149].

Additionally, characterizing of heterogeneous subpopulations of vesicles remains an unsolved issue in particular almost all EV-subtypes including exosomes and microvesicles share common detection markers such as CD63, CD81 and CD9 [134, 140]. However, to resolve this issue there are recent claims to characterize EVs based on their surface protein profiling at large (proteomics) [145, 157], or RNA content profiling (RNAomics) of individual populations of EVs separately for exosomes and microvesicles [158-162].

It is expected that new advances in technologies and optimized protocols for the isolation and characterization will certainly foster progress in obtaining pure vesicles. This will greatly influence the identification of specific biomarkers when intended from various diseases as well as therapeutic utility of EVs [120, 163].

1.2.2 Roles in normal physiology, health, and disease progression

Mounting body of evidence has revealed that EVs are secreted under both physiological and pathological conditions and serve as mediators of cell-to-cell signalling and communication [164]. The biological signals transmitted by EVs include the transport of nucleic acids, lipids, proteins, transcriptional factors, Toll-like receptors (TLRs), and cytokines.

Although, the functions of EVs are not illuminated to full extent, however the most profound effect of EVs that have been invariably established is their participation in cell-to-cell communication and signal transduction allowing the exchange of biological information between cells [132, 164-167]. Since eukaryotic cells needs to communicate continuously in order to keep homeostasis, signalling during biological process, nutrients balance and so forth. Now it is tempting to escalate that EVs could mediate bidirectional communication, and therefore the EV-mediated transport of bioactive molecules could be observed bidirectionally [123]. There is emerging role of EVs in cellular differentiations, stem cell-maintenance and defining cell-fates (reviewed elsewhere [123]). Such evolving roles of EVs are mainly reliant on their features that

mimic and recapitulate stem cell properties in promoting tissue's intrinsic regenerative programs and repair process within recipient cells in paracrine manner [123].

As compared to other paracrine secreted factors such as cytokines and hormones, the EVs are gaining intensive attention due to the fact that they transport a variety of bioactive molecules and elicit enormous biological activities between cells. EVs carrying diverse cargo can move through biological fluids in particular through blood circulation and thus may elicit long distance inter organ communication by dissemination of their cargo from one organ to the other one [118, 168, 169]. In addition to regulation of fundamental biological processes and normal physiological states the roles of EVs have also been increasingly reported in diseases. EVs have attracted much attention in the recent years due to their participation in physiology of the body such as waste management, coagulation, angiogenesis, maintaining homeostasis and host defence [170].

The roles of EVs are emerging in maintaining cellular health and cell survival including self-renewal, differentiation and pluripotency. Moreover, stem cell derived EVs are implicated in healing processes by stimulating and regulating intrinsic regenerative programs in damaged tissues. EV-mediated functional delivery of RNAs (miRNA, lncRNA and mRNA) to the sites of injury could enable regulation of genes responsible for repair processes in injured cells [123, 171]. EVs derived from stem cells could potentially exert immunomodulatory and anti-inflammatory effects, anti-apoptotic and protective effects in order to ameliorate organ functions [122, 163].

Although EVs secretion is a constitutive process, however the process of EVs release is accelerated during altered cellular or organ conditions and disease state, such as against infectious agents, or during inflammatory and immune responses. Due to their natural capacity in transportation and dissemination of biological molecules EVs could spread toxic and misfolded or abnormally expressed proteins, lipids, mutated genes and deregulated nucleic acids. Therefore, they can propagate number of diseases such as neurodegenerative disease [172-175], inflammatory and cardiovascular diseases [176, 177]. The dissemination of ncRNA content via EVs could promote tumour progression and therapeutic resistance [133, 178]. In addition, EVs could mediate host-parasite interactions and may contribute in the progression of infectious diseases by dissemination of virulence factors [179].

1.3 Extracellular vesicles as mediators of inflammation and inflammatory diseases: Roles in arthritis development

Although, the roles of EVs in numerous physiological and pathological conditions such as development of cancer, infectious diseases and immunomodulation including both immune (suppression as well as immune activation) has been well demonstrated [93, 122, 163], their role in autoimmune and inflammatory diseases is just beginning to be emerged [176, 180-182]. Importantly, blood-derived EVs are novel regulators of the human osteoclastogenesis and may offer discrete effector function in distinct inflammatory arthropathies [183].

