Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9283
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v38i4.30572

Distinct strains of *Staphylococcus aureus* lead to different inflammatory response patterns in a murine model of intradermal infection

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ABSTRACT. *Staphylococcus aureus* infection may lead to the development of soft tissue damage. It has been evaluated in other researches using different animal models. In addition, the inflammatory response developed by the host organism facing an infection by this pathogen has been analyzed and neutrophils have been linked to the immune response developed. In this study, we aimed to compare the inflammatory response developed by the host induced by an intradermal infection with a methicillin-resistant strain of *Staphylococcus aureus* (MRSA) or a methicillin-susceptible strain of *Staphylococcus aureus* (MSSA). Mice euthanasia occurred in the following times: 6, 24, 48 and 96 hours of infection; the cell number and the cytokine release were evaluated. Our results showed that infections by different strains of *Staphylococcus aureus* lead to different immune response degrees. Although MRSA infection induces higher neutrophil recruitment to the infection site and higher inflammatory response in the draining lymph node, the increased production of TNF-α, IFN-γ, IL-6 and IL-1β in the lymph node 6 hours after the infection was observed only in MSSA infected animals. Considering the data, MSSA may have mechanisms to prevent neutrophil recruitment to the infection site.

Keywords: Neutrophil, lymph node, MRSA.

Diferentes cepas de *Staphylococcus aureus* levam a diferentes padrões de resposta inflamatória em um modelo murino de infecção intradérmica

RESUMO. Infecções causadas por *Staphylococcus aureus* podem causar o desenvolvimento de lesões no tecido frouxo. Isto tem sido avaliado em estudos que avaliam a resposta imune em diferentes modelos animais. Além disso, a resposta inflamatória desenvolvida pelo hospedeiro frente à infecção por este patógeno tem sido analisada e neutrófilos têm sido associados com a resposta imune desenvolvida. Neste estudo, nosso objetivo foi comparar a resposta inflamatória desenvolvida pelo organismo hospedeiro induzida por uma infecção intradérmica com uma cepa de *Staphylococcus aureus* resistente à meticilina (MRSA) ou uma cepa *Staphylococcus aureus* susceptível à meticilina (MSSA). A eutanásia dos camundongos ocorreu nos seguintes tempos: 6, 24, 48 e 96 horas de infecção; o número de células e a quantidade de citocinas foram avaliados. Nossos resultados mostraram que infecções por diferentes cepas de *Staphylococcus aureus* causam resposta imunológica com diferentes intensidades. Enquanto infecções por MRSA induzem maior recrutamento de neutrófilos para o sítio de infecção e maior resposta inflamatória no linfonodo, o aumento na produção de TNF-α, IFN-γ, IL-6 e IL-1β no linfonodo 6 horas após a infecção foi observado somente nos animais infectados com MSSA. Considerando as análises, MSSA pode possuir mecanismos para prevenir o recrutamento de neutrófilos para o sítio de infecção.

Palavras-chave: Neutrófilos, linfonodo, MRSA.

Introduction

Staphylococcus aureus is a gram-positive bacterial pathogen whose infection can lead to the development of soft tissue damage and other diseases in which the degree and intensity vary depending on the site of infection (Mertz et al., 2007). This bacterium is an important human pathogen in terms of adaptability and susceptibility. Its remarkable feature of acquiring antibiotic resistance and pathogenic determinants are advantageous for its survival due to its genetic plasticity (Zetola, Francis, Nuermberger, & Bishai, 2005). Some strains of *S. aureus* exhibit in their genome the *mecA* gene, which confers resistance to

β-lactam antibiotics due to production of a specific penicillin-binding protein (PBP) called PBP 2 (Ma et al., 2002). In general, those strains are called methicillin-resistant *Staphylococcus aureus* (MRSA) and represent a significant number of nosocomial infections reported by hospitals and emergency departments (Espadinha et al., 2013; Klevens et al., 2007; Miles, Voss, Segedin, & Anderson, 2005).

