

*Original Research Article*

# Nasal Infection by Slime-Producing *Staphylococcus aureus* in a Mouse Model

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Abstract

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*Staphylococcus aureus* is part of the normal microbiota in humans. Its main reservoir is the nostrils, from where it can spread and be responsible for a wide spectrum of diseases. The aim of this study was to evaluate nasal colonization and histopathogenic effects on the nasal mucosa mice infected with slime producing *S. aureus*. Mice C57BL/6 (wild-type) and C57BL/6 deficient in TNFR p55 were infected intranasally with *S. aureus* slime-producing strain. Colony counts from blood and from nasal and internal organs homogenates were evaluated. Histological studies were performed. No significant differences in bacterial counts were found between mice strains after nasal infection with *S. aureus*. In the deficient mouse strain, low respiratory system infection was detected, demonstrated by bacterial counts in the lung homogenates. The histological sections of the nostrils of both strains of infected mice showed an increase in fat cells in relation to controls. The lung of infected C57BL/6 TNFR p55 -/- mice revealed adipose tissue and marked leukocyte infiltration. The passage of bacteria to the lung could be related to a decrease in defence mechanisms against *S. aureus* in C57BL/6 TNFR p55 -/- mice, due to the absence of signalling and consequent lack of action of TNF- $\alpha$ . The local increase in adipocytes may constitute an important defence response against *S. aureus* infection by the host.

**Keywords:** *S. aureus*, nasal carrier, slime, histological studies, C57BL/6 wild-type mice, C57BL/6 TNFR p55 -/- mice

## INTRODUCTION

Most species of the genus *Staphylococcus* are widely distributed in nature and are part of the normal microbiota of the skin, the armpits, the perineum, the anterior portion of the nostrils and the upper respiratory tract of humans. Several studies have shown that the anterior nostrils constitute the main ecological niche for *S. aureus* in humans. From this site, carriers can transmit or disseminate this bacterium to susceptible individuals, constituting an important risk factor for the development of serious infections (Fosch et al., 2012; George et al., 2016; Sakr et al., 2018).

Although the relationship between colonization and the infectious process has not been clearly defined, it has been established that there are intrinsic factors both of

the host and of the strains of *S. aureus* associated with colonization and infection (Archer et al., 2013; Cole et al., 2016). Certain strains of *S. aureus* have the ability to produce an extracellular mucoid (slime) whose main component is a polysaccharide in nature and consists of glycosaminoglycans. It is known that slime production by microorganisms is an important virulence factor responsible for adhesion to living or inert surfaces (Raza et al., 2013). This exopolysaccharide confirms the bacteria greater resistance against host defences and antimicrobial treatment (Mathur et al., 2005; Diamond-Hernandez et al., 2010).

Mice are widely used as staphylococci colonization and infection model, due to various advantages:

numerous strains with knock-out genes are available, their immune system has been well characterized, and they enable a variety of infection routes, including the intranasal (González-Zorn et al., 2005; Kim et al., 2014).

*S. aureus* infections are characterized by a profound inflammatory response, which contributes significantly to the pathogenesis and elimination of bacteria. The complex mechanisms of the host response to invasion by pathogens include the production and release of pro-inflammatory and immunomodulatory cytokines. Among the induced pro-inflammatory cytokines, TNF- $\alpha$  has been shown to be crucial in immunity to bacterial infections, as demonstrated by neutralization experiments and, more recently, by the use of knock-out mice. TNF- $\alpha$  is a pro-inflammatory and immunomodulatory cytokine produced by a broad spectrum of cells such as monocytes, macrophages, B and T lymphocytes, NK cells, as well as non-immune cells such as fibroblasts and keratinocytes (Schluter and Deckert 2000; Carrillo de Albornoz Sainz et al., 2006). Since TNF- $\alpha$  plays an important role in the protection against bacterial infection, its main function is the recruitment and stimulation of neutrophils and monocytes, together with the induction and regulation of mediators of inflammation. This cytokine can be recognized by two different receptors, namely, TNFR1 or p55 and TNFR2 or p75; however, immune reactions in bacterial infections are more effective through TNFR1 signalling, whereas the signalling attributed to the TNFR2 receptor in this sense is lower or even nil (Schluter and Deckert, 2000).