In rheumatic diseases, namely osteoarthritis (OA) and rheumatoid arthritis (RA), EVs have been isolated from synovial fluid, synovial fluid mononuclear cells (SFMCs), and peripheral blood mononuclear cells (PBMCs) from RA patients and have been shown to play pathogenic roles in the progression of both diseases [183-186]. EVs are known to play a significant role in the RA microenvironment and potentially favour the progression of T cell exhaustion [184]. RA models have shown that EVs stimulated with inflammatory cytokines are capable of inducing apoptosis resistance in T cells, presenting antigen to T cells, and causing extracellular damage with matrix-degrading enzymes [186].

It has been argued that the synthesis and release of EV are closely associated with autophagy. At the same time, both EV and autophagy play a role in OA development. Based on the mechanism of EV and autophagy in OA development, EVs may be beneficial in the early diagnosis of OA; on the other hand, the combination of EV and autophagy-related regulatory drugs may provide insight into possible OA therapeutic strategies [187]. Recently it was shown that osteoclast-derived EVs transfer miR-214-3p to osteoblasts to inhibit bone formation [188]. Therefore, inhibition of miR-214-3p in osteoclasts may be a strategy for treating skeletal disorders involving a reduction in bone formation.

EVs have also been associated with joint repair and regeneration [189], and drug delivery vectors for RA [190]. Collectively, EVs may have therapeutic effect via delivery of molecules that may stop disease evolution, whereas the content of EVs from arthritis patients could be used as biomarker discovery.

1.4 Staphylococcus aureus-derived extracellular vesicles

Recently, it has been shown that *S. aureus* secretes EVs from their outer surface, commonly known as outer membrane vesicles that could have role in infection as secretory factors [98, 191]. EVs from *S. aureus* contain pathogenic proteins, RNA, and toxins that could induce atopic dermatitis-like skin inflammation [191], as well as surface-associated or extracellular virulent proteins including β-lactamase, coagulase, hemolysin, IgG-binding protein SbI, and N-acetylmuramoyl-l-alanine amidase. These vesicles derived from *S. aureus* are between 20 and 200 nm in size and are shed from the bacterium's outer membrane (**Figures 12** and **13**) [98, 191].

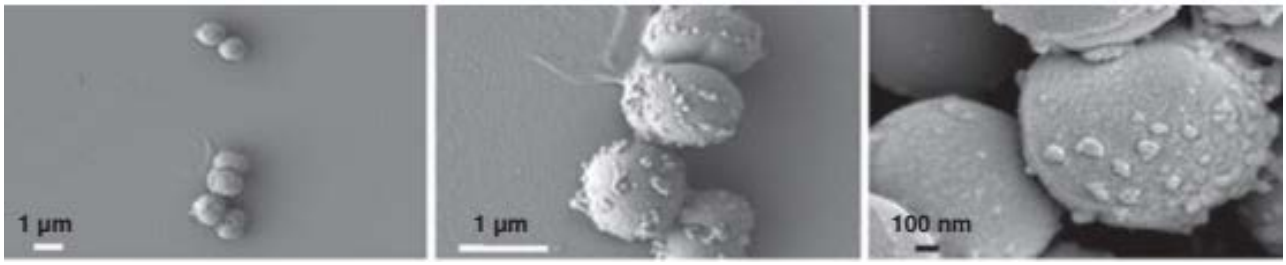


Figure 12. *S. aureus* secreted outer membrane vesicles (EVs) enhance *in vitro* secretion of immune and pro-inflammatory molecules from mouse dermal fibroblasts. A scanning electron microscopic images showing that *S. aureus* secretes EVs (Figure adopted from Hong *et al.*, 2011 [191]).

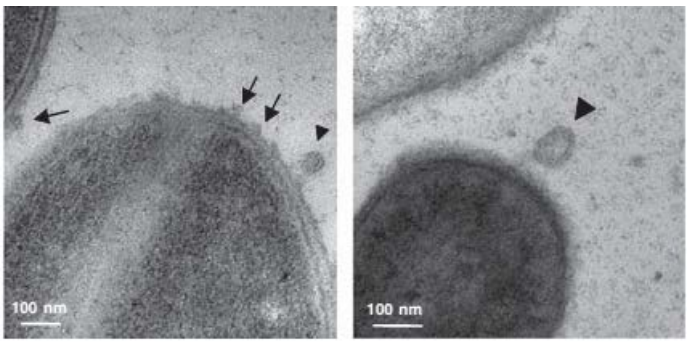


Fig 13. Outer membrane vesicles (EVs) produced from *S. aureus*. Thin section TEM of *S. aureus* shows the formation of EVs (arrows) on the cell surface, and their secretion into the extracellular milieu (arrow head). (Figure adopted from Lee *et al*., 2009, Proteomics [98].

