

Time window of TNF- α in innate immunity against staphylococcal infection

Bachelor thesis in biomedicine 30 hp

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Abstract

Staphylococcus aureus (*S. aureus*) is responsible for many human diseases including septic arthritis and sepsis shock. Tumor necrosis factor alpha (TNF- α) is a pro-inflammatory cytokine involved in inflammation and produced mainly by macrophages and monocytes. It is believed to be involved in pathogenesis of septic arthritis. Time window of TNF- α in innate immunity against staphylococcal infection was studied in this project.

Two experiments were carried out: In the first experiment mice were infected with a low dose (8×10^6 cfu/mouse) of *S. aureus* to induce septic arthritis whereas in the second experiment the mice were infected with a higher dose (8×10^7 cfu/mouse) of *S. aureus* to induce sepsis shock. All mice were divided into three groups. The first group was treated with anti-TNF- α 20 minutes after infection. The second group was treated with the anti-TNF- α three days after infection. The third group served as control and was injected with PBS instead of anti-TNF- α . The mice were regularly weighed and signs of arthritis and mortality were recorded. Two weeks after inoculation bacteria viable counts in different organs was done, as well as histopathological assessment of joints and measurement of cytokines in blood.

We have observed that mice treated with anti-TNF- α had less severe arthritis and also less mortality. However, they had more bacteria accumulated in the kidneys and lost more weight compared to the control group. The results were mostly seen in the group early treated with TNF- α , compared to the late treated group.

We conclude that anti-TNF- α might be potentially used as a therapy against septic arthritis and sepsis shock. This should be combined with antibiotics to eliminate the bacteria while the anti-TNF- α reduces the severity of the inflammation and thus reduce the risk of permanent joint destruction and mortality. We can conclude that blocking TNF- α early on is essential in order to get the best results.

Introduction

Staphylococcus aureus

Although more than 30 different species of Staphylococcus exists, 3 are of major significance because of their ability to cause wide range diseases, and these are Staphylococcus epidermidis, (*S. epidermidis*), Staphylococcus aureus (*S. aureus*), and Staphylococcus saprophyticus (*S. saprophyticus*) (Tarkowski *et al.*, 2001). Of these, *S. aureus* is of most interest and has been the subject of countless studies and research over the years. *S. aureus* is a gram positive, facultative anaerobic, non-motile coccoid bacteria that forms clusters shaped like a bunch of grapes, hence their name staphylococcus derived from the greek word staphyle which means “a bunch of grapes” (Abbas and Lichtman, 2009). *S. aureus* is unique in that it is the only type among its genus colonizing humans that secrete enzyme coagulase (Abbas and Lichtman, 2009). *S. aureus* is also a pathogen responsible for many diseases among human beings and its more resistant form known as methicillin-resistant *S. aureus* is becoming more common nowadays. Most of the populations carry *S. aureus* on their body, especially the skin and the nose. It is estimated that 30% of the human population are carriers of different strains of *S. aureus* (Kluytmans *et al.*, 1997). *S. aureus* causes mild to sometimes major severe diseases, the most common being skin diseases such as abscesses and impetigo. Spread of *S. aureus* in the blood can cause bacteremia and septic arthritis if the bacterium reaches the joints. Pneumonia, gastroenteritis and the fatal toxic shock syndrome are other diseases caused by *S. aureus* (Kluytmans *et al.*, 1997).

S. aureus secretes different toxins, which make the bacteria such a dangerous pathogen. Examples of these toxins are pyrogenic toxin superantigens which causes common food poisoning and the fatal toxic shock syndrome (Schlievert *et al.*, 2000). Other noteworthy substances of the *S. aureus* are Protein A, teichoic acids, and staphylococcus enzymes such as catalase, coagulase and staphylokinase (Abbas and Lichtman, 2009).

Septic arthritis

Septic arthritis, also known as infection arthritis, is a disease caused by microorganisms that invade the joints of patients. *S. aureus* is the most common cause of septic arthritis when it gets into the blood and manages to spread into the joints (Brusch, 2010). Other strains of gram positive cocci causing septic arthritis are the Staphylococcus epidermidis (*S. epidermidis*) and streptococci. These three strains account for almost 70% of septic arthritis while gram negative Neisseria gonorrhoeae (*N. gonorrhoeae*) and Neisseria meningitidis (*N. meningitidis*) account for approximately 20% of the disease (Tarkowski, 2006). There are many causes of septic arthritis ranging from open wound infections to osteomyelitis. An existing wound infection, prosthetic joint surgeries or rheumatoid arthritis significantly increases the risk of acquiring septic arthritis (Tarkowski, 2006). Infection of the skin is also a significant risk factor for septic arthritis (Mathews *et al.*, 2010). Septic arthritis has a prevalence of about 9,2 per 100 000 patients, with older and younger patients (under 15 and over 55) having the