The aim of this study was to evaluate nasal colonization and histopathogenic effects on the nasal mucosa of two mice strains infected with slime-producing *S. aureus*: C57BL/6 (wild-type, WT) and C57BL/6 deficient in p55 TNFR.

## MATERIALS AND METHODS

### Bacterial strains

*S. aureus* ATCC 35556 (SA113) slime-producing strain (*icaA* and *icaD* positive), kindly provided by Dr. Andreas Peschel, University of Tuebingen, Germany.

### Mice

Male mice of C57BL/6 (wild-type WT) and C57BL/6 deficient in *TNFR p55* (C57BL/6 *TNFR p55*  $-/-$ ), of 6 to 8 weeks of age were used. The animals were maintained with sterile water and *ad-libitum* feeding. Mice were provided by Dr Silvia Di Genaro of the Laboratory of Immunopathology of the National University of San Luis (UNSL). The protocol of experimentation with the animal model used was supervised and approved by the Commission of the use of laboratory animals of the

UNSL. All experiences were repeated three times under the same conditions.

### Infection

The mice were infected by instilling 20  $\mu$ l intranasally (10  $\mu$ l in each nostril) with a bacterial suspension of  $1 \times 10^8$  CFU/ml using a sterile pipette. On the second day after the infection, blood was drawn from the submandibular vein and then the animals were sacrificed by the physical method of cervical dislocation. Then, the corresponding homogenates of nasal and internal organs were performed. Each experiment was performed in groups of 4 mice. Uninfected mice were used as controls.

### Sample processing

*Blood*: 50  $\mu$ l of blood was seeded on salty mannitol agar that was incubated at 37°C for 24-48h. Subsequently, colony counts were performed and expressed in CFU/ml of blood.

*Nasal homogenate*: the nose was removed with sterile scissors and a homogenate was made in Eppendorf tubes containing 500  $\mu$ l of physiological solution. Fifty microliters (50  $\mu$ l) were seeded directly from the initial homogenate and 50  $\mu$ l of the first base 10 dilutions into salted mannitol plates to perform colony counts.

*Homogenate of internal organs*: lung and spleen were extracted and placed in tubes containing 1 ml of sterile physiological solution. The homogenate was made by pressing and grinding each organ in a metal mesh. Subsequently, 100  $\mu$ l were plated in salted mannitol plates and incubated at 37°C for 24-48 h for colony counting. The bacterial counts were expressed in CFU/organ decimal logarithm.

*Histological study*: the nostrils were fixed in Bouin's liquid for approximately 12 to 24 hours. Subsequently, the tissues were dehydrated in alcohols of increasing concentration and included in paraffin. Sections 3 to 4  $\mu$ m thick were obtained with a Microm HM 325 rotating microtome and stained according to the Hematoxylin-Eosin (H-E) staining technique.

*Verification strains*: the presence of *S. aureus* ATCC 35556 was confirmed using Gram staining and traditional biochemical tests (Murray et al., 2017); the detection of slime was performed by the qualitative phenotypic method of Congo Red Agar (CRA) described by Freeman et al. with some modifications (Freeman et al., 1989). Colonies of heart-brain agar were seeded on CRA plates and incubated overnight at 37 °C in aerobiosis and then 24-48h at room temperature.

## Statistical Analyses

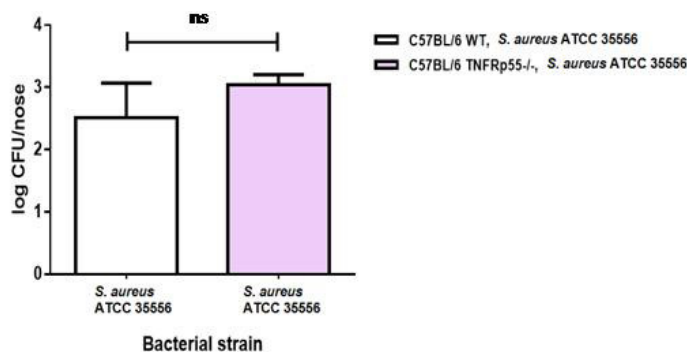
The mean values of the different groups were compared using Student's t-test. Values of  $p < 0.05$  were considered statistically significant. Statistical analyzes were performed using GraphPad Prism 5.0 software.