Proteomic analysis have revealed that the protein expression pattern in these EVs differs from that in whole bacteria from which they were secreted, and that they contain various pathogenic molecules, such as α-hemolysin and cysteine protease [192]. *S. aureus* secreted EVs have been linked with pathogenesis through inducing Th1 and Th17 neutrophilic pulmonary inflammation, mainly in a TLR2-dependent manner [193]. The *in vitro* application of *S. aureus* EVs provokes the production of pro-inflammatory mediators (IL-6, thymic stromal lymphopoietin, macrophage inflammatory protein-1a, and eotaxin). These changes are associated with the enhanced production of IL-4, IL-5, IFN-c, and IL-17 [191]. The inflammatory responses are associated with enhanced production of cytokines [194]. Recently, it was reported that IL-17 can induce IgE production in B cells [195]; suggesting that *S. aureus* secreted EVs could induce systemic immune response. Thus, vesicles from *S. aureus* may play potential role in pathogenesis.

AIM AND OBJECTIVES

1 AIM AND OBJECTIVES

Extracellular-vesicles (EVs) are important messengers of cell-to-cell communication and potentially might cause inflammatory and immunological responses post infection. Although much is known about the inflammatory responses caused by EVs from several bacterial strains, little is known about those secreted by *S. aureus* strains. The major aim of current project (performed at University of Gothenburg Sweden as a part of PhD sandwich program) was to study the secretion of vesicles from *S. aureus* and inflammatory effects induced and disseminated by these vesicles both in mice splenocytes (*in-vitro*) and histological changes in bone in mouse model.

The specific objectives are;

1. Isolation of extracellular-vesicles from Staphylococcus aureus strains Newman
2. To identify the potential role of *S. aureus* derived extracellular vesicles in provoking inflammation responses *in vitro* on splenocytes of variable mice strains.
3. To study the cytokine levels of post EV induction *in-vitro*.
4. Examine the joint inflammation *in-vivo* after inoculation with *S. aureus* excreted vesicles.

METHODOLOGY

2 MATERIALS AND METHODS

3.1 Mice

Female NMRI mice, Balb/c and RAGE knockout, 6–8 weeks old, were purchased from Charles River Laboratories (Sulzfeld, Germany). They were bred and housed in the animal facility of the Department of Rheumatology and Inflammation Research, University of Gothenburg. The mice were kept under standard conditions of temperature and light, and were fed laboratory chow and water ad libitum. The Ethical Committee of Animal Research of Gothenburg approved the study.

3.2 Bacteria culture to produce vesicles

S. aureus Newman strain was cultured in TSB (Tryptic soy broth) growth medium. In short bacterial colony was placed in 5ml TSB and incubated for 5 hours. After that the culture was transferred to 300 mL TSB and left for incubation on shaker overnight.

3.3 Isolation and purification of S. aureus-derived extracellular vesicles (EVs)

S. aureus culture was subjected to centrifugation at 4000 RPM for 10 min at 4C to remove deplete the cells. The resultant supernatant was transferred into new falcon tubes and centrifuge at 5100 RPM for 30 minutes to further remove the debris. Supernatant was then transferred into ultracentrifuge tubes (Quick Seal, Beckman Coulter), and ultracentrifugation was performed at 19400 RPM, 35 minutes, 4C using Ti70 rotor. The supernatant was filtered using 0.22 µm filters and transferred into new ultracentrifugation tubes and ultracentrifugation was performed at 38800 RPM for 70 min at 4C to pellet down the vesicles. The resultant pellets were dried and were dissolved in PBS.

3.4 Estimation of secreted vesicles

10µl of EVs sample solution was used for estimation of EVs. 10µl SDS was added to it and was sonicated at 56C for 5 min. 1µl EV sample solution was dissolved in 198µl of Qubit buffer that was already added with 1µl protein reagent. The readings were made using Qubit 2.0 spectrofluorometer (Thermo Scientific).

3.5 Separation of mice splenocytes

The mice spleens were removed aseptically crushed and filtered using 10mL PBS. Centrifugation was performed at 1500 RPM for 5 minutes at 4C. The resultant pellet was

dissolved in 10 mL Ammonium chloride solution. Later 40 ml PBS was added and centrifugation was performed at 1500 RPM for 10 minutes. The cell pellet was dissolved in 1 mL IMDM medium. Cell counting was performed using hymocytometer (abcam), and 100 µl was transferred into 96 well plates and were stimulated with EVs.