highest frequencies (Tarkowski, 2006). Young children are most prone to develop septic arthritis although the disease can occur at any age (Holder, 2010). Patients who abuse drugs intravenously and rheumatoid arthritis patients as mentioned earlier are very prone to develop septic arthritis and the prevalence incidence in this group is much higher than in other patient groups (Holder, 2010). The mortality rate is quite high in septic arthritis, ranging from 5 – 20% and is worse among rheumatoid arthritis patients (Tarkowski, 2006). Morbidity is also quite high and approximately 40% of patients suffer permanent bone destruction despite antibiotic therapy. The fact that septic arthritis has a high mortality rate and that almost half of the patients will never completely recover makes the disease very interesting to study. However, what is more interesting to some is that, the adaptive immune response contributes to the severity of the disease instead of eliminating it. Many different studies have shown that while the innate immune response is very helpful in fighting septic arthritis, the adaptive immune response has quite the opposite effect. Different animal models treated with immunosuppressive drugs have shown that lack of T lymphocytes reduces the severity of septic arthritis and leads to lower mortality and morbidity. This means that the human adaptive immune response is very highly activated during septic arthritis and starts attacking own tissues and joints thus leading to more severe infection/morbidity (Tarkowski, 2006). In order to diagnose the disease, bacteria in synovial fluid must be detected and/or a thorough check of swelling, erythema, warmth and redness for all the joints, especially the knee, elbows, shoulders, hip, ankle wrists must be performed (Brusch, 2010). The precise time of onset of septic arthritis is highly important in order to be sure of the exact timeframe immunosuppressive drugs should be used and this is why it is very helpful to use animal models for this purpose (Tarkowski, 2006). Septic arthritis is treated with antibiotics and treatment should be started as soon as possible for optimal results (Tarkowski, 2006).

TNF- α

First discovered by Dr. Lloyd J. Old in 1975 (Old, 2008), Tumor necrosis factor is a cytokine involved in inflammation and produced mainly by macrophages and monocytes. TNF- α is responsible for many different functions in the body, primarily modulating the immune cell response (Locksley et al., 2001) by inducing inflammation, apoptotic cell death, neutrophil proliferation as well as neutrophil apoptosis (Murray et al., 1997), and inhibit viral replication (Brian et al., 1999).

The synovium in joints of patients suffering from septic arthritis produce very big amounts of TNF- α which is an immune response designated to eliminate the bacteria, however they cause more damage than good leading to severe inflammation in the joints and attacking the body's own cells and thereby for the most part cause irreversible joint damages. Mice lacking TNF- α had less severe joint damage but increased mortality in a septic arthritis model (Olof et al., 1998).

(Bremell et al., 1992) found that when living *S. aureus* is administered i.v. in mice, TNF in the serum does not appear immediately (6 hours) but is already present at day 1 after infection and continuously increases with time (at day 15 it was 6 times higher than on day 1)

However, when dead bacteria are directly injected into the joints, the levels of TNF peaks very fast (2 hours) and then decreases as shown by (Makoto et al., 1997). Furthermore,

Blocking TNF- α delayed recruitment of lymphocytes to the joint, but had no result on later development of disease.

Animal model

There are different animal models that are used by different research groups to study the effects of anti-TNF- α in septic arthritis. The animal model we used in this study was a mouse model of *S. aureus* arthritis (Bremell *et al.*, 1992) due to its highly similarities to human Septic arthritis. The similarities to human acquisition of septic arthritis in our model is using of living bacteria and hematogenous spread of infection instead of heat killed bacteria as used by other research groups (Makoto *et al.*, 1997).

AIMS

The primary aim of the study was to investigate if anti-TNF- α therapy had any effect on septic arthritis. The second aim was to study the time window of TNF- α in innate immunity against staphylococcal infection.

Material and methods

Mice

NMRI mice (Charles River, Germany) were used in the experiment. The mice were five to six weeks old and were maintained at the animal facility at the Department of Rheumatology and Inflammation Research in Gothenburg University. The mice were fed water and standard laboratory chow ad libitum and were kept 8 in each cage. All experiments were accepted by Animal Research Ethics Committee of Goteborg University.

Bacterial preparation (inoculation)

A frozen suspension of *S. aureus* LS-1 in plastic tube was thawed and 9 ml of PBS was added. The bacteria was afterwards centrifuged for 10 min at 4000 rpm speed. The supernatant was discarded and the bacteria was again mixed with PBS thoroughly and then diluted to the working concentration. The mice were injected intravenously with 0,2 ml of the bacterial solution in PBS. After injection, leftover solution was serially diluted and spread on horse-blood agar plates and incubated for 24 hours before being counted in order to verify the number of bacteria injected in each mouse.

Clinical examination of infected animals.

The infected animals were checked upon every day at least once. The mice were regularly weighed and the signs of arthritis and the mortality recorded. The arthritis was evaluated by checking the joints for any swelling and/or erythema and points were given for each joint according to the level of swelling and erythema. The score was from zero to three with zero representing no visible swelling, 1 representing mild swelling and/or erythema and 3 representing major swelling and erythema. For each mouse arthritic index was counted as the sum of points given to each joint (minimum zero, maximum twelve points per mouse).

Termination of experiment.

The mice were first put to sleep using ketamine hydrochloride (Pfizer AB Sweden) and metedomidine (Orion Pharma Finland) anaesthesia and blood was drawn from the axillary artery. Afterwards the mice were killed and the liver, spleen and kidneys were excised. The blood collected from the mice was left at room temperature for an hour to coagulate and then centrifuged for 10 minutes at 4000 rpm, and the sera was collected and stored at -20°C.

The organs collected were placed in plastic bag and homogenized. Afterwards 10 ml of PBS was added to the plastic bag, and after mixing 100 µl of the solution was spread on Horse-blood agar plates and incubated at 37°C and the bacteria colonies were counted after 24 hours. The solutions with smashed kidneys were serially diluted and then bacteria colonies were counted as mentioned above. This serial dilution was done due to the overwhelming number of bacteria in the kidneys and had to be serially diluted in order to be able to count viable colonies of bacteria. Viable counting was done to compare bacterial elimination from the kidneys.

