PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
Facultad de Ciencias Biológicas
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Mención Genética Molecular y Microbiología

DOCTORAL THESIS:

Evaluation of the ability of neutrophils to induce an anti-inflammatory response in the airways through the recognition of molecular patterns of *Streptococcus pneumoniae.*

by

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DEDICATION

I dedicate this thesis to my dear family: my mother, my father, my sister, my husband, and my beautiful and amazing children. All of them are my reason for living and they make me keep trying to be better every day. Their support and endless love, give me the strength and the courage to wake up every day and continue following my dreams.

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ABBREVIATIONS

AM: Alveolar macrophages

ANOVA: Analysis of variance

BALF: Bronchoalveolar lavage fluid.

CAP: Community-acquired pneumonia

CFUs: Colony forming-units

DAMPs: Damage-Associated Molecular Patterns

DC: dendritic cells

DNA: Deoxyribonucleic Acid

eGFP: Enhanced Green Fluorecent Protein

H: Hours

H&E: Hematoxylin & Eosin

HAP: Hospital-associated pneumonia

HPI: Hours post-infection

IFN-γ: Inferferon gamma

IL-10$^{-/-}$: Interleukin-10 knock out

IL-10: Interleukin-10

IM: Interstitial macrophages

LPS: Lipopolysaccharide

LTA: Lipoteichoic acid

MDSCs: Myeloid derived suppressor cells

MPO: Myeloperoxidase

OD: Optical density

PAMPs: Pathogen-Associated Molecular Patterns

PI3Ks: phosphoinositide-3 kinases

PGN: Peptidoglycan

PRRs: Pattern Recognition Receptors

S. pneumoniae: *Streptococcus pneumoniae*

TEM: Transmission electron microscopy.

THYE: Todd Hewitt broth supplemented with 0.5% yeast extract

TLR: Toll-like receptor

Uninf: Uninfected

WT: Wild-type

RESUMEN

Streptococcus pneumoniae es el principal agente bacteriano asociado a neumonía adquirida en la comunidad a nivel mundial. Este patógeno posee varios factores de virulencia, que se expresan de forma diferencial y permiten la colonización del tracto respiratorio y la evasión de la respuesta inmune, contribuyendo así al desarrollo de la enfermedad pulmonar. La neumonía neumocócica se caracteriza por el reclutamiento de un alto número de neutrófilos a los alvéolos, lo cual se asocia con inflamación y daño pulmonar. Los neutrófilos se han reconocido ampliamente por sus propiedades pro-inflamatorias, sin embargo, estudios recientes han mostrado que estas células son versátiles y tienen capacidad de modular la respuesta inmune, por ejemplo, a través de la producción de IL-10. Se ha demostrado que esta citoquina antiinflamatoria tiene un rol dual durante la neumonía, reiterando la necesidad de conocer los factores involucrados en su regulación. En este trabajo se evaluó qué células están involucradas en la producción de IL-10 durante el proceso infeccioso causado por *Streptococcus pneumoniae*. Se determinó que los neutrófilos contribuyen significativamente al aumento de IL-10 a las 24 horas posteriores a la infección con *Streptococcus pneumoniae*. Se realizó una caracterización de los neutrófilos infiltrantes, lo que evidenció una población heterogénea, en la que se distinguió al menos dos subtipos celulares que difieren en su morfología y en su capacidad para producir IL-10. Para determinar el papel de los neutrófilos productores de IL-10 durante la neumonía neumocócica, se realizaron ensayos de transferencia adoptiva de neutrófilos a ratones incapaces de producir IL-10. Los resultados obtenidos mostraron que la transferencia de neutrófilos de ratones “wild-type” (capaces de producir IL-10) a ratones IL-$10^{-/-}$ (incapaces de producir IL-10 y altamente susceptibles a la infección) promueve la recuperación pulmonar y protege a los ratones IL-$10^{-/-}$ de la neumonía. Por otro lado, se observó que los ratones IL-$10^{-/-}$ transferidos con células “wild-type” mostraron una eliminación bacteriana disminuida en comparación con los otros grupos IL-$10^{-/-}$. Estos resultados indican que los neutrófilos capaces de producir IL-10 podrían estar modulando la respuesta inmune y jugando un papel crítico durante las primeras 48 horas después de la infección pulmonar por *Streptococcus pneumoniae*. Finalmente, para determinar los factores involucrados en la inducción de IL-10 en neutrófilos, se llevaron a cabo pruebas *in vitro* para evaluar si el estímulo proviene del hospedero, de la bacteria o si ambos son necesarios. Los resultados de estos experimentos *in vitro* mostraron que solo la infección por *Streptococcus pneumoniae* induce la producción de IL-10 en neutrófilos. Los resultados de esta tesis en conjunto permiten sugerir fuertemente que *Streptococcus pneumoniae*, a través del reconocimiento de sus patrones moleculares, induce la producción de IL-10 en neutrófilos y este hecho tiene un impacto importante en el resultado de la enfermedad.