3.6 Stimulation of splenocytes

The purified vesicles i.e. EVs (that were isolated from *S. aureus*) were used for stimulating splenocytes *in vitro* to examine whether they have pro-inflammatory properties. In short, 100 µl of variable doses of EVs and LPS as positive control was added to splenocytes as stimuli and incubated for 48 hours. 50 µl solutions from each treated group were transferred into new plate and were saved for cytokine analysis later on. 10ul thymidine was added in the first plate and incubated for 17 hours. Cell were harvested on filter-mat and beta counter (Ortec MPC-900-DP) was used to count the resultant cells after given interval of stimuli.

3.7 Measurement of cytokines levels

After stimulating splenocytes with *S. aureus*-derived EVs, the cytokines TNF-α and IL-6 were measured using DuoSet ELISA Development Kit (R&D Systems Europe), according to manufacture protocols. Briefly, 100ul of capture antibody prepared in PBS was added in the 50 µl cell preparation and incubated overnight. After washing reagent diluent was added and samples were incubated for 1 hour. Again washing was done and respective samples and standards were added in the wells. After further 2 hours incubation, detection antibody is added and samples were measured in ELISA reader and values were adjusted accordingly.

3.8 In-vivo mice model

To further study whether vesicles can induce the joint inflammation, intra articularly injected the *S. aureus*-derived EVs into knee joints of mice via *intra* articularly injections. Two doses of EVs were used. Higher dose of 9.76 ug /ml and lower dose of 0.97 ug /ml. As a control right knee was injected with PBS and left knee with either highe or lower dose of EVs.The knee joints were scanned by µCT scan, and later on to be processed for histopathological analysis. The different types of immune cells will be stained by immunohistopathology.

RESULTS

3 RESULTS

4.1 Isolation and optimization of *S. aureus* extracellular vesicles

Isolation of EVs from *S. aureus* Newman strain was carried out by modified conventional ultracentrifugation method. Comparable amounts of EVs were isolated from *S. aureus* Newman strain culture. Qubit based quantification at the end of each isolation determined that the EV isolation method has been optimized (**Figure 14).**

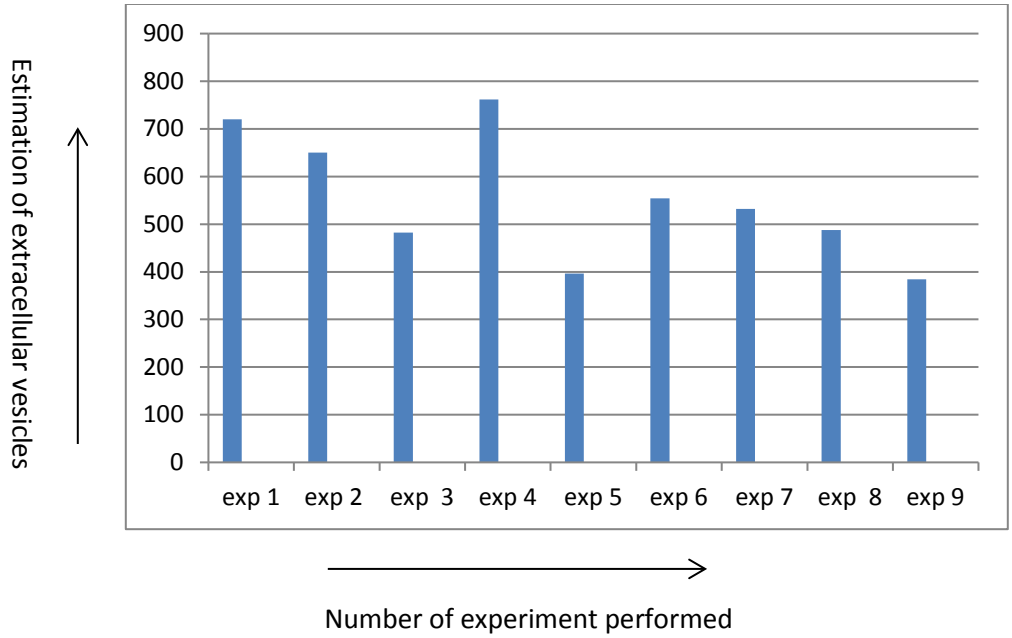


Figure 14. Optimization of EVs isolation: Repeated EVs isolation experiments displaying comparable amount of EV been isolated from *S. aureus* Newman strain culture.

