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Interleukin 10 modulation of neutrophil subsets infiltrating lungs during *Streptococcus pneumoniae* infection

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ABSTRACT

Interleukin-10 production and lung neutrophil infiltration are two essential components of the balanced immune response to pneumonia caused by *Streptococcus pneumoniae*. Here we describe the existence of two neutrophil subsets in lungs during experimental *S. pneumoniae* infection in mice, which have different size, granularity and expression of activation markers. During infection, both neutrophils subsets were increased in the lungs of IL-10 producing mice, however this increment was significantly higher in the absence of this cytokine. These results suggest that IL-10 is a key cytokine that regulates lung inflammation during bacterial infection caused by specific neutrophil subsets infiltrating the lungs.

1. Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is a pathogenic extracellular Gram-positive bacterium that colonize and invade the respiratory tract [1]. This bacterium affects mainly children under five years of age and the elderly, remaining as a major health concern worldwide [2,3]. *S. pneumoniae* expresses several virulence factors used to evade the host immune response [1]. The immune response against *S. pneumoniae* starts with the bacterial recognition by alveolar macrophages and epithelial cells [1,4], which actively secrete chemokines and cytokines to promote a massive recruitment of monocytes, neutrophils and lymphocytes to the lungs [1,4]. Neutrophils are the main host defense against *S. pneumoniae* [1]. This first neutrophil influx is lately enhanced by the secretion of IL-17 by γδ T cells and $CD4^+$ T cells [1,5]. As lungs comply a vital function for host survival, the immune response must be tightly regulated to avoid an excessive lung inflammation that leads to damage and loss of function. In this context, interleukin-10 (IL-10) is a major anti-inflammatory cytokine that modulates the inflammatory immune response during bacterial infections in several organs, including lungs [6]. In a previous study, we described that the production of IL-10 is essential for host survival after an infection with *S. pneumoniae*, because IL-$10^{-/-}$ mice presented a significant increased mortality rate [7]. This phenotype is probably due to elevated lung damage caused by higher neutrophils recruitment to lungs [7].

Neutrophils have been viewed traditionally as effector cells that clear the pathogen and contribute to a pro-inflammatory state and immunopathology. However, new properties of neutrophils have been identified in acute infectious diseases. These properties include antigen presentation to $CD4^+$ and $CD8^+$ T cells [8,9]; assistance in the process of Ig class switching, somatic hypermutation and antibody production by B cells in the spleen [10], modulation of other immune cellular properties such as antimicrobial activity, cytokine production, survival and activation [11]. Since neutrophils participate in diverse processes of the immune response, it is possible that subsets of neutrophils with specific phenotypes play an important role in bacterial infections. In agreement with this idea, in a murine model, at least two different subsets of neutrophils have been identified [9,12]. However, whether these subsets are present in lungs during *S. pneumoniae* infection remains unknown. Here, we describe the existence of two different subsets of neutrophils infiltrating lung tissue with different size, granularity and activation capacity that depend on the production of IL-10. We observed that IL-$10^{-/-}$ mice presented higher amounts of both subsets in lungs, although the activation state of IL-$10^{-/-}$ and WT neutrophils were equivalent.

2. Material and methods

2.1. Mice

C57BL/6 wild-type (WT litter-mates) mice and B6.129P2-Il10tm1Cgn/J (IL-$10^{-/-}$) mice were obtained from The Jackson