ABSTRACT

Streptococcus pneumoniae is the bacterial agent mostly associated with community-acquired pneumonia in the world. This microorganism has several virulence factors, differentially expressed to colonize the respiratory tract and evade the immune response, contributing to the development of lung disease. Pneumococcal pneumonia is characterized by the induction of a high influx of neutrophils into the alveoli, and this phenomenon is associated with inflammation and lung tissue damage. Neutrophils, widely recognized for their pro-inflammatory properties, have been highlighted in recent studies for being versatile cells able to modulate the immune response, for example, through the production of IL-10. IL-10 is an anti-inflammatory cytokine, which has been shown to have a dual role during pneumococcal pneumonia, reiterating the need to know the factors involved in its regulation. To determine the factors involved in the regulation of IL-10 production, during this thesis, the cells that are involved in IL-10 production during the infectious process were evaluated and it was determined that neutrophils contribute significantly to the increase of the levels of expression of IL-10 observed in the 24 h after infection with *S. pneumoniae*. A characterization of neutrophils was performed, which evidenced that these cells constitute an heterogeneous population, in which at least two subtypes are distinguished that differ both morphologically and in their ability to produce IL-10. Besides, adoptive neutrophil transfer assays were performed in mice unable to produce IL-10, to determine the role of neutrophils producing this cytokine during pneumococcal pneumonia. The results obtained in these experiments demonstrated that neutrophil transfer from wild-type (capable of producing IL-10) to IL-10$^{-/-}$ mice (highly susceptible to infection) promoted lung recovery and protected IL-10$^{-/-}$ mice from pneumonia. On the other hand, it was observed that IL-10$^{-/-}$ mice transferred with WT cells showed a reduced bacterial clearance when compared with the other IL-10$^{-/-}$ groups. These results indicate that neutrophils capable of producing IL-10 could be modulating the immune response and playing a critical role during the first 48 h after *S. pneumoniae* lung infection. Finally, to determine the factors involved in the induction of IL-10, *in vitro* tests were performed to assess whether the stimulus comes from the host, the bacteria, or if both were necessary. The results of these experiments showed that *in vitro*, only molecules from *S. pneumoniae* induce the production of IL-10 in neutrophils. Taken together, the results of this thesis suggest that *S. pneumoniae*, through the recognition of its molecules, induce the production of IL-10 in neutrophils and this fact plays a crucial role in the outcome of pneumonia.

1. INTRODUCTION

1.1 PNEUMONIA

Pneumonia definition has been a challenging issue for several years due to the lack of a consensus among all the authorities worldwide (Mackenzie, 2016); however, it can be described as an acute inflammatory condition usually caused by infectious agents that affects lung parenchyma and therefore interferes with the correct air exchange occurring at the alveoli level (Scott et al., 2008; Mackenzie, 2016). Among the infectious diseases, pneumonia is considered the first cause of death in children under five years old causing more than 800,000 deaths in one year (WHO 2018, O'Brien et al., 2019), and also represents a major problem in the elderly, being associated to more than one million of deaths in the world (O'Brien et al., 2019).

The etiology of pneumonia covers a wide repertoire of microorganisms, including viruses, bacteria, and fungus. The prevalence of each etiologic agent has shown a strong association with specific conditions related to age, the individual´s immune status, and the environment where people get infected. Regarding the environmental conditions, pneumonia can be generally classified as hospital-associated pneumonia (HAP – including ventilator-associated pneumonia and healthcare-associated pneumonia) or community-acquired pneumonia (CAP) (Anand and Kollef, 2009). HAP is defined as pneumonia occurring inside healthcare settings, therefore affecting hospitalized patients or people with comorbidities (Lanks et al., 2019). This type of pneumonia is usually associated with Gram-negative bacteria

like *Pseudomonas aeruginosa*, *Klebsiella pneumoniae,* and *Acinetobacter baumanii*, all of them frequently carrying antibiotic resistance, as the highly prevalent Gram-positive bacteria Methicillin-resistant *Staphylococcus aureus* (Weiner et al., 2016). On the other hand, CAP is the disease related to all cases occurring outside of any hospital setting (Lanks et al., 2019). Although CAP can affect anyone, morbidity and mortality are higher in young children and the elderly (Fong, 2020). Likewise, there is a strong association of certain microorganisms to age. For example, some viruses like the Respiratory Syncytial Virus and the human Metapneumovirus are more common in children under five years old (Bradley et al., 2011; Jain et al., 2015a), while pneumonia in adults is more associated to bacterial agents, rhinovirus and influenza virus (Jain et al., 2015b). Among bacterial agents, *S. pneumoniae,* non-typeable *Haemophilus influenzae,* and *Moraxella catarrhalis* are the main bacterial agents usually associated with CAP worldwide (Chochua et al., 2016; Park et al., 2017). However, *Streptococcus pneumoniae* has shown to be associated with higher morbidity and mortality rates, mainly in developing countries (Varon and Guntmann, 2010; Wahl et al., 2018).

1.2 *STREPTOCOCCUS PNEUMONIAE*

1.2.1 General characteristics

S. pneumoniae, also called pneumococcus, is a Gram-positive capsulated bacterium usually found arranged in pairs or short chains as part of the microbiota of the nasopharynx of children and some adults (Mackenzie et al., 2010; Simell et al., 2012; Adegbola et al., 2014). This colonization of the upper respiratory tract is asymptomatic and considered the pre-requisite to the appearance of several clinical

manifestations results from the dissemination of the bacteria to other niches (Bogaert et al., 2004; Henriques-Normark and Tuomanen 2013). In agreement with this, *S. pneumoniae* is considered an opportunistic microorganism because is able to cause disease depending on the host's immune status and the integrity of the mucosal barriers (Henriques-Normark and Tuomanen 2013). When pneumococcus access beyond the upper respiratory tract, it is able to cause an array of diseases, ranging from mild diseases like otitis media and sinusitis, to life-threatening conditions like pneumonia and Invasive Pneumococcal Disease (IPD) (Wahl et al., 2018; Cilloniz et al., 2018). IPD is a term used to refer to the more severe clinical syndromes product of the dissemination of the *S. pneumoniae* to sterile anatomic places, causing meningitis, cardiopathies, and sepsis (Backhaus et al., 2016; Feldman et al., 2018). This disease is an important cause of death worldwide, affecting children, the elderly, and people with co-morbidities (Yildrim et al., 2015; Backhaus et al., 2016). The last report of IPD shows that more than 3 million children presented severe episodes, and 300,000 died because of this disease (Wahl et al., 2018).