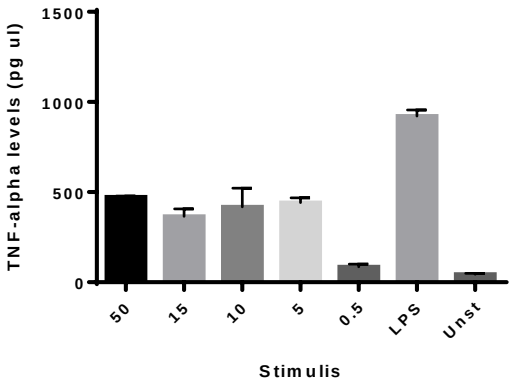
4.2 Assessment of inflammatory properties of *S. aureus* EVs by cell proliferation assay

The purified *S. aureus* EVs were used for stimulating splenocytes (from different strains of mice) *in vitro*, to identify whether they have pro-inflammatory properties. Three separate stimulation experiments were performed (n=2-3), using EV doses in the range of 0.5-67 µg/ ml. TSST-1 and LPS were used as positive controls. Affirmative results were obtained only in the range of 6.7-15 µg/ml EV stimulants.

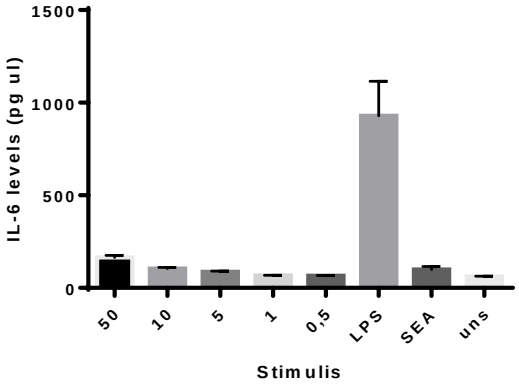
4.3 Measurement of pro-inflammatory cytokines level post EV induction in vitro

Levels of pro-inflammatory cytokines including TNFα and IL-6 were analyzed both 24 hours and 48 hours post EV stimulation *in vitro* on different wild type and knockout mice spleen cells. Serum levels of wild type NMRI mice spleens, stimulated with different doses of EVs were analyzed for IL-6 and TNF-α, which showed dose dependent pattern according to the doses of EVs. Similar dose dependent pattern was also observed in wild type RAGE and knock out strain with wild type RAGE showing slightly higher levels compared to the knockout strain (**Figure 15**).

A



B



C

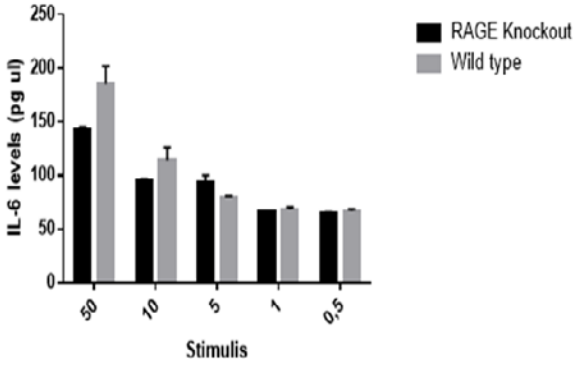


Figure 15. Mice splenocytes stimulation with EVs and measurement of cytokines: (A) levels of TNF-a (pg/µl) after stimulation of EVs. (B) Levels of IL-6 post EVs stimulation. (C) IL-6 levels in RAGE knockout versus wild type mice stimulated with EVs.

4.4 Induction of joint inflammation by intra articular injection of *S. aureus* EVs: Pilot Study

To further investigate whether *S. aureus* EVs can induce the joint inflammation, pilot study was performed using two doses of EVs (High-9.76 ug /ml, Low- 0.97 ug /ml) were intra-articularly injected into knee joints of 5 NMRI mice. The knee joints were processed for histopathological analysis which showed signs of inflammation (**Figure 16**).