## RESULTS

### Nasal colonization, Bacteriological study

Both strains of mice presented *S. aureus* in the homogenates of their nostrils two days after infection. As shown in Figure 1 no significant differences were obtained in the nostril *S. aureus* load between C57BL/6 WT mice and mice deficient in the gene coding for the p55 receptor of TNF.

The study of internal organs showed that the passage of bacteria to the lower respiratory tract (lung) occurred only in C57BL/6 *TNFR p55*<sup>-/-</sup> mice, detected by the positive count of the homogenate (average counts: 50 CFU/lung). The culture of spleen homogenates and peripheral blood was negative for both mice strains studied.



**Figure 1.** Average count of *S. aureus* obtained from nasal homogenates of C57BL/6 WT and C57BL/6 *TNFR p55*<sup>-/-</sup> mice 2 days after infection with ATCC 35556. ns: not significant.

### Histological study

The structural organization of the mucosa in the nasal cavity of non-infected C57BL/6 WT mice showed a flattened keratinized stratified epithelium and a lamina propria with numerous hair follicles and sebaceous glands. No adipose tissue or leukocyte infiltrate was observed in the deep region of the mucosa. Non-infected C57BL/6 *TNFR p55*<sup>-/-</sup> mice showed a structural organization of the nasal mucosa similar to the one described above. (Figure 2A, 2B).

In the group of C57BL/6 WT mice infected with *S. aureus* ATCC 35556, the histology showed an organized mucosa with abundant adipose cells in the different regions of the lamina propria. (Figure 3).

The histological study of the nasal mucosa in infected C57BL/6 *TNFR p55*<sup>-/-</sup> mice showed some morphological changes in the epithelial lining and connective tissue. In the lamina propria, an abundant presence of adipose cells and some leukocytes was observed. (Figure 4A, 4B, 4C).

The lung histological study of non-infected C57BL/6 *TNFR p55*<sup>-/-</sup> mice showed normal pulmonary architecture of spongy appearance with numerous alveolar sacs and wide and empty ducts, delimited by fine septa or alveolar septa. The different types of bronchioles were identified according to their typical characteristics: the lobular bronchiole, of irregular light, lined with cylindrical epithelium, the terminal bronchiole, of regular light with simple cubic epithelium and the respiratory bronchiole, with irregular light and discontinuous flat epithelium. (Figure 5A, 5B, 5C).

On the other hand, the lung study of infected C57BL/6 *TNFR p55*<sup>-/-</sup> mice revealed an infiltrated and collapsed lung parenchyma. In addition, tissue of compact appearance, alveolar sacs of reduced light, presence of adipose tissue and marked leukocyte infiltration were identified, forming lymphatic nodules of various sizes. Cartilaginous structures were also observed, typical of the intrapulmonary bronchiole, surrounded by numerous muscle fibres, nerves and dilated blood vessels. (Figure 6A, 6B, 6C).

Figure 2: A) A flat stratified epithelium (Ep) and normal morphology of the anterior nasal fossa are observed, with abundant hair follicles (f) and a fibromuscular stroma (fm) in the depth of the lamina propria; mg: mucous gland; oc: olfactory corpuscle. 40X. H-E. B) C57BL/6 *TNFR p55*<sup>-/-</sup>. Structural organization of the mucosa of the anterior nasal fossa in mice without infection. A flattened keratinized stratified epithelium (Ep) and a compact lamina propria (Lp) with mucosal glands (mg) and hair follicles (f) are observed a: adipocytes; mf: skeletal muscle fibre. 100X H-E.

Figure 3: Flat stratified epithelium (Ep) and a lamina propria (Lp) organized with the presence of hair follicles (f) and mucous glands (mg) are observed. a: adipocyte; mf: muscle fibre. 40X. H-E.

Figure 4: A) A flattened keratinized stratified epithelium (Ep) and a compact lamina propria (Lp) without morphological alterations are observed. a: adipocytes; mf: muscle fibres; mg: mucosal glands; f: hair follicles. 100 X H-E. B) Image showing the presence of blood vessels and some leukocytes (arrow). mf: muscle fibres. 400X. H-E. C). This image shows abundant adipose tissue in the lamina propria a: adipocytes; mf: muscle fibres; Bv: blood vessels. 400X. H-E.