Currently, more than 90 serotypes of *S. pneumoniae* have been characterized based on their capsular polysaccharides, which also have become a key target for vaccine development against pneumococcus (Geno et al., 2015). In recent years, the availability of conjugated pneumococcal vaccines (PCVs) has significantly reduced the pneumococcal burden worldwide (Wahl et al., 2018). However, *S. pneumoniae* still represents a serious problem, partly due to a serotype replacement phenomenon, which has allowed an increase in the revival of the IPD related to the not-vaccinated serotypes (Weinberger et al., 2011; Balsells et al., 2017). Two

mechanisms of serotype replacement have been described: the first is related to the establishment and expansion of the serotypes not included in the vaccine (Loman et al., 2013) and the second one explains how the replacement of genes can give rise to important changes in types and quantities of polysaccharides present in the capsule (Geno et al., 2015). This serotype replacement, added to the continuous emergence of antimicrobial resistance in pneumococcal strains, has raised the need to look for new resources to counteract this opportunistic pathogen (Lo et al., 2019).

1.2.2 *Streptococcus* virulence factors

S. pneumoniae is characterized by having a plethora of virulence factors, differentially expressed to colonize or invade the host (Andre et al., 2017). Among them, the capsule is considered one of the most important, given the strong association with the infective capacity of this bacterium (Hyams et al., 2010). The capsule is a structure made up of complex polysaccharides surrounding the pneumococcus cell wall, that can vary in composition at a molecular and structural level, giving rise to more than 90 serotypes nowadays (Geno et al., 2015). Within the mechanisms required by *S. pneumoniae* to colonize the nasopharyngeal epithelium, one of the most relevant is the regulation of the capsule expression (Hammerschmidt et al., 2005; Arai et al., 2011). This regulation is translated in a reduction or capsule elimination, with subsequent exposure of adhesion proteins that allow pneumococcus to remain attached to the nasal epithelium, thus avoiding the elimination mediated by mechanical protection mechanisms of the host (Li and Zhang, 2019). On the other hand, when favorable conditions for dissemination are

present, the pneumococcus brings its capsule back and evades the recognition of its proteins by the immune cells (Li and Zhang., 2019).

Pneumococcus also has a wide variety of surface proteins that will be expressed depending on the phase of infection. Some of these proteins are characterized by their immunogenic capacity, which makes them candidates for being targets in new vaccine prototypes (Gamez and Hammerschmidt, 2012; Pichichero et al., 2012). Among proteins involved in adhesion to host cells, we found the pneumococcal adhesion proteins PavA and PavB, CbpE, BgaA, and PspC (Nieto et al., 2013). Additionally, the PspC protein interferes with the immune response against the pathogen by recruiting the factor C4BP, a negative regulator of the classical complement pathway (Dieudonné-Vatran et al., 2009). The metalloprotease ZmpA also plays a key role against bacterial recognition by the complement system by cleaving mucosal IgA (Roche et al., 2015). Other relevant proteins involved in the mechanisms to evade the complement pathways are the StrH, the neuraminidase A (NanA), β-galactosidase (BgaA), and the pneumolysin (Ply) (Dalia et al., 2010). Ply is a cytolytic toxin that also stands out as a pivotal virulence factor due to its relationship with the development of an unspecific pro-inflammatory response as well as its ability to form pores in cell membranes of host cells and for capturing proteins of the complement, thus reducing the availability of its components for the recognition of pneumococcus (McNeela et al., 2010; Zafar et al., 2017). Furthermore, there are proteins with autolytic activity. The most important is LytA, considered one of the determining virulence factors in infection due to its

relationship with the release of Ply and highly pro-inflammatory bacterial fragments, contributing in this way to the development of the disease (Eldholm et al., 2009; Kietzman et al., 2016). LytC is a lysozyme belonging to this group, that seems to act in the upper respiratory tract, due to its optimum temperature (30ºC) and its contribution to biofilms formation (García et al., 1999; Eldholm et al., 2009,). Other components of the pneumococcal membrane considered virulence factors are lipoproteins given their participation in modulation of the immune response (Tomlinson et al., 2014; Nguyen and Götz, 2016).

1.2.3 Pathogenesis

In the process of *S. pneumoniae* pathogenesis, the key stages are the colonization and invasion. Colonization occurs asymptomatically in most cases, where the host in its role as a carrier, becomes a source of transmission for other people and also a source of self-infection since the state of “carriage” is essential and precedes the subsequent development of the different pathologies associated with pneumococcus (Simell et al., 2012; Sharma et al., 2013). Given its nature as an opportunistic pathogen, *S. pneumoniae* can become a part of the individual's nasopharyngeal microbiota, where it remains hidden for days or months (Henriques-Normark and Normark, 2010). This colonization occurs more frequently in children, probably due to the immaturity of their immune system, allowing a more prolonged bacterial colonization (Simell et al., 2012). Another important factor that makes children the perfect carriers is their attendance at nurseries and schools, where they are in close contact with a high number of individuals (Simell et al., 2012).