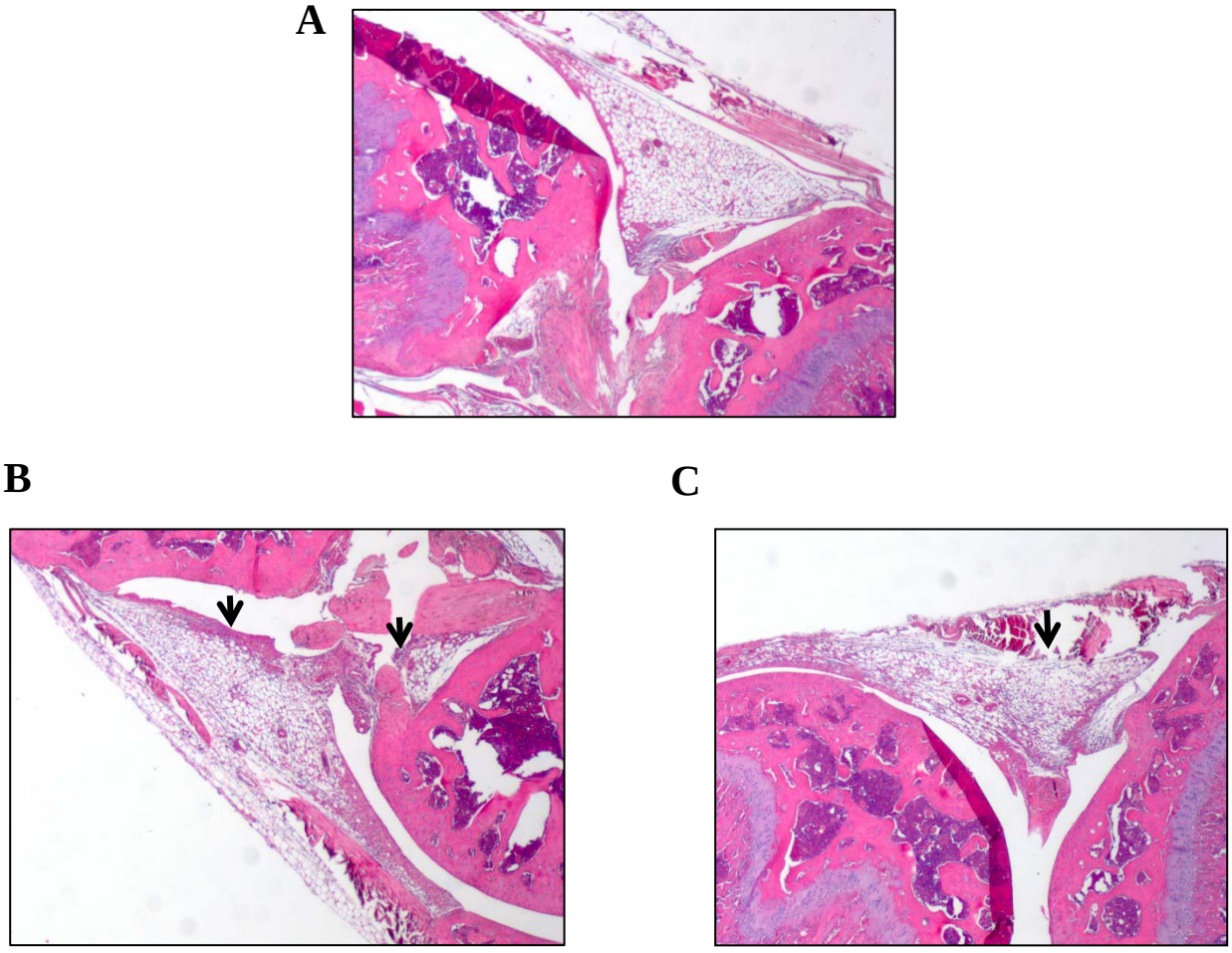


Figure 16. Histochemical analysis after induction of inflammation with different doses of EVs in wild type NMRI mice: A-Control knee joint with PBS injection**. B-** High dose of EVs (9.76 µg /ml) **C-** low dose injection **(**0.97 µg /ml) showing knee joint intact. (B and C) Showing slight signs of inflammation (i.e. joint erosion) in knee joints (indicated by arrow)

DISCUSSIONS

5 DISCUSSIONS

Extracellular vesicles (EVs) are considered important messengers of cell-to-cell communication which potentially might cause inflammatory and immunological responses and are considered potential vehicles of pathogenicity implicated in a wide range of diseases including infectious ones.

Although, the roles of EVs in numerous physiological and pathological conditions and immunomodulation been well demonstrated [93, 122, 163], nevertheless their role in inflammatory diseases is just beginning to be emerged [163, 176, 180-182]. Blood-derived EVs have been recently considered as novel regulators of the human osteoclastogenesis and may offer discrete effector function in distinct inflammatory arthropathies [183]. EVs have been detected in synovial fluid, synovial fluid mononuclear cells and PBMCs of arthritic patients [183-186]. RA models have shown that EVs stimulated with inflammatory cytokines are capable of inducing apoptosis resistance in T cells, presenting antigen to T cells, and causing extracellular damage with matrix-degrading enzymes [186].