Figure 5: A) Panoramic image showing the histological organization of the lung parenchyma. The presence of terminal (Tb) and respiratory (Rb) bronchioles is observed. Note abundant alveolar sacs (As) and blood vessels forming spongy tissue. ca: capillary. 40X. H-E. B) Region of the lung tissue characterized by the

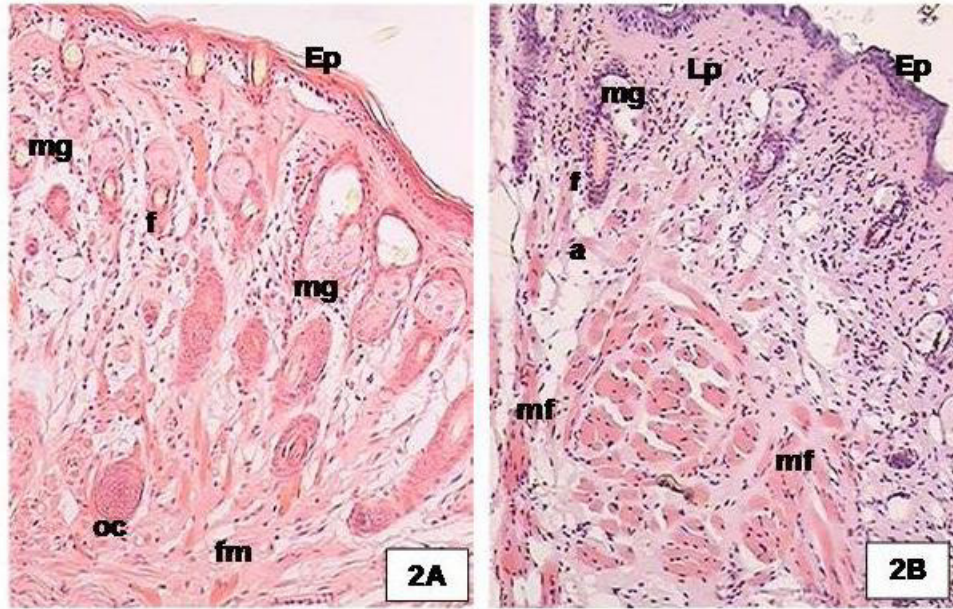


Figure 2. Nose histology in mice without infection. A) C57BL/6 WT.

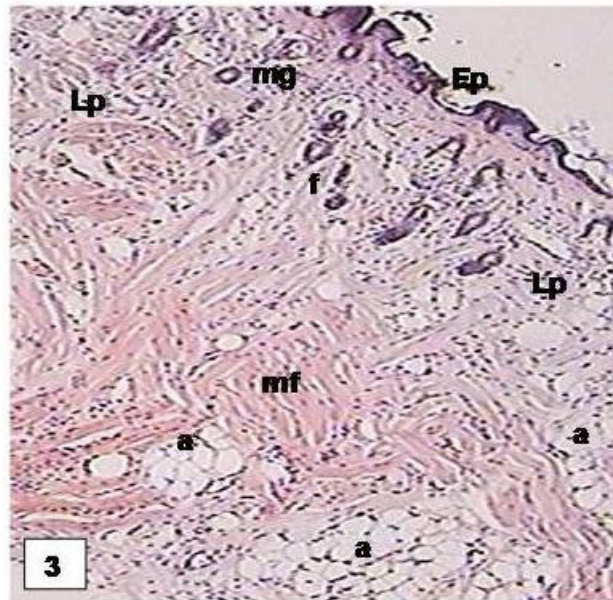


Figure 3. Nose histology in C57BL/6 WT mouse infected with *S. aureus* ATCC 35556.