The first step for pneumococcus entry into the individual is the adhesion to the nasopharyngeal epithelium, mediated mainly by interaction of the pathogen with host cells (Voss et al., 2012). A clear example of this interaction is that, once the pneumococcus avoids being trapped by the mucus present in the nasopharynx, the density of its capsule undergoes a reduction, which as previously mentioned, occurs in order that some surface proteins of the pathogen become exposed and can adhere to components of the nasopharyngeal epithelium, thus eluding its elimination (Chao et al., 2015; Kietzman et al., 2016). During the first part of this phase, pneumococcal proteins act on glycosylated residues, allowing the pneumococcus to access and adhere to receptors in host cells using specialized adhesion proteins such as PavA and PavB (Jensch et al., 2010; Kadioglu et al., 2010). Another factor that plays an essential role in pneumococcal adhesion is pili (Bagnoli et al., 2008). Subsequently, the pneumococcus deploys its arsenal of proteins that interfere with the complement pathway, including ZmpA, PspA, and neuraminidase, and in this way, they remain undetected until their subsequent transmission to another host or spread within the same individual (Dockrell and Brown, 2015).

It should be noted that dissemination is not an easy process for pneumococcus. This microorganism does not possess the ability to adhere to the lower respiratory tract´s epithelium, without being eliminated by the host's protective response (Melvin and Bomberger, 2016). For this reason, the change from commensal to a pathogen mainly depends on the integrity of the epithelium of the respiratory tract, as well as on the immunological status of the host. This means that the change in the bacterial status is probably due to external and internal

predisposing factors, among which viral infections stand out (Pittet et al., 2010; Kash et al., 2011; Melvin and Bomberger, 2016). A great example is the influenza virus infection, which usually affects the epithelium integrity, increases the availability of nutrients for bacteria, and provides a pro-inflammatory environment suitable for the spread of pneumococcus into the lower respiratory tract (Kash et al., 2011; Damjanovic et al., 2013). Once it succeeds, recognition of pneumococcus initially results in a pro-inflammatory type response, characterized by excessive recruitment of neutrophils to the site of infection and subsequent induction of tissue injury, which added to the virulence factors of the pathogen, allows damage to the epithelium and endothelium enabling the proliferation and dissemination of the pathogen by the hematogenous route (Bordon et al., 2013; Peñaloza et al., 2015). Interestingly, it has been described that pneumococcus can simultaneously induce the implementation of an anti-inflammatory response characterized by the proliferation of regulatory T cells (in people who have had previous contact with the serotype) and the production of IL-10 and TGF-β by macrophages (Neill et al., 2014, Subramanian et al., 2019a). This anti-inflammatory response allows pneumococcus to modulate the response and establish itself at the pulmonary level (Subramanian et al., 2019b). Another described modulation mechanism is the Ply-mediated inhibition of dendritic cell maturation. This inhibition has been shown to occur at the intracellular level, affecting DCs ability to produce cytokines and activate the inflammasome. This mechanism has been observed in alveolar macrophages and DCs, but not in neutrophils or monocytic-derived macrophages (Subramanian et al., 2019a).

There is another way to reach various anatomical sites, based on the invasion through the hematogenous route. The term invasion refers to the pneumococcus ability to cross the epithelial barrier and reach organs that are usually sterile. Among invasion mechanisms used by the pneumococcus, stands out the one involving phosphorylcholine of the bacterium that has a structure similar to the platelet activation factor (PAF) and therefore can be recognized by the receptor of this factor present in epithelial and endothelial cells. This virulence factor favors the increase of the endothelium permeability, allowing the passage of the microorganism into the blood (Thornton et al., 2010). In this sense, it is interesting to highlight the PAF receptor overexpression observed after infection with the influenza virus or respiratory syncytial virus, which explains the high incidence of secondary IPD following these infections (McCullers, 2014; Nakamura et al., 2011).

1.2 IMMUNE RESPONSE DURING *S. PNEUMONIAE* LUNG INFECTION

As mentioned before, *S. pneumoniae* possesses several virulence factors, which are selectively expressed depending on the phase of infection, allowing the pneumococcus to evade and modulate the host immune response. Some of them display regulatory effects, while others contribute to an intensified pro-inflammatory response elicited during lung infection (Nieto et al., 2013; Andre et al., 2017). In agreement with the above, virulence factors are fundamental in the contribution to lung disease, considering that in *S. pneumoniae* lung infection, host immune response plays a critical role and determines the course of the infection in a short and long-term (Chang et al., 2013; Andre et al., 2017). The immune response against *S. pneumoniae* is characterized by the release of pro-inflammatory cytokines

and the recruitment of innate immune cells to the lungs, and this process is considered crucial to control the infection. At first, epithelial cells and alveolar macrophages recognize the pathogen, inducing cytokine and chemokine production, required to kill the pneumococcus and to recruit phagocytic cells to the site of infection (van der Poll et al., 2009; Nieto et al., 2013). Additionally, antigen-presenting cells migrate to lymph nodes to initiate the antigen presentation process required for the development of adaptive immune response and the enhanced neutrophil recruitment via IL-17 secretion by $CD4^+$ T cells (Kirby et al., 2007, Nieto et al., 2013). In brief, the consequences of this response converge in a high neutrophil influx to the site of infection, which, although necessary for pathogen elimination, have shown to contribute to excessive tissue inflammation and damage (Kadioglu et al., 2008; Nieto et al., 2013). Taking into consideration that lungs are critical for host survival, the immune response against *S. pneumoniae* must be tightly regulated to guarantee pathogen elimination and, at the same time, prevent excessive inflammation and tissue damage. In response to this requirement, the host immune response usually displays strategies to impair pro-inflammatory response, mainly coordinated by interleukin-10 (IL-10).