Histopathology examination

All four limbs of the mice were removed after the mice were sacrificed, fixed in formalaldehyde and decalcified. Afterwards the limbs were dehydrated and embedded in paraffin. The joints tissues were then stained with hematoxylin and eosin. Hematoxylin stains the nucleus blue while eosin stains the cytoplasm pink. Finally the sections were evaluated microscopically (Bremell *et al.*, 1992). The same scoring method mentioned above was used to evaluate the synovial hypertrophy and degradation of bones. The score was from zero to three with zero representing no visible synovial hypertrophy, 1 representing mild synovial hypertrophy and 3 representing major synovial hypertrophy and joint damage. For each mouse a histological index was counted as the sum of points given to each joint (minimum zero, maximum twelve points per mouse) (Jonsson *et al.*, 2008).

Representative pictures of a healthy joint and damaged joints are shown in figures 1-3. Figure 1 is a perfect example of a healthy knee joint with no synovitis or joint damages. On the other hand, figure 2 shows a picture of a inflammated knee joint due to inflammatory cells accumulated in the synovial fluid with a minor bone damage. A severe synovitis and bone damage is illustrated in figure 3 with overwhelming inflammatory cells accumulated in the synovial fluid leading to severely degradation of the bones.

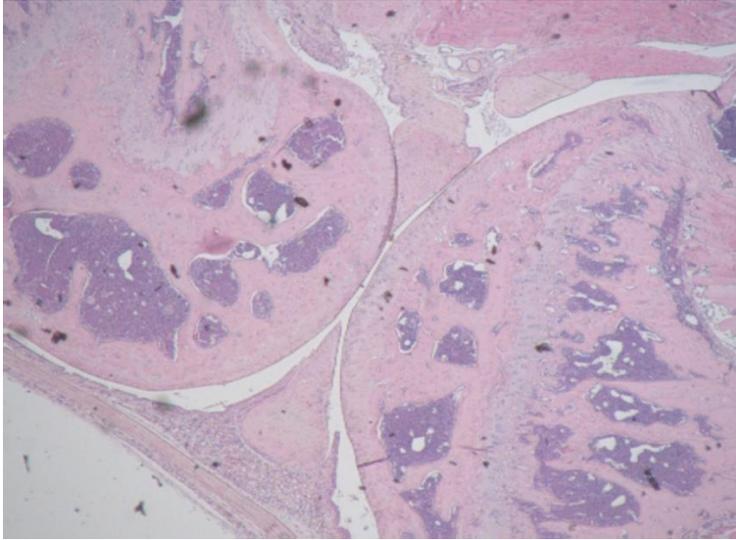


Figure 1. A picture of a healthy knee joint with no synovitis or joint damage.

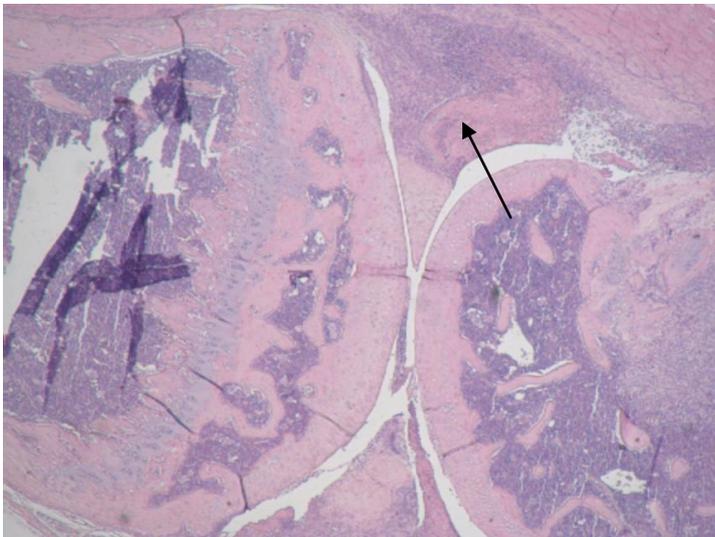


Figure 2. A picture of a knee joint with 2 points of synovitis and 1 point bone damage. The small purple dots between the joints indicate inflammatory cells, bone damage can be seen on the top left with many inflammatory cells accumulated there.

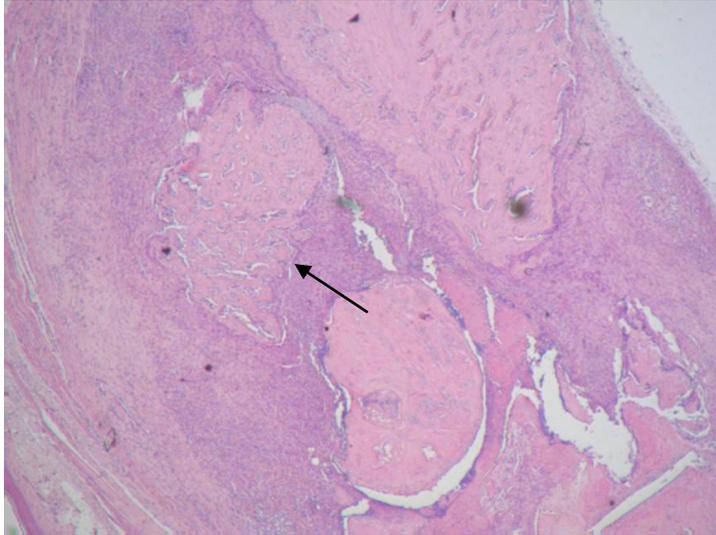


Figure 3. A picture of a wrist with 3 points synovitis and 3 points bone damage. As can be seen inflammatory cells have completely surrounded and eroded the bone on the upper left side and cause also severe synovitis.