The host initiates an innate immune response mediated primarily by neutrophil when infected by *S. aureus* (Nippe et al., 2011). Several studies have shown that hosts who have a neutrophil deficiency are more susceptible to be infected by *S. aureus*; once infected, diseases tend to be more severe (Mölne, Verdrengh, & Tarkowski, 2000). As a virulence factor, this bacterium presents several mechanisms that might induce neutrophil lyses or reduction of their migration to the infection site (Gresham et al., 2000; Mölne et al., 2000). Some molecules involved in that process are the Chemotaxis-Inhibitory Protein of *Staphylococcus aureus* (CHIPS) and the Extracellular Adherence Protein (EAP), which inhibits the neutrophil migration to the infection site; and the Panton-valentin leukocidin, an exotoxin which activates neutrophils to commit apoptosis (Haggar, Ehrnfelt, Holgersson, & Flock, 2004; Voyich et al., 2005). Other studies have seen that *S. aureus* also developed mechanisms that would prevent its recognition by neutrophils as well as its production of several molecules aiding in evasion of the immune response developed by the host (Rigby & DeLeo, 2012). Therefore, *S. aureus* structure induces the formation of abscesses preventing the immune system cells to reach the infection site prolonging the infection (Cheng et al., 2009; Tzianabos, Wang, & Lee, 2001).

Mice usually is the first choice as a model for pathogenesis and immunity studies due to their well-defined strains and the wide range of immunological reagents available. Moreover, there is a great range of infection routes including intraperitoneal, subcutaneous and intradermal (Tscharke & Smith, 1999). Studies with *S. aureus* infection models should select an infection route that reproduce common routes of infection in humans. Considering humans are infected by *S. aureus* commonly through the skin, intradermal infection appears as the most suitable model for this role (Alabi et al., 2013). In this study, we aimed to compare the inflammatory response developed in an intradermal infection model induced by two different *Staphylococcus aureus* strains.

Material and methods

Bacterial culture

The ATCC 25923 strain of *S. aureus* susceptible to methicillin (MSSA) and a Community-acquired MRSA strain obtained from the Microbiology Laboratory at *Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia*, were reactivated and grown in brain-heart infusion agar for 24 hours at 37°C. After growth, colonies of bacteria were collected, diluted in sterile saline and the number of colony forming unit (CFU) was determined based on analysis of spectrophotometric absorbance at 660nm (value equivalent to 10^8 CFUs in the McFarland scale).

Animals

Male BALB/c mice aged between eight and 10 weeks were kept in the *Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia* facilities, with food and water *ad libitum*. The protocol was approved by the Ethic Committee on the use of Animals of the *Universidade Estadual de Feira de Santana* (UEFS).

Infection model

The animals were divided into three groups (n = 7-9 per experimental time point), and each group received a different treatment. In the first group, the animals were intradermally infected in the right ear with 10^5 CFU of the MSSA strain resuspended in 10 μL normal saline. The animals of the second group received the same treatment but with the MRSA strain. In the third group, the control group, the animals received 10 μL of saline. After 6, 24, 48 and 96 hours the animals were euthanized. The ear and blood were collected and assessed for cell number and profile. The right lateral cervical draining lymph node was collected for analysis of cell count and cytokine levels.

Lymph node cell and cytokines level analysis

The draining lymph nodes were evaluated at 6, 24, 48 and 96 hours after infection, macerated into 1 ml of RPMI medium (HIMEDIA) supplemented with 10% (v/v) fetal bovine serum (GIBCO) inactivated by heat. For cell number assessment, 100 μL of the macerated solution were collected and resuspended in 900 μL of RPMI medium. The cell number was analyzed in a Neubauer chamber using light microscope. For the cytokines level analysis, 900 μL of the macerated solution were diluted with 100 μL of RPMI medium and centrifuged at 12000 g for 15 minutes at 4ºC. To perform the analysis, the supernatant of the centrifuged draining lymph node

was collected and the ELISA Ready-SET-Go! (eBioscience) was performed to measure the levels of tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin-1 beta (IL-1β), IL-10 and IL-6 produced in response to the infection.