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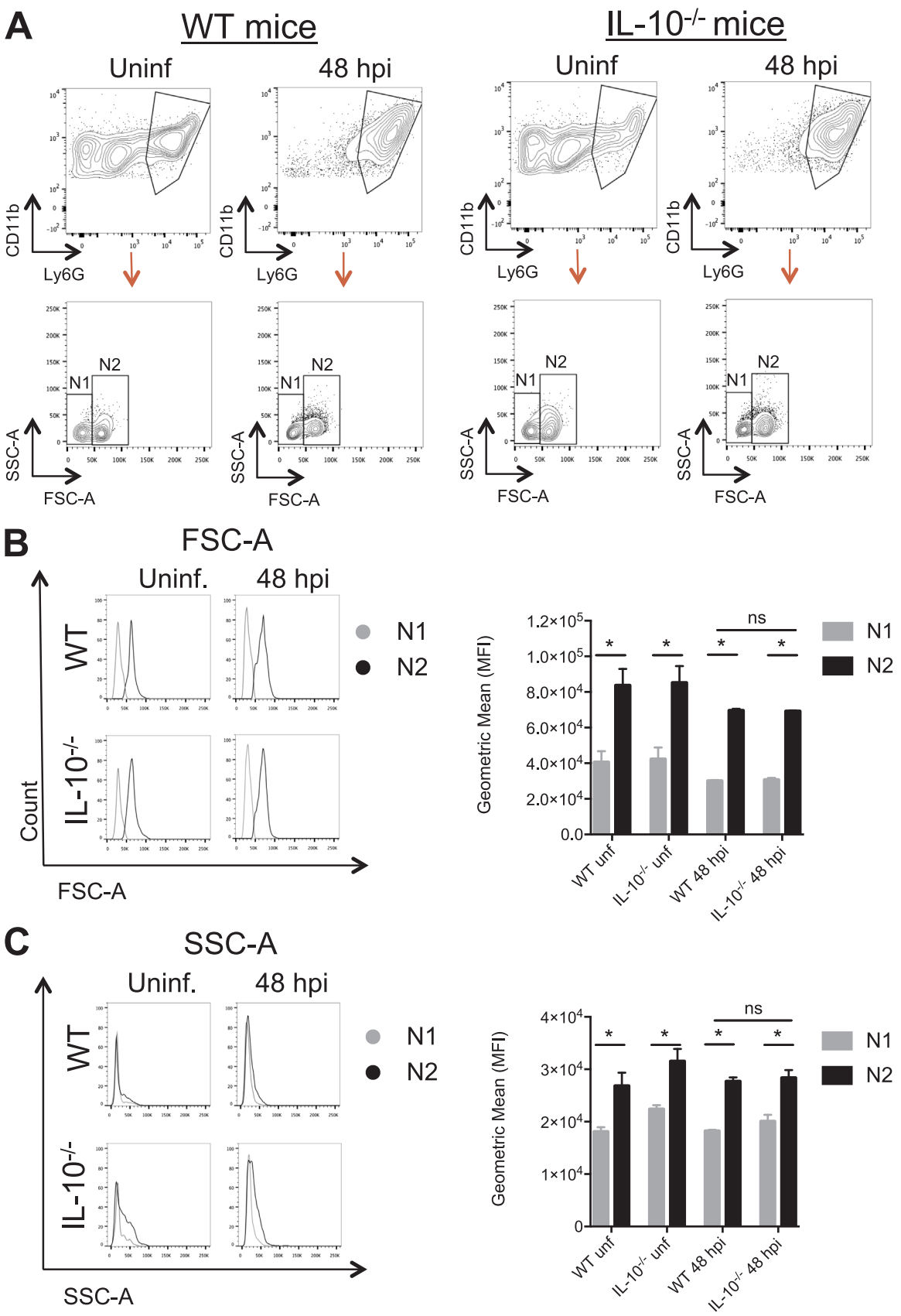

Fig. 1. Two neutrophil subsets, N1 and N2, are found in lungs of WT and IL-10$^{-/-}$ mice during *S. pneumoniae* infection. A. N1 and N2 neutrophils subsets were identified through FSC-A/SSC-A from CD45^{+}CD11b^{+}Ly6G^{+} neutrophils in lungs from uninfected and *S. pneumoniae* infected WT and IL-10$^{-/-}$ mice by flow cytometry. B. Size (Geometric mean of FSC-A) and C. Granularity (Geometric mean of granularity SSC-A) were analyzed by flow cytometry in uninfected and *S. pneumoniae* infected WT and IL-10$^{-/-}$ mice. Two-way ANOVA followed by Sidak's multiple comparison test was performed. P value < 0.05 was considered statistically significant.