ELISA Kits

IL-10 ELISA was measured using the kit Mouse IL-10 DuoSet ELISA development systems from R&D Systems.

TNF- α ELISA was measured using the kit Mouse TNF- α /TNFSF1A DuoSet ELISA development system from R&D Systems.

IFN- γ ELISA was measured using the kit Mouse IFN- γ DuoSet ELISA development systems from R&D Systems.

IL-6 ELISA was measured using the kit Mouse IL-6 DuoSet ELISA development systems from R&D Systems

Experiments

In experiment 1, 7-8 mice per group were used and they received a single intravenous injection of 8×10^6 *S. aureus* bacteria to induce septic arthritis. In experiment 2, the mice were injected with a dose of 8×10^7 *S. aureus* bacteria in order to induce sepsis shock. The mice were then treated with 100 μ l anti-TNF- α , injected subcutaneously. The early treatment group received the injection 20 minutes after infection while the late treatment was first injected on day 3. Both groups were injected every three days for two weeks. The control group was injected subcutaneously with PBS 20 minutes after infection and every three days for two weeks.

Statistics

All statistics were analyzed using the GraphPad prism 5 software. Mann-Whitney test was used to compare different levels of compounds between the groups whereas log rank (Mantel-Cox) test was used for the survival statistics. All result values are reported as means if not stated otherwise. The significance level was set to $p < 0, 05$.

Results

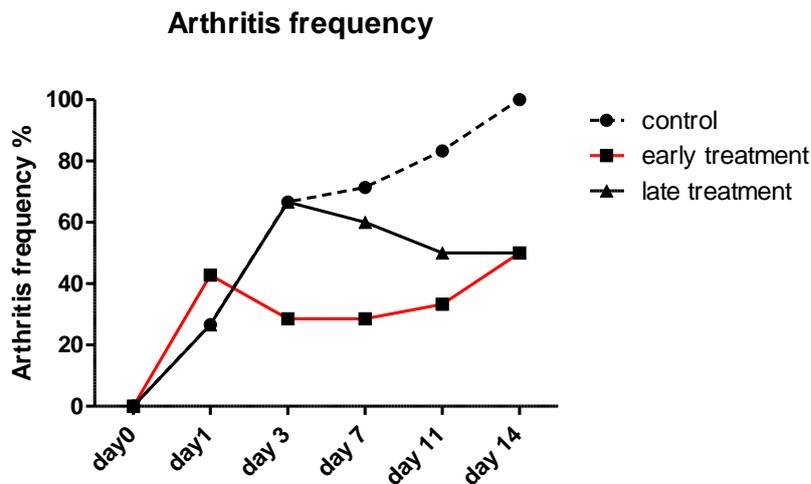


Figure 4. Arthritis frequency occurrence in mice injected intravenously with 8×10^6 *S. aureus*.

Figure 4 illustrates reduced frequency in arthritis in both early and late treatment, compared to the control group which was injected with PBS. The late treatment group shows somewhat higher frequency from day 3 when it peaks compared to the early treatment group but stabilizes going forward until day 14 when both the early and late treatment groups have the same arthritis frequency.

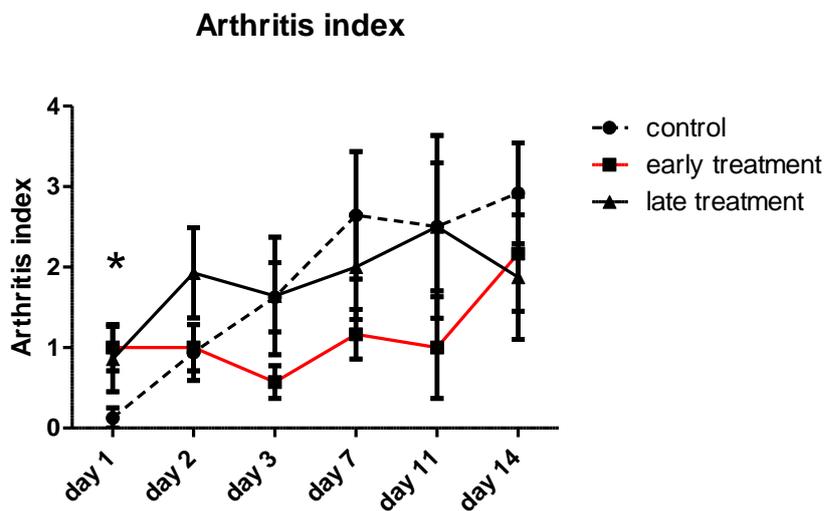


Figure 5. Severity of arthritis for the different groups after intravenous injection of 8×10^6 *S. aureus*.

No statistical significant difference in severity in arthritis was found between groups as shown by arthritis index in figure 5, except for day 1. However, some trend was seen towards reduced severity in the early treatment.