Histopathology

Ear tissue fragments were fixed in 10% formalin overnight and then transferred to 95% ethanol until be embedded in paraffin, sectioned (5 μm) and stained with hematoxylin and eosin (HE). The stained sections were visualized in light microscopy and the analysis was performed in photomicrographs obtained with the software AnalySIS getIT 5.1 (Olympus Soft Imaging Solutions).

Statistical treatment

For statistical analysis, Kruskal-Wallis one-way ANOVA was performed using GraphPad Prism 5.1 Software. Differences were considered significant at a p value less than 0.05 in Dunn's posttest. Data were expressed as mean ± standard error of the mean (SEM).

Results and discussion

Staphylococcus aureus is the major pathogen associated to nosocomial infections around the world (Klevens et al., 2007). Nowadays, with the rising number of *S. aureus* antibiotic-resistant strains, it is necessary to develop new alternative prophylactic methods to treat infected patients. The main problem is that this pathogen has a high number of mechanisms that allows it to overcome the host immune system, making the development of new infection control methods harder than for other similar pathogens (DeDent, Kim, Missiakas, & Schneewind, 2012). In this way, it is necessary to understand the immune response developed against distinct *S. aureus* strains to set a strategy that could be used to treat infections caused by this pathogen.

In order to evaluate the systemic response to different strains of *S. aureus* in an intradermal model of infection, total and differential white blood cells were assessed after infection. The total number of circulating cells showed no statistical differences between groups MRSA, MSSA and control (data not shown). However, the analysis of blood smears showed differences in the profile of circulating leukocytes between the groups. There was not observed increasing numbers of neutrophils in either infected groups when compared to control group at any experimental time points (data not shown). In spite of this, when the relative number of neutrophils was analyzed, we observed a higher percentage of neutrophils in the MRSA groups when compared to the control group at 24, 48 and 96 hours after infection (Figure 1). It is well known that the PMNs are the first cells to migrate to the infection site performing an essential role to control bacterial infections. As demonstrated in other studies, during a bacterial infection, cytokines and chemokines are released to stimulate the PMN differentiation, proliferation and migration to the infection site, which permits the evaluation of the lesion intensity. This result was directly correlated with the inflammatory cell number at the infection site, showing that these cells can be mostly represented by PMNs (Durando et al., 2011; Liard et al., 2012).

In the major evaluations no significant differences were observed between the group infected with the MSSA strain and the control group; despite the inflammatory response developed by those groups. It could be associated to the genotype differences between both strains. Preview studies have shown that MRSA strains have more virulence genes than MSSA strains, which can explain the higher intensity of the inflammatory response developed by the MRSA strain (Lim, Yeo, Suhaili, & Thong, 2012). Moreover, the MRSA strain studied in this paper was analyzed and its virulence factors were identified as Spa, PVL and Sea genes as well as it is a strong producer of biofilm (Souza et al., 2014).