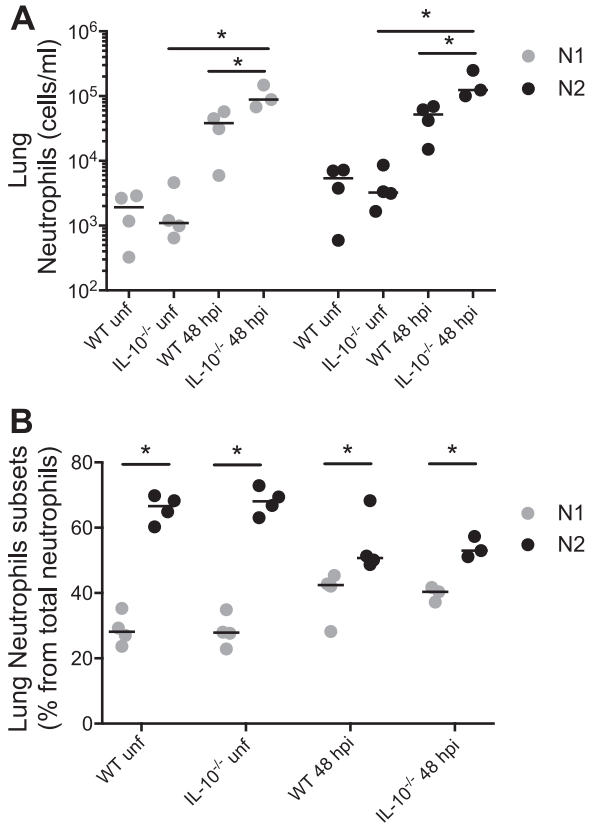

Fig. 2. IL-$10^{-/-}$ mice have increased levels of both neutrophil subsets in lungs during *S. pneumoniae* infection. A. N1 and N2 neutrophil total numbers and B. Percentage from total neutrophils, were quantified in lungs from uninfected and *S. pneumoniae* infected WT and IL-$10^{-/-}$ mice by flow cytometry through CountBright absolute counting beads. Two-way ANOVA followed by Sidak's multiple comparison tests was performed. P value < 0.05 was considered statistically significant.

Laboratory and maintained in specific pathogen-free animal facility at the Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile. All animal work was performed according to the Guide for Care and Use of Laboratory Animals (National Institute of Health, Bethesda, MD) and Institutional guidelines. Mice were overseen daily by personnel trained in animal welfare. All experimental procedures requiring animals and biohazards were revised and approved by the Scientific Bioethics Committee of the Pontificia Universidad Católica de Chile (CBB-245/2012).

2.2. Pneumococcal infection

S. pneumoniae D39 (serotype 2) strain was grew on Todd Hewitt Yeast extract (THYE) medium at 37 °C with 5% CO_2, until reach an optical density (O.D.) of 0.4 at 600 nm. Then, bacterial aliquots were frozen at −80 °C on THYE containing 10% glicerol. By the time of the infection, aliquots were thawed and diluted in THYE to reach the final concentration of 3×10^7 CFUs in 30 uL. Six-to-eight weeks old WT and IL-$10^{-/-}$ mice were anaesthetized intraperitoneally with a ketamine 16%/xylazine 4% solution and intranasally infected with 30 µL of THYE broth containing 3×10^7 colony-forming units (CFUs) of *S. pneumoniae* D39. Body weight was recorded daily from the day of infection (day 0) until 48 h post infection (Day 2). Mice that lost more than 25% of their original weight were euthanized. To corroborate that all mice used in this study were properly infected, bacterial inoculum was checked by seeding serial dilutions on blood agar plates and incubating them overnight at 37 °C with 5% CO_2.

2.3. Flow cytometry

To evaluate neutrophils infiltration in lungs, infected mice were sacrificed at 48 h post infection and lungs were recovered and minced with sterile scissors. Next, the tissue obtained was incubated in a PBS/collagenase (1 mg/ml)/ DNAse I (50 µg/ml) mixture for 1 h at 37 °C, with agitation. Homogenized lungs were filtered using a 70-µM size pore cell strainer. Cells were recovered by centrifugation (10 min at 1800 rpm), washed once with ACK lysis buffer (5 min at room temperature) and twice with PBS (5 min at 1800 rpm). Then, cells were resuspended in PBS/2% Fetal bovine serum (FBS) and stained with the following antibodies: Anti-CD45-PerCP (Biolegend, clone 30-F11, Catalog number 103130), Anti-CD11b FITC (BD, clone M1/70, Catalog Number 553310), Anti-Ly6G- APC (Biolegend, clone 1a8, Catalog number 127614). After staining, cells were washed twice with PBS (5 min at 1800 rpm), fixed with paraformaldehyde 2% (PFA 2%), resuspended in 100 µL of PBS and saved at 4 °C. Before analysis, CountBright absolute counting beads (Invitrogen) were added to quantify neutrophils population. All samples were analyzed in a BD FACSCanto II Flow cytometer and obtained data were analyzed using Flowjo V.10.0.7.