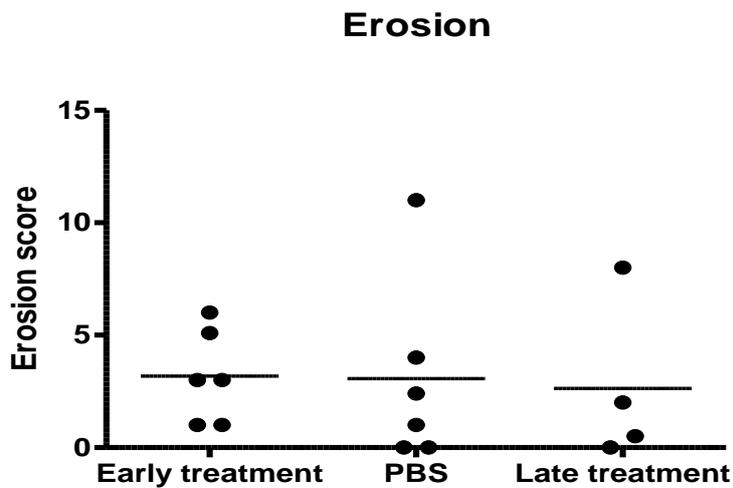


Figure 6. Histopathological assessment for erosion of the joints in the different groups.

No significant changes in joint bone erosion were observed between the groups as figure 6 illustrates.

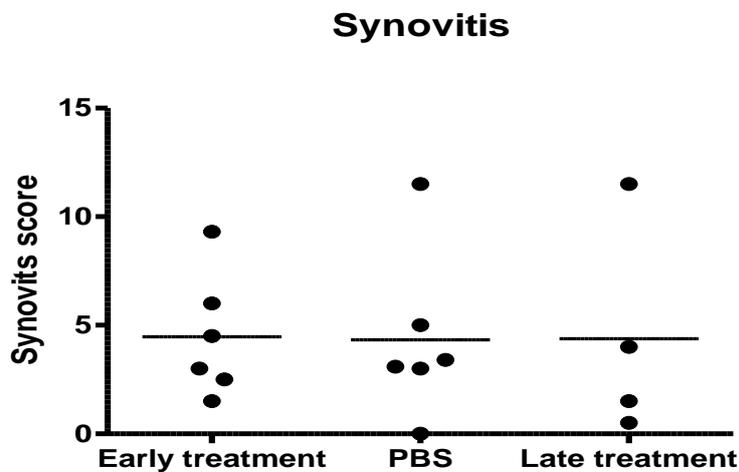


Figure 7. Histopathological assessment for synovitis of the joints in the different groups.

No significant changes in joint histopathology (synovitis) were observed between the groups as figure 7 shows.

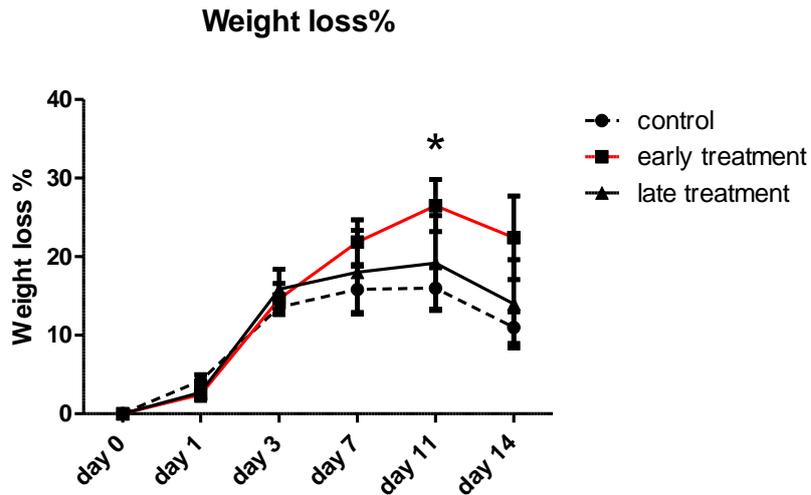


Figure 8. Percentage weight loss observed in the different groups.

Figure 8 shows that early treatment significantly increased weight loss while late treatment had no effect on the weight development.

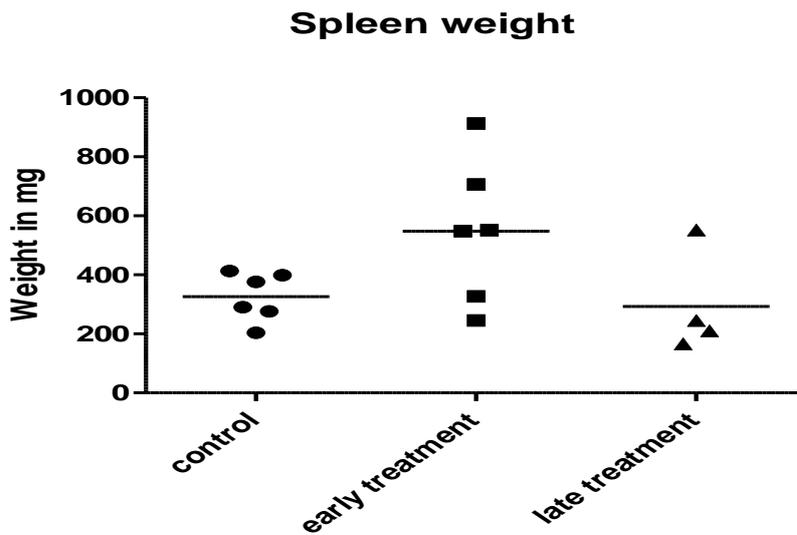


Figure 9. Spleen weight of the different groups 2 weeks after initiation of the experiment.