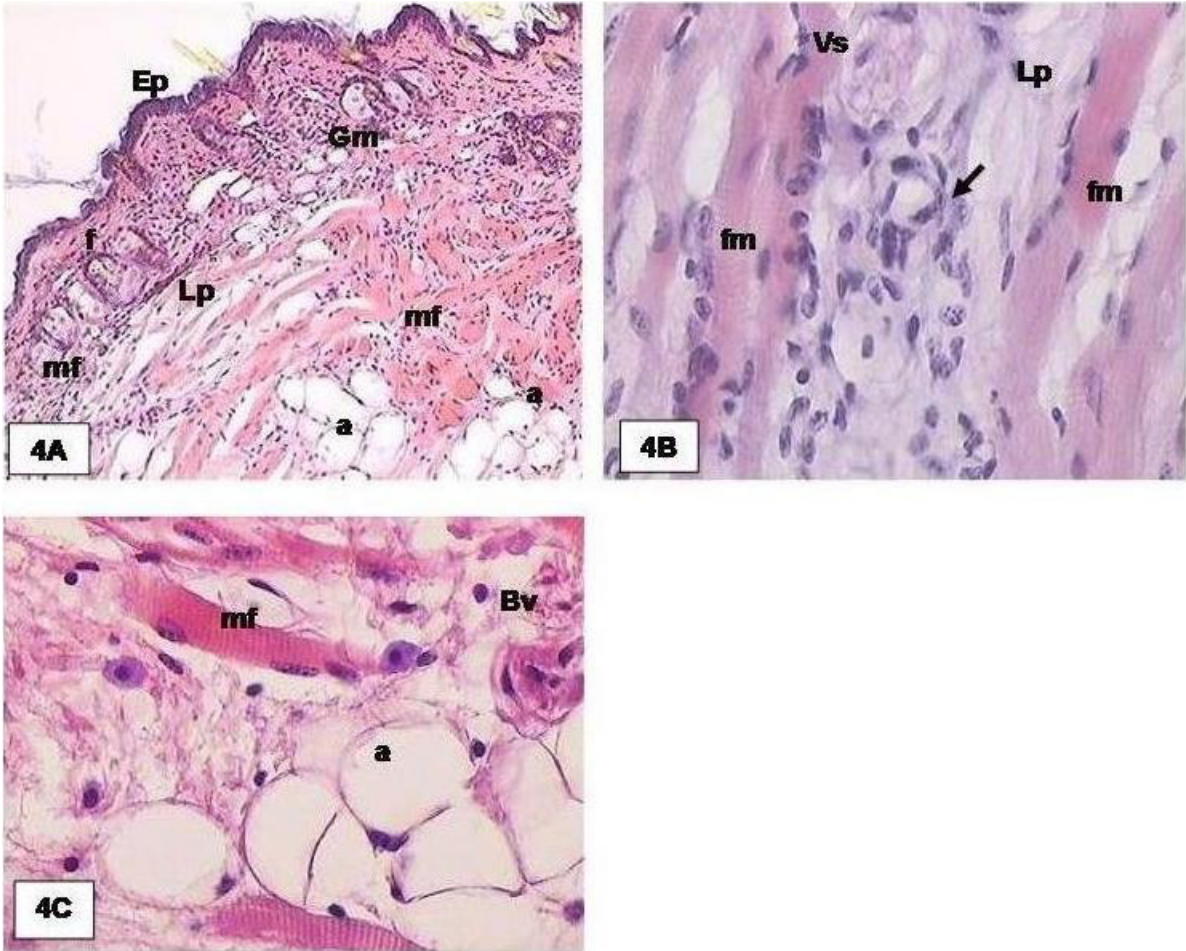
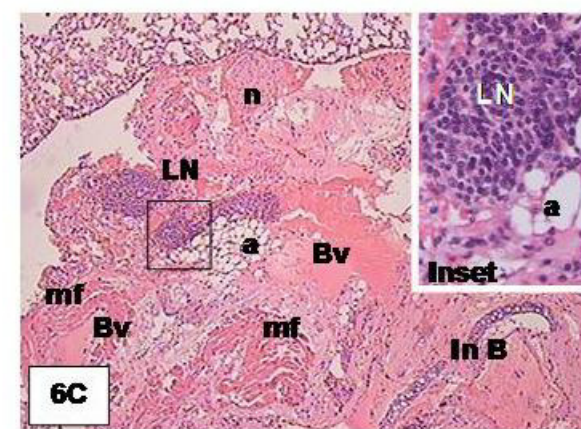
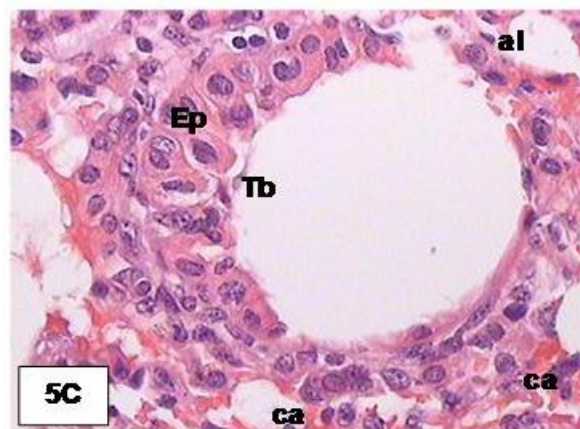
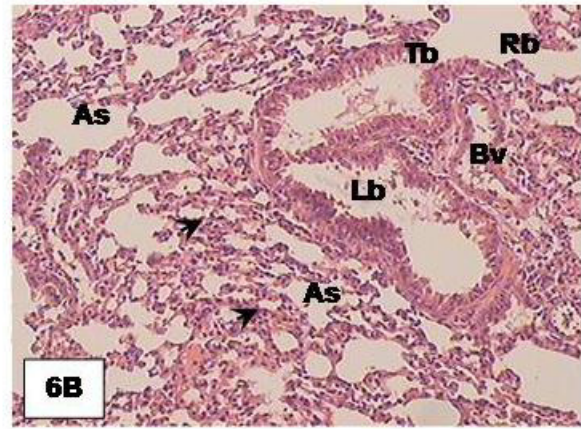