1.3 THE ROLE OF IL-10 IN *S. PNEUMONIAE* INFECTION

IL-10 is a cytokine with anti-inflammatory properties that modulate the immune response to restore the homeostasis and counteract excessive tissue damage during inflammatory processes (Ouyang et al., 2011). This cytokine is released by innate and adaptive immune cells, and its production initiates with the recognition of ‘‘pathogen-associated molecular patterns’’ (PAMPs) or “damage-associated

molecular patterns" (DAMPS) (Saraiva and O'Garra 2010; Duell et al., 2012). Among the 13 TLR known receptors in mammals (Nie et al., 2018), TLR2 is one of the most versatile receptors due to its ability to conform heterodimer complexes with other TLRs, such as TLR1 and TLR6, which is translated in the possibility to recognize a wide number of PAMPs (de Oliviera Nascimento et al., 2012). TLR2 has shown to be involved in cell activation by molecules found in *S. pneumoniae* cell wall and membrane such as peptidoglycan (PGN), Lipoteichoic Acid (LTA), and lipoproteins (Moreira et al., 2008, Nguyen and Götz, 2016). Further, there is evidence that TLRs in monocytes and macrophages recognize cell wall components of *S. pneumoniae*, inducing IL-10 production (Moreira et al., 2008).

To date, there are several studies confirming that *S. pneumoniae* induces IL-10 during pneumonia. Given the traditional link between the downregulation of inflammatory processes with host wellness, it is reasonable to think that IL-10 production during pneumonia, could improve the symptomatology and a better prognosis. Peñaloza and colleagues showed that IL-10 signaling is a key component in the regulation of lung inflammation and contributes to IL-10 knockout mice survival (Peñaloza et al., 2015). However, the same study also showed that IL-10 seems to impair bacterial clearance, given the increased bacterial loads found in lungs and blood in WT mice, contrasting with the same parameter obtained from IL-10 knockout mice (Peñaloza et al. 2015). This last result is in line with previous studies suggesting that IL-10 produced during pneumonia might be related with the impaired innate and acquired cellular immunity observed in mice (Bogaert et al., 2009; Ota et al., 2011), indicating that induction of IL-10 production might inhibit immune

responses required to protect the host, contributing to persistence and pathogen dissemination (Liu et al., 2012). In infectious diseases, this fact makes IL-10 a cytokine with contradictory roles that depend directly on the infected tissue, as well as the pathogen involved (Peñaloza et al., 2016). Considering the dual role of IL-10 during pneumonia, it becomes imperative to study all the mechanisms involved in IL-10 production during the infection. To contribute to this knowledge, we have studied the cells involved in IL-10 production during pneumococcal pneumonia in a murine model, and in contrast with current information supporting that macrophages and monocytes are the main producers, it seems that the neutrophils could be the main IL-10 producing (unpublished data).

1.4 NEUTROPHILS AS IMPORTANT MODULATORS OF THE IMMUNE RESPONSE

Neutrophils are the most abundant immune cells found circulating in human blood and infiltrating tissues during inflammatory processes and infection. Neutrophils are produced in the bone marrow, enter into the circulation, and are recruited to the tissues, where they become fully activated. These cells present a multi-lobed nucleus, many antimicrobial proteins in their cytoplasm, and a wide variety of receptors that recognize opsonized bacteria or molecular patterns (Segal et al., 2005). During infectious processes, neutrophils are the first responders and rapidly extravasate from the vasculature into infected tissue to fight intruding microorganisms. These cells have different mechanisms to generate an efficient bacterial killing as phagocytosis, NADPH oxidase-derived reactive oxygen species

(ROS), degranulation of cytotoxic components, and antimicrobial peptides (Segal et al., 2005; Teng et al., 2017).

During the phagocytosis, the recognized bacterium is surrounded by a plasma membrane, activating the oxidative burst by the NADPH oxidase and the concomitant production of ROS, myeloperoxidase (MPO) and nitric oxide (NO) into the microbe-containing vacuole. Further, the degranulation process occurs, in which phagosome fuses with different types of neutrophil granules that contain proteinases and peptidases, all in order to kill the containing pathogen (Segal et al., 2005; Teng et al., 2017). In this context, neutrophils present four different types of granules which differ in their content: azurophilic or primary granules; specific or secondary granules; gelatinase or tertiary granules; and secretory vesicles (Segal et al., 2005, Yin and Heit, 2018). Besides, during the last years, another mechanism for the elimination of extracellular bacteria has been described. This mechanism is the formation of an extracellular structure composed of extracellular DNA, histones, and primary and secondary granular proteins, as MPO and neutrophil elastase called Neutrophil Extracellular Traps (NETs) (Mayadas et al., 2014; Papayannopoulos, 2018).