Spleen groups weight was somehow increased in the early treatment compared to late treatment and control groups as observed in figure 9. The differences however between the groups were not statistically significant.

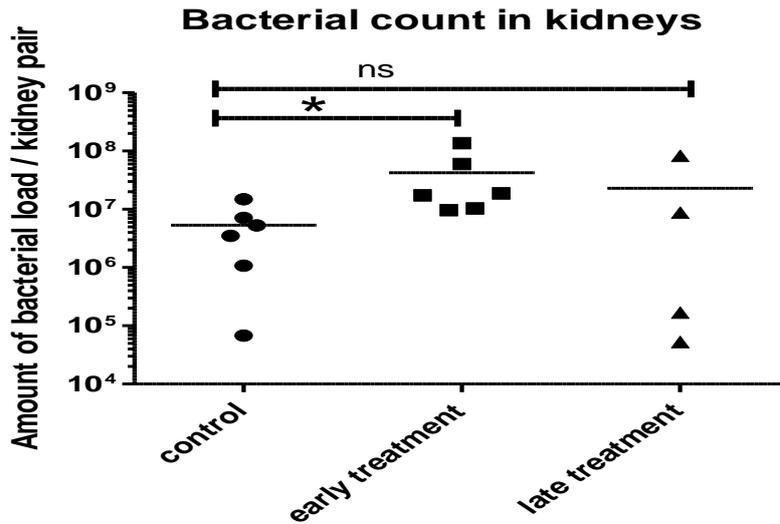


Figure 10. Bacterial load in kidneys observed in the different groups.

Early treatment diminished bacterial clearance as shown by the bacterial load in kidneys in figure 10, while late treatment had no effect on the bacterial clearance from the kidneys. The difference in bacterial clearance in the early treatment was of statistical significance.

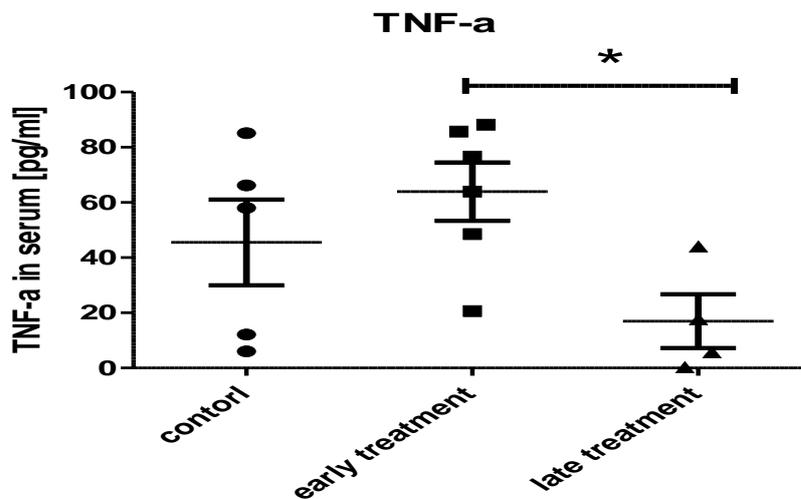


Figure 11. Levels of TNF- α in serum measured 2 weeks after initiation of the experiment.

The TNF- α level in the early treatment group was much higher than in the late treatment group and the difference was of statistical significance (figure 11). Figure 11 also illustrates that the late treatment group had lower TNF- α level than the control although this difference was of no statistical significance.

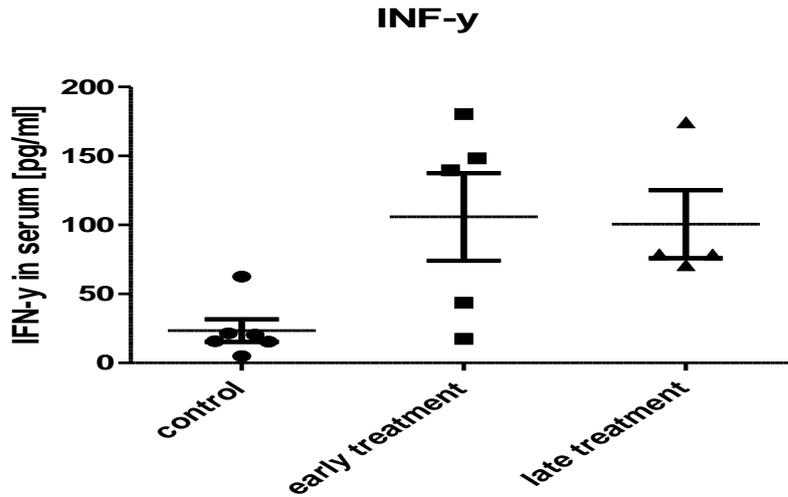


Figure 12. Levels of IFN- γ in serum measured 2 weeks after initiation of the experiment.

As can be observed in figure 12, both the early and late treatment exhibited high levels of IFN- γ but the difference was of no statistical significance.