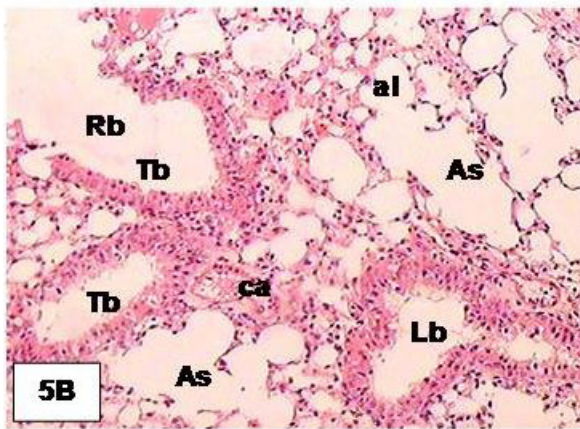
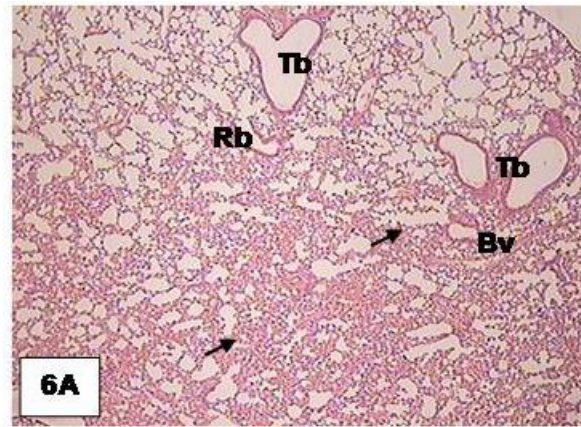
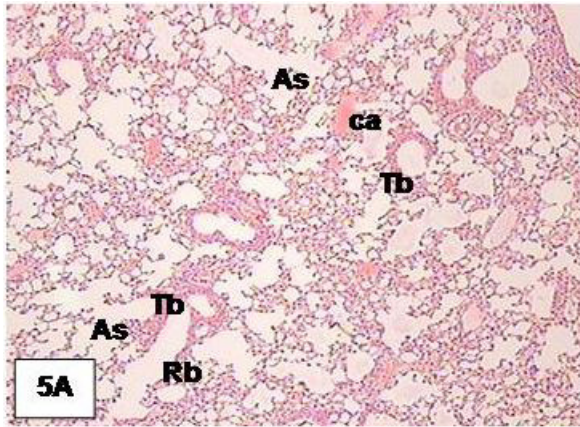