2.4. Statistical analyses

Two-way ANOVA followed by Sidak's post-test were performed to quantify N1 and N2 neutrophils in lungs of WT and IL-$10^{-/-}$ mice, as well as to compare their size, granularity and surface expression levels of Ly6G and CD11b. P value < 0.05 was considered statistically significant. All comparisons were calculated using the GRAPHPAD PRISM 7.0a software for Macintosh.

3. Results

3.1. Two neutrophil subsets are present in lungs of WT and IL-$10^{-/-}$ mice

To evaluate the neutrophils subsets [9,12] present in healthy condition and during a *S. pneumoniae* infection in lungs of C57BL/6 mice, total lung neutrophils ($CD45^+CD11b^+Ly6G^+$) were analyzed by flow cytometry. As shown in Fig. 1A, we identified two different subsets of neutrophils, that were called N1 and N2 neutrophils. Although N1 and N2 neutrophils share the typical extracellular markers, N1 are smaller (Fig. 1B) and have less granularity (Fig. 1C) as compared to N2 neutrophils. The presence of these populations is independent of the production of IL-10, since no differences in size and granularity were observed between WT and IL-$10^{-/-}$ N1 and N2 neutrophils (Fig. 1B-C). Importantly, these two neutrophil subsets were also present in lungs of both uninfected WT and IL-$10^{-/-}$ mice during a pneumococcal pneumonia.

3.2. IL-$10^{-/-}$ mice have higher numbers of both neutrophil subsets at 48 h post infection with S. pneumoniae

In a previous study, we described that during an infection with *S. pneumoniae*, IL-$10^{-/-}$ mice present higher neutrophil amounts in lungs at 48 h post infection [7]. Thus, we evaluated whether this increment in the total number of infiltrating neutrophils is due to a specific neutrophil population, or due to an equivalent increment of both subsets. As shown in Fig. 2A, lungs of uninfected WT and IL-$10^{-/-}$ mice have equivalent levels of N1 and N2 neutrophils. At 48 h after *S. pneumoniae* infection, IL-$10^{-/-}$ and WT mice showed an increment of both neutrophil subsets, as compared to uninfected groups. However, IL-$10^{-/-}$ mice showed higher numbers of both neutrophils subsets in lungs at 48 h post infection, as compared to WT mice (Fig. 2A). As show in Fig. 2B, the proportion of both subsets were equivalent between uninfected IL-$10^{-/-}$ and WT mice, as well as between infected IL-$10^{-/-}$ and WT mice.