In agreement with the previously cited functions, traditionally, neutrophils have been considered as simple pro-inflammatory short-lived effector cells required to act in response to any inflammatory stimuli (Kolaczkowska and Kubes, 2013; Mantovani et al., 2011). This is supported by the fact RNA in neutrophils is lower compared to other myeloid cells (Tamassia et al., 2014). However, that perception of neutrophils

has been changing in the last years, and neutrophils started to be recognized as versatile cells with other capabilities involved in immune modulation. Indeed, it has been described now that neutrophils have a half-life larger than 24 h (5.4 days in humans) (Pillay et al., 2010) and the ability to express several molecules, including cytokines and chemokines (Jablonska and Granot, 2017; Tamassia et al., 2018). Among the cytokines produced by neutrophils in mice and humans are the IL-1α–β, IL-12p40, IL-6, IL-22, IL-23, TNF-family members, TGF-β, and IL-10 (Tamassia et al., 2018). Neutrophils are also able to develop different phenotypes according to the microenvironment. In tumor milieu, neutrophils exert anti-inflammatory roles by releasing iNOS and arginase-1, and more recently they have shown to induce angiogenesis required for tumor growth and metastasis (Deryugina et al., 2014; Mollinedo 2019). In lymph nodes, they express high levels of MHC-II, showing the ability to act as an antigen-presenting cell (Abi Abdallah et al., 2011; Lok et al., 2019). Considering the high numbers of neutrophils recruited during inflammatory processes and their ability to produce different modulatory molecules, it is possible that beyond effector cells, these cells are fundamental actors in the establishment in the acute and reparative response. In line with this assumption, there is evidence that after exposure to bacterial components, neutrophils preferentially produce anti-inflammatory cytokines and play a regulatory role, affecting macrophages, dendritic cells and T cells in a paracrine or cell-to-cell contact manner (Zhang et al., 2009, Perobelli et al., 2015).

Several studies describe the presence of neutrophils with anti-inflammatory properties reported as low-density granulocytes (LDGs), in antitumor responses, seropositive HIV patients, and autoimmune diseases (Cloke et al., 2012; Carmona-Rivera and Kaplan, 2013). Among LDGs, we can highlight a population that fits in the regulatory phenotype, recently described as granulocytic myeloid-derived suppressor cells (G-MDSCs), an important sub-population with granulocytic morphology expressing $CD11b^{+}Ly6C^{low}Ly6G^{+}$ in mice (Peranzoni et al., 2010; Youn et al., 2012). The G-MDSC phenotype is characterized by a high ROS production and lower expression of nitric oxide synthase (NOS). These cells can inhibit T cell responses modifying the microenvironment by secreting IL-10, H_2O_2, arginase-1, and/or ROS or by contact with T cells through PD1-PDL1 (receptor-ligand) complex (Bowers et al., 2014). In inflammatory pathologies, neutrophils exhibit clear differences in their morphology, as compared to circulating neutrophils. For instance, these cells show important changes in nucleus shape and segmentation, granularity, and size (van Hout et al., 2015; Hesselink et al., 2019). This evidence confirms that two or more different types of neutrophils could be found in mammals. Moreover, accumulated evidence suggest that neutrophils are a heterogeneous population of cells and the presence of “neutrophils-like” cells have been described in several conditions (Tsuda et al., 2004; Carmona-Rivera and Kaplan, 2013; Ssemaganda et al., 2014; Shaul et al., 2016). For example, in acute mycobacterial infection, the inflammatory response of monocytes and macrophages is damped by the secretion of large amounts of IL-10 from neutrophils (Zhang et al., 2009), a phenomenon also observed in a model of septic peritonitis where the adoptive transfer of WT but not $IL\text{-}10^{-/-}$ neutrophils decreases the release of TNF by monocytes (Ocuin et al., 2011).

Moreover, in a *Trypanosoma cruzi* infection model, a dual role of neutrophils was highlighted: the neutrophils first displayed an inflammatory response against the parasite, and then, after activation by IL-17A, migrated to the spleen and liver to release IL-10 (Boari et al., 2012). More recently, Peñaloza and colleagues described the presence of two neutrophils subsets infiltrating lungs after *S. pneumoniae* infection. These subsets were characterized according to their size, complexity, and surface markers (Peñaloza et al., 2018). While it has been demonstrated that a cell--cell contact with LPS-activated regulatory T cells triggers the IL-10 production by human neutrophils (Lewkowicz et al., 2016), the mechanism is still poorly understood. Although now it is well known that there are different subsets of neutrophils, a concrete classification remains undone, and it has not been defined whether the subsets of neutrophils come from different cell lineages or if they get differentiated in response to a host or pathogen stimulus (Pillay et al., 2013). Additionally, studies of IL-10-producing neutrophils in bacterial infections are scarce. In fact, despite the relevance of pneumococcal pneumonia, data regarding "regulatory neutrophils" characterization and contribution to disease are not currently available. Given the results obtained in our laboratory, where we observed an important contribution of neutrophils to IL-10 production during *S. pneumoniae* infection, we propose in this thesis to determine which is the main source of IL-10 during the first h of infection and reveal how much neutrophils contribute to IL-10 levels produced in mice lungs. Moreover, we propose to deepen in the characterization of the two subsets previously described in our laboratory (Peñaloza et al., 2018), and determine the role of neutrophils able to produce IL-10. Finally, we expect to contribute to identify receptors and ligands involved in neutrophils change