Figure 4. Nose histology in C57BL/6 *TNFR p55*<sup>-/-</sup> mouse infected with *S. aureus* ATCC 35556.



**Figure 5.** Lung histology in C57BL/6 *TNFR p55*<sup>-/-</sup> uninfected mice.

**Figure 6.** Lung histology in C57BL/6 *TNFR p55*<sup>-/-</sup> mouse infected with *S. aureus* ATCC 35556.

presence of spongy parenchyma with small alveolar ducts. Lobular bronchioles (Lb) and terminal bronchioles (Tb) continue with the respiratory bronchioles (Rb). Note the arrangement of the alveoli (al) and alveolar sacs (As); ca: capillary. 100 X H-E. C) Higher magnification image showing a terminal bronchiole (Tb), with normal histology, surrounded by some capillaries (ca). Ep: epithelium. 400X. H-E.

Figure 6: A) Areas of lung tissue showing infiltration of

leukocytes (arrows). Bronchioles and blood vessels (Bv) show wide clear and irregular shapes. Tb: terminal bronchioles; Rb: respiratory bronchioles. 40X. H-E. B) The photo-micrograph shows lung parenchyma with marked leukocyte infiltration (arrow). Note the presence of narrow alveolar sacs (As) and collapsed alveoli (arrowheads). Lb: lobular bronchiole; Tb: terminal bronchiole; Bv: blood vessel. 100X H-E. C) Image showing a lung area near the intrapulmonary bronchiole

(in B). Note a large development of muscle fibres (mf), nerves (n), and adipose tissue (a), associated with numerous lymph nodes (LN). Bv: blood vessels. 20X. H-E. Inset: Detail at higher magnification of a lymphatic accumulation (LN) close to adipose cells (a). 400X. H-E.

## DISCUSSION

It has been established that some intrinsic factors of the host and specific factors of the *S. aureus* strains can influence both colonization and infection (Archer et al., 2013; Cole et al., 2016; Sakr et al., 2018; Ceccarelli et al., 2019). The upper respiratory tract seems to be an ideal target for bacterial colonization and the growth of bacteria in biofilm because it is a warm and humid environment and easily accessible to airborne pathogens. Infections of the upper respiratory tract caused by biofilm-forming bacteria often have a persistent course, are more resistant to antimicrobials, and are hardly eliminated by the immune system, which affects their treatment and control (Bjarnsholt et al., 2011; Emaneini et al., 2015; Morris 2007; Mohamed et al., 2020).

In this study, nasal infection in C57BL/6 WT and C57BL/6 *TNFR p55*<sup>-/-</sup> mice with ATCC 35556 showed no significant difference in bacterial counts between strains.

On the other hand, the histology of the infected C57BL/6 WT mice nostrils showed an organized mucosa with an abundant presence of adipose, while cells and nostrils of C57BL/6 *TNFR p55*<sup>-/-</sup> showed some morphological changes, highlighting leukocytes in the lamina propria and abundant presence of adipocytes.

The histological sections of the nostrils of both strains of infected mice showed an increase in fat cells in relation to uninfected mice. Likewise, the histological study of the lung in infected C57BL/6 *TNFR p55*<sup>-/-</sup> mice in which infection of the lower respiratory system was detected revealed the presence of adipose tissue.

No other studies have been found so far that relate the effect of nasal colonization and respiratory infection by *S. aureus* with the expansion of adipose tissue. However, the important role of adipocytes in the innate immune response against skin infections by this bacterium is well known. A local increase in adipocytes constitutes an important defense response by the host, thanks to the secretion of a variety of bioactive adipokines and cytokines released by adipocytes that mediate the immune response, in addition to producing the antimicrobial peptide cathelicidin with a bactericidal effect (Desruisseaux et al., 2007; Zhang et al., 2015; Qian et al., 2016). Further in-depth studies should be carried out to describe the response of adipocytes against a staphylococcal nasal infection and to elucidate the influence of different virulence factors on adipogenesis in this type of infection.

In addition, it is worth noting that the passage of may

bacteria to the lung in C57BL/6 *TNFR p55*<sup>-/-</sup> mice, may be related to a decrease in the defence against *S. aureus* of the deficient mouse strain due to the absence of signalling and the following lack of action of TNF- $\alpha$  in these mice.