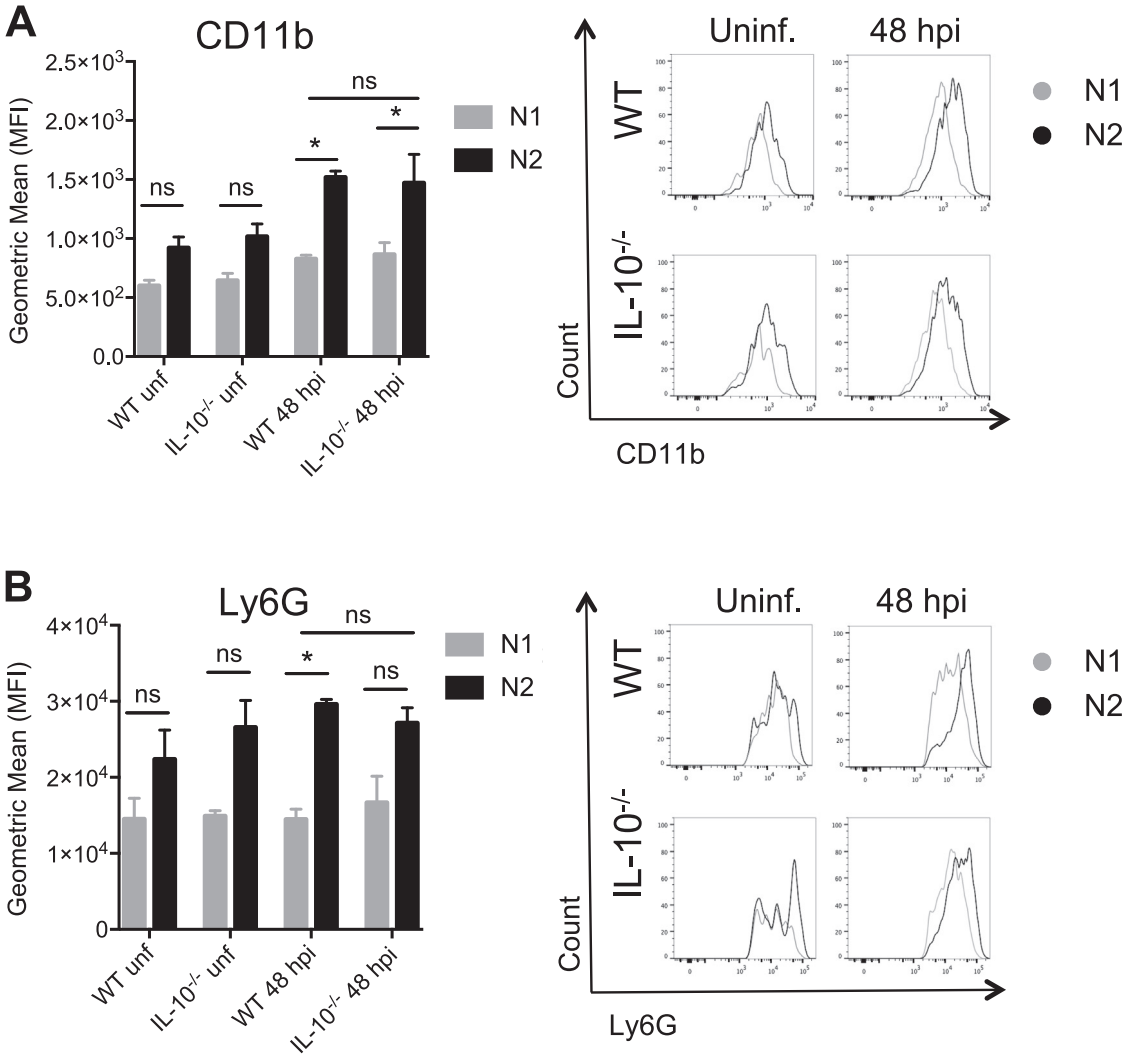

Fig. 3. N2 neutrophils from WT and IL-10$^{-/-}$ mice show no differences in activation during an *S. pneumoniae* infection. A. Activation (Geometric mean of CD11b) and B. Maturity (Geometric mean of Ly6G) were analyzed by flow cytometry in N1 and N2 from uninfected and infected WT and IL-10$^{-/-}$ mice. Two-way ANOVA followed by Sidak's multiple comparison tests was performed. P value < 0.05 was considered statistically significant.

3.3. Neutrophils from IL-10$^{-/-}$ and WT mice presented similar activation markers during S. pneumoniae infection

The expression levels of some surface markers, such as CD11b, are increased in neutrophils due to activation. Therefore, we measured neutrophil activation pattern of N1 and N2 neutrophils from WT and IL-10$^{-/-}$ mice, before and during *S. pneumoniae* infection. In uninfected mice, lung N1 neutrophils from WT and IL-10$^{-/-}$ mice showed similar expression levels of CD11b (Fig. 3A) and Ly6G (Fig. 3B), and no changes were observed after an infection with *S. pneumoniae*. On the other hand, N2 neutrophils from uninfected mice showed elevated levels of CD11b expression (Fig. 3A) and Ly6G (Fig. 3B) as compared to N1 neutrophils, with an increment of CD11b expression after 48 h post infection with *S. pneumoniae*, suggesting that N2 neutrophils are those which respond to *S. pneumoniae*. Notably, there were no differences between the expression levels of CD11b and Ly6G in N2 neutrophils from lungs of WT and IL-10$^{-/-}$ mice, in both infected and uninfected groups.