Although much is known about the inflammatory responses caused by EVs from several bacterial strains, little is known about those secreted by S. aureus strains. Current study reports the secretion of vesicles from *S. aureus* and their inflammatory effects. Since, the surface of *S. aureus* secretes several virulence factors that cause septic arthritis (discussed in chapter I of this thesis); we sought that *S. aureus* may also secrete EVs that may induce and disseminated infection directly or by induction of cytokine secretion.

Stimulation of splenocytes with EVs (*in-vitro*) and histological changes in bone in mouse model after injecting different doses of EVs it was at least, observed that different doses of EVs were intra-articularly injected into knee joints, and these effects apparently mimic the *S. aureus* –based induction. It means that *S. aureus* uses EVs as extended factors to spread the infection. However, it is important to consider EVs are not the only type of factors. These EVs may act alone or may act with other virulence factors secreted from *S. aureus.* However, their detailed role needs to be investigated in further studies.

Current study provides initial data that EVs released from *S. aureus* have the potential to provoke inflammatory responses *in vitro* as well as *in vivo*. Cytokine levels and their possible

contribution in joint inflammation post EV induction *in-vitro* could provide a supporting knowledge for disease management. As recent studies have shown that EVs contribute potential role joint repair and regeneration [189], and drug delivery vectors for RA [190], it would be interesting to note that EVs from *S. aureus* secreted EVs may add a supporting the knowledge in better understanding disease mechanisms and monitoring septic arthritis, whereas the content of EVs (e.g. rigorously validated cytokines) from arthritis patients could be used as biomarker discovery.

6. CONCLUSION

Current study provides initial data that EVs released from *S. aureus* have the potential to provoke inflammatory responses *in vitro* as well as *in vivo*. Moreover the pro-inflammatory cytokine levels *in-vitro* could provide a supporting knowledge for disease management and their possible contribution in joint inflammation post EV induction. Further studies regarding content of these EVs from arthritis patients could be used as biomarker discovery in future.

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6 REFERENCES

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ANNEX

ANNEX

Annex A

Ethical approval certificate

FACULDADE DE MEDICINA DE RIBEIRÃO PRETO
DA UNIVERSIDADE DE SÃO PAULO

DEPARTAMENTO DE PATOLOGIA E MEDICINA LEGAL

Ribeirão Preto, 22 de maio de 2017.

DECLARAÇÃO

Declaro, para os devidos fins, que a aluna de doutorado do Programa de Pós-Graduação em Patologia, Farah Fatima, fica dispensada da apresentação de autorização de uso de animais no Brasil, uma vez que foram utilizados animais somente durante parte do projeto desenvolvido na Suécia, com a respectiva autorização pelo comitê daquele país.

Prof. Dr. Fernando Chahud

Coordenador do Programa de Pós-graduação em Patologia

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Annex B

List of Publications, Book Chapter and Editorials

Publications

1. **Farah Fatima,** Ying Fei, Abukar Ali, Majd Mohammad, Malin C. Erlandsson, Maria I. Bokarewa, Muhammad Nawaz, Hadi Valadi, Manli Na, Tao Jin *. *Radiological features of experimental staphylococcal septic arthritis by micro computed tomography scan*. PLoS One. **2017**, 12(2):e0171222.

2. **Farah Fatima**, Irina Nazarenko, Marco Maugeri, Hadi Valadi, Andrew F. Hill, Giovanni Camussi, Muhammad Nawaz*. *Non-coding RNAs in MesenchymalStem Cell-Derived Extracellular Vesicles: Deciphering Regulatory Roles in Stem Cell Potency, Inflammatory Resolve, and Tissue Regeneration.* Frontiers in Genetics, **2017**, 8:161.

3. **Farah Fatima**, Muhammad Nawaz*. *Long Distance Metabolic Regulation through Adipose-Derived Circulating Exosomal miRNAs: A Trail for RNA-Based Therapies?*. Frontiers in Physiology, **2017**, 8:545.

4. Muhammad Nawaz*, **Farah Fatima.** *Extracellular Vesicles, Tunneling Nanotubes, and Cellular Interplay: Synergies and Missing Links.* 2017, Frontiers in Molecular Biosciences 4:50.

5. **Farah Fatima,** Muhammad Nawaz*. *Nexus between extracellular vesicles, immunomodulation and tissue remodeling: for good or for bad?* Annals of Translational Medicine, **2017**, 5(6):139.