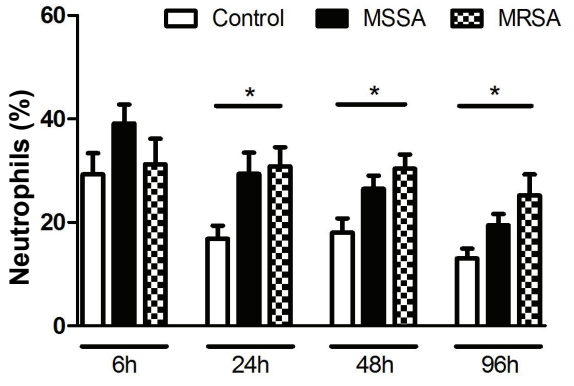

Figure 1. Relative neutrophil count in peripheral blood. Peripheral blood samples of mice in the following study groups: control, infected by methicillin-susceptible *Staphylococcus aureus* (MSSA) and infected by methicillin-resistant *S. aureus* (MRSA), were collected after euthanasia; blood smears were obtained and stained with panoptic dyes. Neutrophil counts were conducted on light microscopes. From the values obtained, the relative number of neutrophils was found. Data presented are the means with SEM of three independent experiments (n = 7-9 animals per group in each experimental point). *$p < 0.05$.

Otherwise, it does not explain why the MSSA strain could not induce an intense inflammatory response. As shown in several studies, some *S. aureus* strains express virulence factors that present

inhibitory effects in the leukocytes recruitment and PMNs phagocytosis. Factors such CHIPS, Efp (Extracellular fibrinogen binding protein) and adenosine synthase A, an enzyme linked to the synthesis of adenosine, an immunosuppressant (de Haas et al., 2004; Ko et al., 2013; Thammavongsa, Kern, Missiakas, & Schneewind, 2009).

Histopathological analysis was correlated to the neutrophil number present in the blood; the MRSA group demonstrates an increased inflammatory infiltrate comparing to the control group in all experimental time points. Moreover, while it was noted the incidence of necrotic tissue with formation of abscesses at the infection site at 24, 48 and 96 hours in MRSA groups, no necrosis was observed in the other groups (Figure 2). Analysis of inflammatory cells number showed an enhancement of the cell number in the MRSA group infection area at all experimental time points. (Figure 3a). Neutrophil was the main cell type present in abundance at the infection site (data not shown). Besides that, the duration of the inflammatory cells recruitment developed at the infection site was also analyzed. It was observed, that the cell number of the MRSA group decreased significantly over time, showing that in this group the inflammatory response was higher than the one developed in the MSSA (Figure 3b).

In was possible in this report to observe that different strains of *S. aureus* can induce distinct inflammatory response in the intradermal infection model. In the MRSA infected group, even after 6 hours of infection it was observed a higher inflammatory cell number at the infection site and this number was significantly higher over time. This data, however, was not observed in the MSSA infected group. Moreover, was possible to observe in all experimental points the development of sustained abscesses in the infection site, in which the most present cell type were neutrophils. In addition, in the differential analysis of blood smears, a higher number of PMNs in the blood of *S. aureus* infected groups was observed when compared to the control group.

The results of our analysis point to a possibility that the MSSA strain expresses at least one of these inhibitory virulent factors, which may explain the lower intensity of the inflammatory response developed against MSSA strain. In Figure 2 and Figure 3 can be observed that MSSA strain presented lower number of inflammatory cells at the infection site and lower cells at the lymph node when compared to MRSA strain.