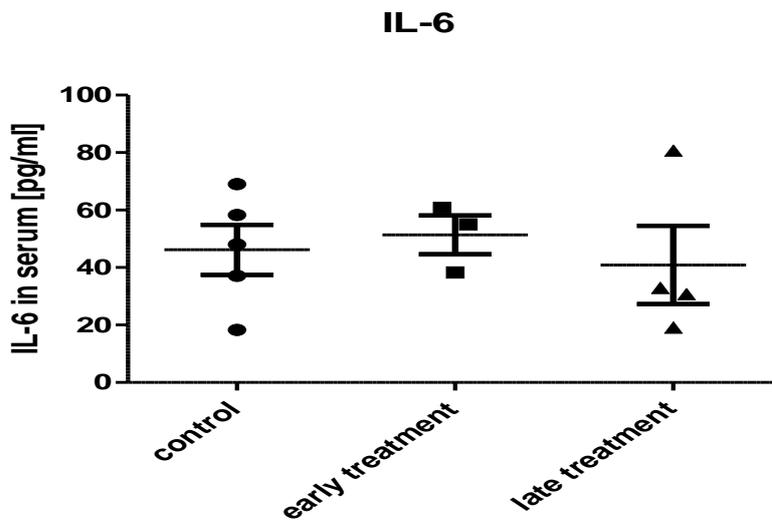


Figure 13. Levels of IL-6 in serum measured 2 weeks after initiation of the experiment.

No significant difference of IL-6 levels were observed within the groups as seen above in figure 13.

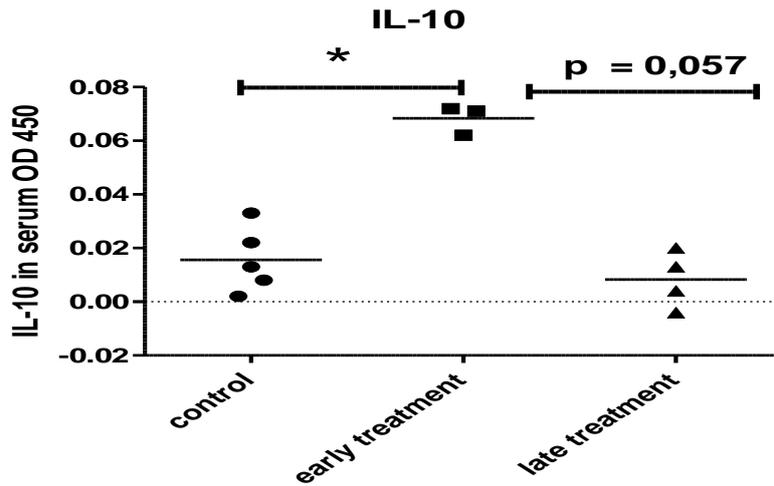


Figure 14. Levels of IL-10 in serum measured 2 weeks after initiation of the experiment.

Early-treatment had much higher IL-10 levels than both the late treatment and control groups, and the difference between the early treatment and control groups was of significant difference (figure 14). An almost ($p = 0,057$) statistical significant difference was also seen between early and late-treatment groups.

Survival of Septic arthritis: Survival proportions

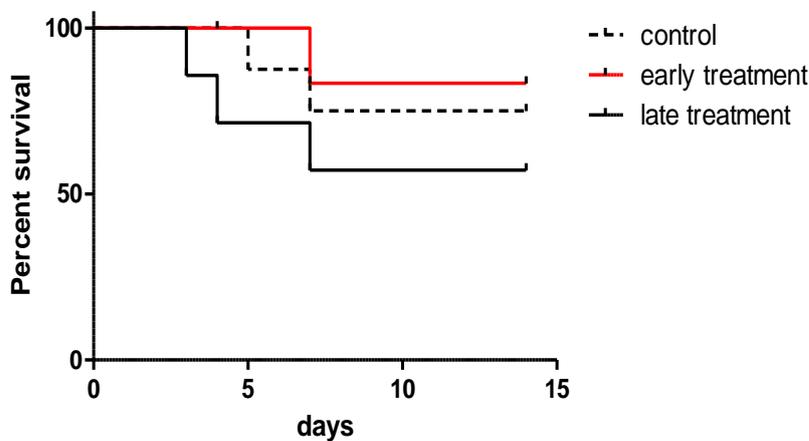


Figure 15. Survival rate for mice induced with septic arthritis.

The mortality reached about 40% (for late treatment) and no statistical significant differences were observed between groups as seen in figure 15. However, early treatment had the best survival rate. To study the effects of anti-TNF- α on survival, another experiment was performed with higher a dose of *S. aureus*.

Survival of Sepsis shock: Survival proportions

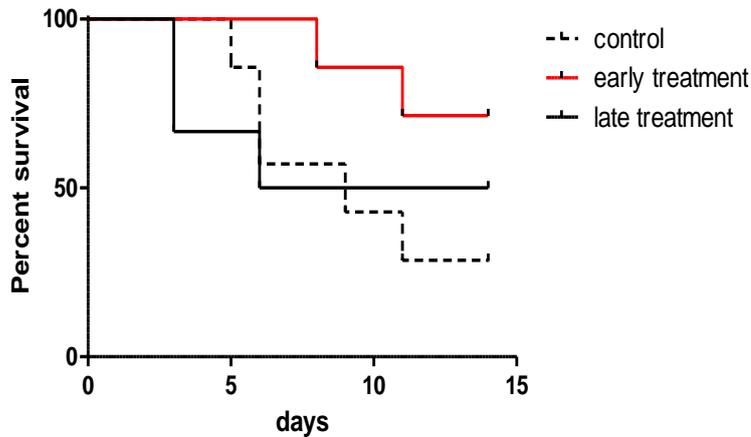


Figure 16. Survival rate for mice induced with sepsis shock.

In the sepsis experiment the mortality reached 70% in the control group as figure 16 above illustrates, and similar to the septic arthritis experiment, the early treatment showed improved survival rate and this was of almost statistical significant difference.