6. **Farah Fatima**, Muhammad Nawaz*. *Vesiculated Long Non-Coding RNAs: Offshore Packages Deciphering Trans-Regulation between Cells, Cancer Progression and Resistance to Therapies*. Non-Coding RNA, **2017**, 3 (1): 10.

7. Wei-Jie Tian, Jing-Ping Yun, Ming-Yuan Chen, Yaxiong Zhang, Li Zhang, Wei Bu, Jeffrey I. Cohen, Jilong Yang, Chunlin Ou, **Farah Fatima,** Muhammad Nawaz. *The 150 most important questions in cancer research and clinical oncology series: questions 31-39: Edited by Chinese Journal of Cancer*. Chinese Journal of Cancer, **2017**, 36:48.

8. Muhammad Nawaz*, **Farah Fatima**, Krishna C. Vallabhaneni, Patrice Penfornis, Hadi Valadi, Karin Ekström, Sharad Kholia, Jason D. Whitt, Joseph D. Fernandes, Radhika Pochampally, Jeremy A. Squire, Giovanni Camussi*. *Extracellular Vesicles: Evolving Factors in Stem Cell Biology*, Stem Cells International, **2016**, (2016): 1073140.

9. Muhammad Nawaz*, **Farah Fatima**, Irina Nazarenko, Karin Ekström, Iram Murtaza, Mariam Anees, Aneesa Sultan, Luciano Neder, Giovanni Camussi, Hadi Valadi, Jeremy Squire, Thomas Kislinger*. *Extracellular vesicles in ovarian cancer: applications to tumor biology, immunotherapy and biomarker discovery*. Expert Review of Proteomics, **2016,** 13(4): 395-409.

10. **Farah Fatima** and Muhammad Nawaz*. *Stem cell-derived exosomes: roles in stromal remodeling, tumor progression, and cancer immunotherapy*. Chinese Journal of Cancer, **2015,** 34(46): 1-13.

11. Muhammad Nawaz, Giovanni Camussi, Hadi Valadi, Irina Nazarenko, Karin Ekström, Xiaoqin Wang, Simona Principe, Neelam Shah, Naeem M Ashraf, **Farah Fatima**, Luciano Neder, Thomas Kislinger*. *The emerging role of extracellular vesicles as biomarkers for urogenital cancers*, Nature Reviews Urology, **2014,** 11(12): 688-701.

12. Muhammad Nawaz, **Farah Fatima**, Zanetti BR, de Lima Martins I, Schiavotelo LN, Mendes ND, Silvestre RN, Luciano Neder*. *Microvesicles in Gliomas and Medulloblastomas: An Overview*, Journal of Cancer Therapy, **2014.** 5(2): 182-191.

Editorials

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1. Wei-Jie Tian, Jing-Ping Yun, Ming-Yuan Chen, Yaxiong Zhang, Li Zhang, Wei Bu, Jeffrey I. Cohen, Jilong Yang, Chunlin Ou, **Farah Fatima,** Muhammad Nawaz. *The 150 most important questions in cancer research and clinical oncology series: questions 31-39: Edited by Chinese Journal of Cancer*. Chinese Journal of Cancer, **2017**, 36:48

2. **Farah Fatima,** Muhammad Nawaz*. *Nexus between extracellular vesicles, immunomodulation and tissue remodeling: for good or for bad?* Annals of Translational Medicine, **2017**, 5(6):139.

Book Chapter

Muhammad Nawaz*, **Farah Fatima**, Jeremy A. Squire. *Mining extracellular vesicles for clinically relevant non-invasive diagnostic biomarkers in cancer, in Novel Implications of Exosomes in Diagnosis and Treatment of Cancer, J. Wang, Editor. 2017, InTechOpen.* dx.doi.org/10.5772/intechopen.69406

Annex C

Abstracts published in conference proceedings

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1. Luciano Neder, Benilton Carvalho, Muhammad Nawaz, **Farah Fatima,** VC de Oliveira, H Lima, R Haddad, RA Panepucci, Gilberto Carlotti Jr. *MicroRNAs and gene expression profile differentially expressed in grade III and grade II oligodendrogliomas*. Brain Pathology, v. 24. p. 95. September, **2014**.

2. **Farah Fatima,** Majd Mohammad, Abukar Ali, Muhammad Nawaz, Hadi Valadi, Manli Na, Tao Jin. *The inflammatory and immunological roles of S. aureus derived exosome-like vesicles in septic arthritis.* Journal of extracellular vesicles, **2017**, 6.