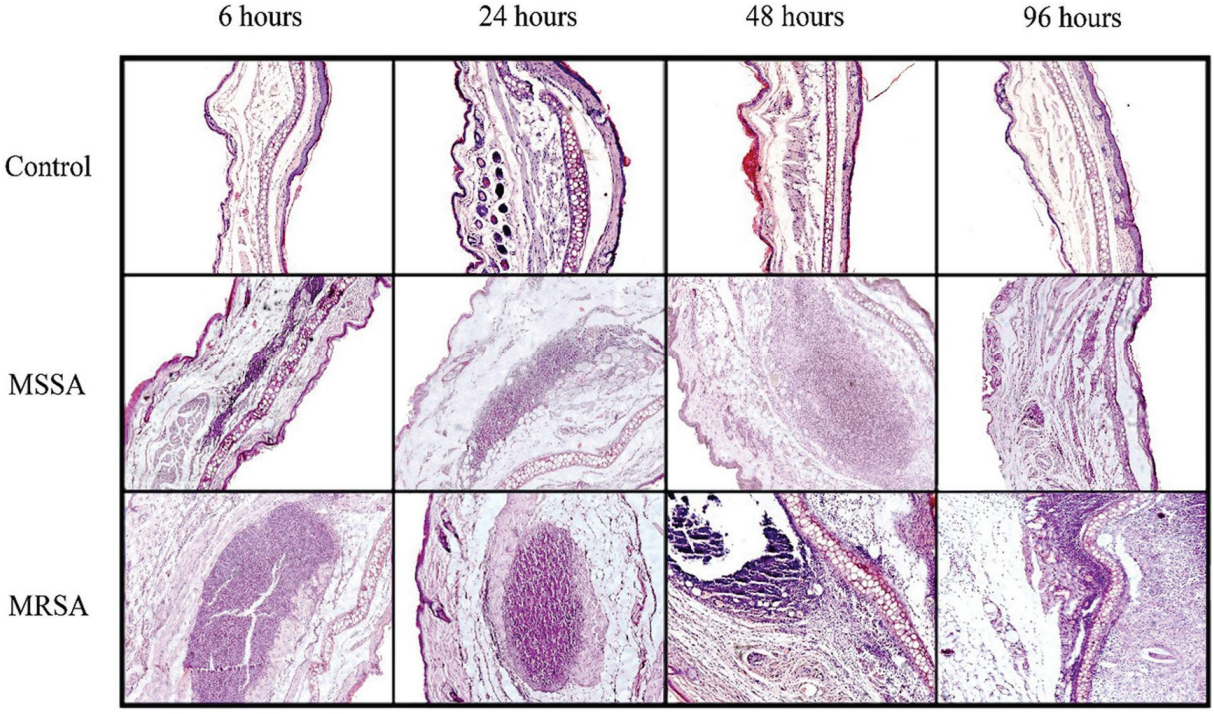

Figure 2. Histopathological analysis of mouse ears. Mice were infected intradermally in the right ear with methicillin-susceptible *Staphylococcus aureus* (MSSA) or methicillin-resistant *S. aureus* (MRSA) 6, 24, 48 and 96 hours before euthanasia. Control group received saline intradermally in the ear. The right ear of each animal group was collected after euthanasia, fixed in formalin and embedded in paraffin. Sections (5μm) were prepared and stained with hematoxylin and eosin. After preparation, the slides were analyzed using light microscope (Magnification 40x). Each group presented n = 7-9 animals in each experimental point.