Discussion

During the course of the experiments, the mice infected with *S.aureus* were divided into three groups: 1) Anti-TNF- α early on, (20 minutes after infection); 2) Anti-TNF- α three days after infection; 3) PBS as controls. Of these three groups, clear distinctions could be observed on several areas between the early treated group and the control group. Few differences were also observed between the early and late treatment and late treatment and control groups. Blocking TNF- α early on reduced the arthritis frequency from the first days and the trend continued until at the end of the experiment. Late treatment also reduced the arthritis frequency compared to the control, however, not as much as early treatment. The severity of the arthritis was also reduced more on early treatment than it was for late treatment even though both groups showed reduced severity compared to the control. These results point to the fact that anti-TNF- α treatment has some effect in reducing both the severity and the frequency of arthritis in septic arthritis. The results also indicate that treatment with anti-TNF- α early on alleviates septic arthritis in both frequency and severity more than starting the treatment later. In a similar experiment (Makoto *et al.*, 1997) where heat killed *S. aureus* bacteria was injected directly into the mouse joint, TNF- α peaked 2 hours after infection (Makoto *et al.*, 1997). If bacteria were injected together with anti-TNF- α , leukocyte infiltration into the joint was inhibited during the first 12 hours. However, after 24 hrs and onwards, there was no inhibition observed, and therefore they concluded that the effect of TNF- α was only limited to the first few hours after infection and thus may not be effective in treating septic arthritis induced by *S. aureus*. The results obtained from our experiment dispute this claim since the

severity, frequency and mortality from septic arthritis were reduced on both the early and late treatment groups, even though the results were much better for the early treatment group.

Another interesting point was the bacterial load in the kidneys which was very high in both treatment groups. The results were expected since there is less TNF- α which means there will be less phagocytes stimulated to eliminate the bacteria and thus more bacteria are accumulated in the kidneys.

Both early, and to some extent late treatment, had more weight loss during the course of the infection. This is surprising, as TNF- α is an important mediator of weight loss (cachexia) during inflammation, since theoretically, TNF- α induces catabolism in muscles and fat tissue, leading to weight loss (in this way the organism has more energy to fight off the infection). One of the mechanisms behind it is that TNF- α increases insulin resistance and the cells instead take up glucose from the blood and leave the glucose in circulation so that the immune system may use it. However, it is difficult to say, if those theoretical mechanism really play much role in infection setting. In our experiments we saw that early inhibition of TNF- α caused more weight loss which is contrary to the theory above (that TNF- α is a mediator of weight loss during inflammation). (Argiles., 1997). Most likely, this weight loss was induced by overwhelming bacterial load in treatment groups (especially early treatment). But we also cannot exclude, that the role of TNF- α in weight loss during infection is simply not as big as it was previously speculated.

Results from spleen weight of the different groups were also very interesting in that the early treatment group had significantly bigger spleen than both late treatment and control groups. This can be explained by the fact that the early treatment group had more bacteria accumulating in the kidneys which in turn were stimulating more cytokines to ward off the bacteria and thus also enlarged the spleen. Weight loss was measured primary because it is a part of this model: weight loss reflects the general condition of the mouse during infection. Additionally, as TNF- α is said to be inducing weight loss during inflammation, monitoring weight was a reasonable choice during TNF- α inhibition.

The early treatment group had better survival rate in both the septic arthritis (lower injected bacterial dose) and the sepsis shock (high bacterial dose) experiments. These are very significant findings, especially in regard to potential human therapy. The late treatment group also had slightly better survival rate compared to the untreated mice, even though this was of no statistical significance. Apparently, inhibiting TNF- α activity not only prevents local joint damage, but also protects the entire organism from shock. On the other hand, these findings are in clear contrast to the experiment done by Olof *et al.*, 1998. While the results from that project showed that mice lacking TNF- α , had lower arthritis frequency, the mortality was higher than the wild type. Those differences might be caused by different bacterial doses and timeframes used in their experiments, however, this issue needs further clarification.

The TNF- α level in the serum was quite high in the early treatment group and as figure 8 shows, the difference was of statistical significance compared to the late treatment group. The amount of cytokines is connected to inflammation and high levels of TNF- α indicates severe inflammation due to more bacteria which stimulates production and recruitment of TNF- α . It might also be an effect of feedback loop, when the more cytokine activity is inhibited; the more the organism produces it trying to fight the inhibition. Even though the levels of TNF- α

in circulation increased, the treatment with anti-TNF- α was able to block its activity. Measuring other cytokines gives us more information as to what is happening inside the body, how intensive inflammation is and if we are interfering with some other inflammatory pathways by inhibiting TNF- α . IFN- γ and IL-6 are pro inflammatory, IL-10 is primary anti-inflammatory, but it is always produced during inflammation, so we can treat all of them as markers of inflammation intensity. And as all the inflammation pathways are somehow connected, it was reasonable to expect that interfering with one pathway (TNF- α) might also somehow affect other pathways. Checking for that helps us to understand inflammatory mechanisms better.

Conclusion

In conclusion, anti-TNF- α can be potentially used as a therapy against septic arthritis and sepsis shock. However, this should be combined with antibiotics to eliminate the bacteria while the anti-TNF- α reduces the severity of the inflammation and thus reduce the risk of permanent joint destruction and mortality.

It can also be concluded that blocking TNF- α early on is essential in order to get the best results. However, this study needs to be repeated in order to confirm the results.

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