of phenotype. In agreement to previous studies performed to determine IL-10 production in neutrophils with other bacterial pathogens (Moreira et al., 2008, Zhang et al., 2009), we consider that pathogen PAMPs might be mainly involved in the rapid induction of IL-10-producing neutrophils in pneumococcal pneumonia and that this switch in neutrophils phenotype contributes to disease outcome modulating the immune response to *S. pneumoniae*. As mentioned before, a wide range of cells produce IL-10, and the mechanisms of induction are well characterized, mainly in macrophages, monocytes, dendritic cells, and T cells (Saraiva and O'garra 2010). This observation suggests that this pathogen might be using this process as a strategy to down-regulate innate immune response and guarantee its survival and dissemination (Moreira et al., 2008, Griss et al., 2016). Finally, the results obtained in this work will allow us to a better understanding of pneumonia pathogenesis. Furthermore, our findings could be useful to guide future treatment strategies aimed at modifying inflammatory responses or cellular immunity in order to approach a balanced response that guarantees a better disease outcome.

1 HYPOTHESES STATEMENTS

2.1 HYPOTHESIS 1

"IL-10 producing neutrophils prevent excessive inflammation of lungs after *Streptococcus pneumoniae* infection in mice"

2.2 HYPOTHESIS 2

"Structural elements of *Streptococcus pneumoniae* cells promote the generation of anti-inflammatory neutrophils in infected lungs"

2 OBJECTIVES

2.2 GENERAL AIMS

- To determine the ability of neutrophils to produce IL-10 during *S. pneumoniae* infection preventing excessive lung damage.
- To determine bacterial PAMPs involved in the IL-10 production by neutrophils during *S. pneumoniae* infection.

2.3 SPECIFIC AIMS FOR THE HYPOTHESIS 1

- To characterize the population of neutrophils in the lungs during *S. pneumoniae* infection.
- To determine the impact of IL-10 producing neutrophils in the outcome of pneumonia caused by *S. pneumoniae*.

2.4 SPECIFIC AIM FOR THE HYPOTHESIS 2

- To determine whether *S. pneumoniae* PAMPs are involved in the induction of IL-10 production in neutrophils

3 SUBMITTED MANUSCRIPT

Characterization of the immune-modulatory capacity of IL-10-producing neutrophils during *Streptococcus pneumoniae* infection

Running title: IL-10-producing neutrophils in pneumococcal pneumonia

Authors

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3.2 ABSTRACT

Neutrophils are immune cells historically defined as pro-inflammatory effector cells. However, current accumulated evidence indicate that neutrophils are more versatile having immune-modulatory properties. Here we further characterized this phenotype in a murine model of pneumonia, assessing how IL-10 producing neutrophils influence the progression of pneumonia caused by *Streptococcus pneumoniae*. *In vivo* assays showed that neutrophils produce high amounts of IL-10 during the first 48 h post-infection and *in vitro* assays showed that the production of this anti-inflammatory cytokine is particularly induced by the recognition of pneumococcal antigens. Furthermore, one neutrophil subpopulation present in infected lungs exhibited clear morphological differences and higher production of IL-10 at 48 h post-infection. Additionally, we analyzed the relevance of neutrophils as a source of IL-10 during pneumococcal pneumonia in neutrophil adoptive transfer assays in IL-$10^{-/-}$ mice, which are highly susceptible to *S. pneumoniae* infection. These experiments showed that the transfer of wild-type neutrophils into wild-type and IL-$10^{-/-}$ mice decreased lung damage and also had a significant impact in bacterial growth and dissemination. These results suggest that an increased regulatory population of neutrophils induced by *S. pneumoniae* during lung infection is able to provide enough IL-10 to decrease the inflammatory immune response, contributing to reduced tissue injury.

3.3 INTRODUCTION

Pneumonia is defined as an inflammatory condition affecting the lower respiratory tract and is considered the leading cause of death due to infections in children under five years old (WHO, 2018; O'Brien et al., 2019). Among bacteria able to cause pneumonia, *Streptococcus pneumoniae* is one of the agents associated with high mortality rates (Varon and Guntmann, 2010, Wahl et al., 2018). *S. pneumoniae* is a Gram-positive encapsulated bacterium considered the major cause of bacterial community-acquired pneumonia and one of the most important causative agents of invasive disease (Cilloniz et al., 2012; Wahl et al., 2018). This microorganism has been known to display numerous virulence factors, usually involved in modulation and evasion of the immune response, which can lead to the survival of this pathogen as part of the commensal microbiota of the nasopharynx for several weeks (Nieto et al., 2013; Andre et al., 2017). Once the immune response and the mucosal barriers of the host become compromised, *S. pneumoniae* is able to spread to other organs causing a wide range of diseases that vary from mild infections -such as otitis and sinusitis- to more severe manifestations such as Invasive Pneumococcal Disease (IPD) (Mackenzie et al., 2010; Simell et al., 2012; Adegbola et al., 2014).

IPD comprises bacteremic pneumonia, meningitis and sepsis and is considered an important cause of mortality in children and the elderly, representing approximately 12% of deaths every year (Kaplan et al., 2013). Although there has been a consistent decrease in the prevalence of pneumonia in children under five years old due to immunization programs (Whitney, 2016), in 2015 an estimated of 3.7 millions of severe episodes of pneumococcal disease and more than 300.000

deaths were reported in children (Wahl et al., 2018). Furthermore, poor coverage of vaccination programs in some countries, serotype replacement of strains that are not included in current pneumococcal vaccines and the continuous emergence of antibiotic resistant strains make *S. pneumoniae* a worldwide concern (Chibuk et al., 2010; Chang et al., 2013; Darrieux et al., 2015; Balsells et al., 2017).