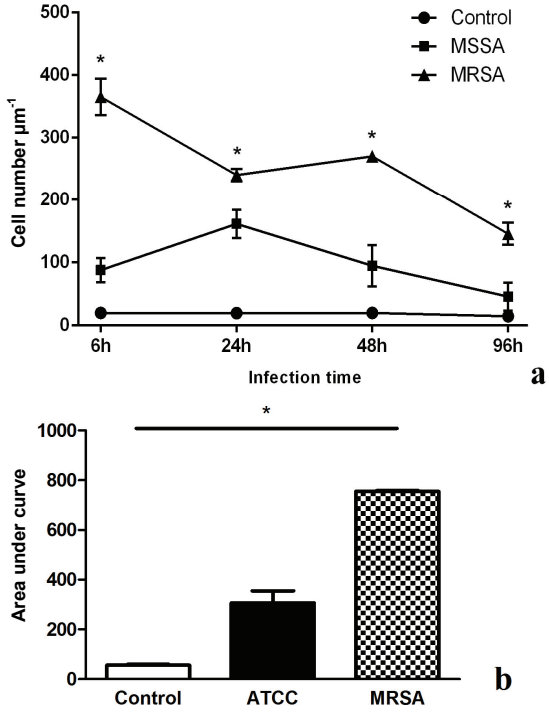

Figure 3. Number of inflammatory cells at the infection site reduce over the time. Mice were infected with 10^5 CFUs of methicillin-susceptible *Staphylococcus aureus* (MSSA) or methicillin-resistant *S. aureus* (MRSA) 6, 24, 48 and 96 hours intradermally in the right ear. Control group received saline intradermally in the ear. The right ears were collected after euthanasia, fixed in formalin and embedded in paraffin. Sections (5 μm) were prepared and stained with hematoxylin and eosin. After preparation, the slides were analyzed and the cell number at the infection site was counted using light microscope and accessed using photomicrographs. Cell number over the time (a). Area under curve (b). Data presented are the means with SEM of three independent experiments (n = 7-9 per group in each experimental point). *p < 0.05.

To analyze the pathogen capacity to stimulate the proliferation of cells in lymph node, the cell number at this site was evaluated by light microscopy. The analysis showed a significant increase of cell number in MRSA group comparing to the control group in all experimental time points, suggesting that MRSA strain is capable to stimulate an immune response more persistent than MSSA strain in this animal model (Figure 4). However, when the lymph node hyperplasia was evaluated over the experimental time points, it was observed a similar pattern of proliferation in MSSA and MRSA groups (data not shown). The analysis of cytokines production in lymph node showed an increase in the production of TNF-α, IFN-γ and IL-1β in the draining lymph node collected 6 hours after infection with both strains. (Figure 5). In the other experimental time points, significant differences were not observed between the groups (data not shown).

In preview studies there is a relation between NOD-like receptors deficient mice, a receptor required for activation of NF-κβ, and the compromising inflammatory response. It leads to a reduced release of IL-1β and IL-6, inhibiting the activation of neutrophils and macrophages during an infection caused by *S. aureus*, favoring the infection permanence (DeLeo, Diep, & Otto, 2009; Hruz et al., 2009). It is possible that the MSSA strain has a mechanism that could inhibit the activation of NF-κβ, reducing the activation of these cells, avoiding the phagocytosis by PMNs. The MRSA strain, on the other hand, appears not to show these chemotactic inhibitory mechanisms due to the persistent inflammatory response developed. However, further studies are still needed to determine this correlation.

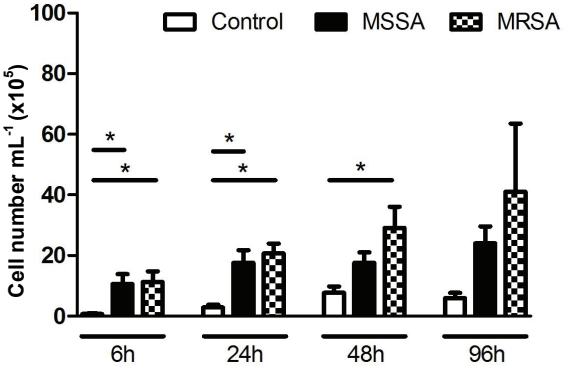

Figure 4. Cell number in lymph node after infection with different strains of *Staphylococcus aureus*. Mice were infected with 10^5 CFUs of methicillin-susceptible *S. aureus* (MSSA) or methicillin-resistant *S. aureus* (MRSA) 6, 24, 48 and 96 hours intradermally in the right ear before euthanasia. Control group received saline intradermally in the ear. After the euthanasia, the right lymph node was collected, centrifuged and the cell number was determined in Neubauer chamber. *p<0.05. The data are pooled from three independent experiments (n = 7-9 animals per group in each experimental point).

In peripheral organs such the skin, when an infection occurs, the Antigen Presenting Cells (APCs), mostly dendritic cells, phagocytize the bacterium and present the antigens to lymphocytes located in the lymph node near the infection site, inducing the development of an adaptive response against the pathogen. This might induce the release of Th1 cytokines that are related to the control of the infection in a long term (Banchereau & Steinman, 1998; Lanzavecchia & Sallusto, 2001; Liard et al., 2012). However, some studies have noticed that some *S. aureus* strains are capable to resist the phagocytosis mechanism, which might reduce the protective role of the antigen. This mechanism involves the induction of a reversible phenotype that can occur following bacterial internalization facilitating the intracellular survival (Tuchscherr et al., 2011).