Numerous studies carried out in experimental models using recombinant TNF- $\alpha$  have shown the contribution of this cytokine in the initial defence of the host against *S. aureus* (Vaudaux et al., 1992) and the role of the TNFR1 receptor (p55) in the recognition of this bacterium by protein A associated with its cell wall (Bluml et al., 2012). On the other hand, the inhibition of endogenous TNF- $\alpha$  has been reported to increase mortality during *S. aureus* infection (Nakane et al., 1995; Fei et al., 2011; Ceccarelli et al., 2019) which demonstrates the important role of this cytokine during the initial response of the host (Giai et al., 2013).

In C57BL/6 mice *p55 TNFR*<sup>-/-</sup> histological examination of the lungs revealed significant changes in relation to the description in lungs from uninfected mice, of this same strain. An infiltrated and collapsed parenchyma, alveolar sacs of reduced light, presence of adipose tissue and leukocyte infiltration with dilated blood vessels were observed in the lungs of infected C57BL/6 *TNFR p55*<sup>-/-</sup> mice. Similar results were found by other authors in the histological study of the lung of C57BL/6 mice infected intranasally with *S. aureus*, including loss of alveolar architecture, necrosis, haemorrhage, infiltration of immune cells and consolidation of the lung parenchyma, associated with pneumonia caused by this bacterium (Parker and Prince, 2012).

An important factor in the pathogenesis of staphylococcal pneumonia is the intensity of the innate immune response to the aspirated organisms. Initial immune signalling is achieved by airway epithelial cells that immediately recruit several types of immune cells, including dendritic cells, macrophages, and T cells (Parker and Prince, 2012). On the other hand, TNF- $\alpha$  plays a very important role in bacterial eradication since it supports the host defences through several mechanisms, such as the potentiation of bacterial death by neutrophils and the positive regulation of adhesion molecules necessary for the recruitment of these polymorphonuclear cells (Tuchscherer et al., 2011). Numerous studies carried out in experimental models using recombinant TNF- $\alpha$  have demonstrated the contribution of this cytokine in the initial defence of the host against *S. aureus* (Giai et al., 2013). Fei et al., noted that inhibition of endogenous TNF- $\alpha$  increases mortality during *S. aureus* infection, revealing an important role of this cytokine (Fei et al., 2011).

Also, this cytokine and its receptors have been investigated in a variety of bacterial infections, such as those caused by *Corynebacterium parvum*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Yersinia enterocolitica* and *S. aureus*, demonstrating the importance of TNFR1 in the optimal elimination of these

infectious agents (Schluter and Deckert, 2000).

The absence of signalling and consequent lack of action of TNF- $\alpha$  in these mice deficient in *TNFR p55*<sup>-/-</sup> could explain the decrease in defence against *S. aureus*, evidenced by the passage of bacteria to the lung.

## CONCLUSIONS

Bacterial counts of nasal homogenates from C57BL/6 WT and C57BL/6 *TNFR p55*<sup>-/-</sup> mice nasally infected with *S. aureus* ATCC 35556 did not show significant differences between the two mice strains. However, in the deficient mouse strain, the passage of bacteria into the lung was detected. The absence of signalling and the consequent lack of action of TNF- $\alpha$  in these deficient mice was related to a decrease of defences against *S. aureus*. In the histological sections of the nostrils of both infected mice strains an increase in fat cells was detected. The histological study of C57BL/6 *TNFR p55*<sup>-/-</sup> mice lung showed leukocyte infiltration and the presence of adipose tissue. The local increase in adipocytes may constitute an important defence response against *S. aureus* infection by the host.

## Future perspectives

Some of our future studies will be: to investigate the role of tumour necrosis factor (TNF- $\alpha$ ) in nasal colonization, studying the concentration of this cytokine in serum and homogenates from the nose and lung of mice C57BL/6 WT, C57BL/6 *TNFR p55*<sup>-/-</sup> and BALB/c infected intranasally with *S. aureus* ATCC 35556 (SA113) slime-producing strain (*icaA* and *icaD* positive), and *S. aureus* ATCC 35556 knockout (SA113<sup>-/-</sup>) (*icaA* and *icaD* negative). Evaluate the histopathogenic effects produced.

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## Authors contributions

Satorres S. and Echenique D. conceived of the presented idea planned and carried out the experiments. Aguilera Merlo C., Cruceño A. and Mattana C. contributed to the preparation and analysis of experiments. All the authors supervised the findings of this work and contributed to the final manuscript.

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## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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