4. Discussion

In this study, we demonstrate the existence of two different subsets of neutrophils in the lungs of healthy mice: N1 and N2, which are also present during *S. pneumoniae* infection. Between these two subsets, N1 neutrophils are smaller and have less granularity, as compared to N2 neutrophils. These data are consistent with a previous report that describes the existence of two different neutrophil subsets in blood after an LPS challenge in mice [12]. In this experimental setting, circulating N1 neutrophils were more abundant than circulating N2 neutrophils, and the levels of Ly6G and CD11b expression were lower than N2 [12]. Our data, however, show that in lungs, N2 neutrophils are more abundant than N1 neutrophils. At 48 h post infection with *S. pneumoniae*, phenotypic characteristics of N1 neutrophils, such as size, granularity, activation state (expressed as the level of surface CD11b [13]), and maturation (expressed as the level of surface Ly6G [14]), remained low and constant, suggesting that these cells are immature neutrophils which are not responding to the infection. Some studies have suggested that lungs are a natural reservoir of neutrophils [15]. In this context, it is possible that N1 neutrophils are immature resident neutrophils that continue their maturation process in the lungs. This hypothesis explains why there are more N2 neutrophils than N1 in lungs. During an infection with *S. pneumoniae*, neutrophils rapidly infiltrate the lung to kill the bacteria [1]. Thus, granulopoyesis of mature neutrophils, but also of immature neutrophils from bone marrow, is a crucial process to increase circulating neutrophils that can reach the lungs [16]. According to this, it is highly possible that the increased amount of N1 neutrophils observed during *S. pneumoniae* infection in lungs is a consequence of an increased granulopoyesis process and not an active host strategy to kill

S. pneumoniae. N2 neutrophils, in contrast to N1, are more represented in healthy lungs, are larger, have a higher granularity and presented higher expression of CD11b and Ly6G, suggesting that they are mature cells with higher activation potential. More important, CD11b expression of N2 neutrophils were higher after 48 h of *S. pneumoniae* infection, which means that N2 neutrophils are getting activated during a *S. pneumoniae* infection.

One of the most important component that sustain a successful immune response during an infection is the cytokine milieu. The environment generated by cytokine production drives the development of a proper immune response. One of the most important cytokine that guides the immune response during different bacterial infections is the anti-inflammatory cytokine IL-10 [6]. In lungs, IL-10 production allows the development of an inflammatory response with limited self-tissue damage, improving host survival. The importance of IL-10 has been observed in different lung bacterial models of bacterial pneumonia, including *S. pneumoniae* [7], *Francisella tularensis* [17,18], *Mycobacterium tuberculosis* [19–21], *Pseudomonas aeruginosa* [22,23]. In a previous study, we described that during an infection with *S. pneumoniae*, the absence of IL-10 leads to an aberrant neutrophil recruitment at 48 h post infection that results in an excessive lung inflammation and host death [7]. Here, we show that compared to WT mice, lungs of IL-$10^{-/-}$ mice have increased amounts of both neutrophil subsets in response to *S. pneumoniae*. Although as observed in WT mice, phenotypic characteristics of IL-$10^{-/-}$ N1 neutrophils did not changed during *S. pneumoniae* infection, supporting the hypothesis that the increase of N1 neutrophils in lungs in response to *S. pneumoniae* infection is a consequence of an increased granulopoyesis. In contrast, N2 IL-$10^{-/-}$ neutrophils presented a slightly increment of CD11b expression during *S. pneumoniae* infection, indicating that this subset is getting activated and is responding to the infection. Whether the lung damage and higher mortality rate observed in IL-$10^{-/-}$ mice is due to a higher activation of neutrophils, or whether the increased lung damage lead to a higher neutrophil activation remains unknown. IL-$10^{-/-}$ N2 and WT N2 neutrophils showed equivalent levels of granularity and expression levels of CD11b and Ly6G, indicating that the production of IL-10 does not affect neutrophil activation but modulates neutrophil migration into the lungs. Therefore, during an infection with *S. pneumoniae*, IL-10 modulates the infiltration of activated neutrophils in the lungs, but doesn't affect their activation state. Deeper functional analyses to dissect the role of each neutrophil subset in healthy conditions and during bacterial pneumonia are needed, however, since both subsets share the common neutrophils extracellular markers, functional analysis to identify the function of each subsets cannot be done properly.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.11.004.

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