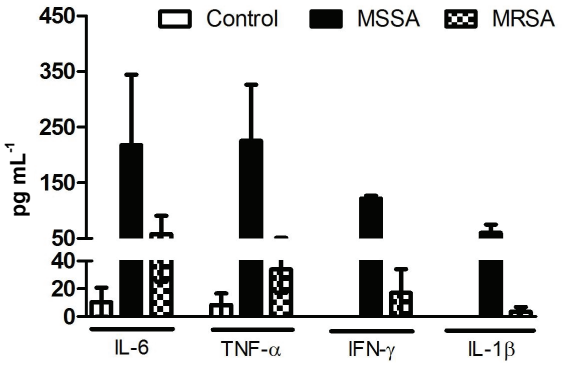

Figure 5. Cytokines levels in draining lymph node after 6 hours of infection with different strains of *Staphylococcus aureus*. Mice were infected with methicillin-susceptible *S. aureus* (MSSA) or methicillin-resistant *S. aureus* (MRSA) 6, 24, 48 and 96 hours intradermally in the right ear. Control group received saline intradermally in the ear. After euthanasia, the right draining lymph node was collected, macerated, centrifuged and the supernatant was used for analysis of IL-6, TNF-α, IFN-γ and IL-1β production by ELISA. The data were pooled from a single independent experiment (n=7-9 animals per group in each experimental point).

Our results showed that both infected groups presented lymph node hyperplasia. However, the MRSA strain showed a sustained hyperplasia until 48 hours after the infection, whereas the MSSA exhibited hyperplasia only until 24 hours after the infection. These data suggest that MRSA strain does not show mechanisms to avoid the phagocytosis accomplished by APCs, which could explain the greater lymph node hyperplasia. On the other hand, the less pronounced hyperplasia observed in mice infected with the MSSA strain could be explained by the presence of mechanisms to avoid the phagocytosis by APCs in the infection site. It, consequently, could reduce the migration of APCs to lymph node avoiding the hyperplasia of this organ. The results are similar to our findings in the infection site, in which the MSSA strain induced a less pronounced inflammatory response.

It is well known that during the inflammatory response against *S. aureus* the production of pro-inflammatory cytokines, such as IL-1β, IL-6, IFN-γ and TNF-α, has an important role in the control of bacterial infections (Nakane, Okamoto, Asano, Kohanawa, & Minagawa, 1995). These cytokines induce the migration and activation of inflammatory cells to control the infection. In our study, however, we observed that in the MSSA infected group the level of IL-1β, IFN-γ and TNF-α was higher in comparison to the MRSA group after 6 hours of infection. It suggests that the hyperplasia observed after the infection with MSSA is not due to the release of pro-inflammatory cytokines in the lymph node. It is possible that these data are linked to the phagocytosis resistance mechanism of the MSSA strain. This resistance mechanism could induce the APCs to release neutrophil-attractive chemokines such as IL-8 and MIP-2 (Burdon, Martin, & Rankin, 2005; Kobayashi, 2008) instead of chemokines, stimulating the influx of inflammatory cells to the draining lymph node promoting the observed hyperplasia.

Conclusion

Our results suggest that both strains may be harmful to the host in different ways. MSSA seems to be able to modulate the immune system inducing the survival of the strain in a long term; it may unleash a systemic infection. On the other hand, the MRSA strain is capable of inducing an intense inflammatory response harming the infection site in a short term. Depending on the infection site, it can be the main cause of the infection complications. Despite knowing that those strains have different mechanisms of infection and survival, further analysis are needed to determinate the genotypic differences between MSSA and MRSA strains responsible for those different immune responses and patterns during infection.

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Received on January 14, 2016.
Accepted on September 29, 2016.