During *S. pneumoniae* lung infection, the host immune response plays a critical role and determines the course of the infection in the short and long-term (Chang et al., 2013; Andre et al., 2017). The immune response against *S. pneumoniae* is characterized by the release of several cytokines and chemokines required for the recruitment of innate immune cells to the lungs and this process is considered crucial to control the infection. Neutrophils are the primary cells recruited into infected lungs and are essential for the elimination of bacteria (Craig et al., 2009; Domon et al., 2018; Steck et al., 2019). However, taking into consideration that the lungs are critical for host survival, immune response against *S. pneumoniae* must be tightly regulated to guarantee pathogen elimination and, at the same time, prevent excessive inflammation and tissue damage. As a mechanism to guarantee this requirement there are several strategies to regulate the inflammatory responses mainly coordinated by IL-10. This cytokine exhibits anti-inflammatory properties that exert modulatory mechanisms to promote the restoration of the homeostasis of the host's immune response (Saraiva and O'garra, 2010; Duell et al., 2012).

Previous data published by our group suggested that IL-10 is induced in the lungs during the early phase after pneumococcal exposure and the peak of IL-10

production is produced during the first 48 h after pneumococcal exposure (Peñaloza et al., 2015). Results obtained in this study highlight that IL-10 plays a crucial role during the infectious process because its presence is required to control lung inflammation, but it also might favor bacterial dissemination (Peñaloza et al., 2015). These results are consistent with previous studies describing IL-10 production during pneumococcal pneumonia in mice (Saraiva and O'garra, 2010; Nieto et al., 2013). It has been reported that almost all innate immune cells are able to produce IL-10 during bacterial pneumonia and most of these aforementioned studies suggest that macrophages and Myeloid Derived Suppressor Cells (MDSCs) are the main contributors to the increased levels of this cytokine observed in the acute phase (Poe et al., 2013; Peñaloza et al., 2016; Peñaloza et al., 2019). In addition, we have observed that during pneumococcal pneumonia, neutrophils also produce IL-10 in lungs of infected mice (unpublished data). These findings agree with recent publications, showing that neutrophils are able to produce this cytokine during infection, indicating that these cells might be playing a role beyond pathogen clearance in pneumonia (Zhang et al., 2009; Kasten et al., 2010; Boari et al., 2012; Lewkovicz et al., 2016).

Indeed, neutrophils have been mostly considered as pro-inflammatory, short-lived effector cells that react to infections and different inflammatory stimuli (Kolaczkowska and Kubes, 2013). That view has changed in recent years, due to the observation that neutrophils have a half-life that is longer than 24 h (5.4 days in humans) (Pillay et al., 2010), and that these cells may significantly contribute to downregulating the inflammatory immune response. In fact, there is evidence that

after exposure to bacterial components, various neutrophil populations with anti-inflammatory profiles expand and play regulatory roles, decreasing the pro-inflammatory activity of macrophages, dendritic cells and T cells (Perobelli et al., 2015; Lewkovicz et al., 2016). Based on this information, here we evaluated the contribution of myeloid immune cells to IL-10 production by measuring the expression of IL-10 at 48 h post *S. pneumoniae* infection. In addition, we determined the contribution of neutrophils in IL-10 production during *S. pneumoniae* infection and determining the impact of these IL-10-expressing neutrophils during the acute phase of pneumonia. We found that neutrophils contribute significantly to IL-10 production during the acute phase of *S. pneumoniae* infection in a murine pneumonia model, displaying a regulatory role and influencing disease outcome. Additionally, in agreement with previously published data from our laboratory, we also found the presence of at least two neutrophil populations that display different phenotypic features and IL-10 production capabilities, indicating possible separated roles for each subset during infection.

3.4 MATERIALS AND METHODS

3.4.2 Mice.

Adult (6-8 weeks) male C57BL/6 (WT littermates), B6.129P2-Il10tm1Cgn/J (IL-10$^{-/-}$) and B6(Cg)-Il10tm1.1Karp (IL-10::eGFP) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). All mice were housed in specific pathogen-free conditions in the animal facility (CIBEM) at the Pontificia Universidad Católica de Chile. All experimental protocols were reviewed and approved by the Scientific Ethical Committee for Animal and Environment Care of the Pontificia Universidad Católica de Chile (protocol number 160822002). All animal experiments were performed by trained personnel according to the Guide for Care and Use of Laboratory Animals (NRC, 2010). A clinical score was used to supervise mouse wellness, which includes parameters such as weight loss, pain signs, and respiratory and neurologic changes (Supplementary data, Table 1).

3.4.3 Bacterial growth and preparation.

Streptococcus pneumoniae D39 (capsulated) and R6 (non-capsulated) strains were grown in Todd Hewitt broth supplemented with 0.5% yeast extract (THYE), without agitation and under anaerobic conditions at 37ºC, until an OD_{600} 0.5 was reached. Then, the bacterial suspension was stored in 10% glycerol at -80ºC until use. Colony-forming units (CFU) of bacterial suspensions were determined by serial dilutions on blood agar. Before use, bacterial suspensions were centrifuged at 4,700 g for 7 min at 4ºC and washed with PBS or 0.5% THYE broth. For infections, the bacterial pellet was resuspended in 0.5% THYE broth. For in vitro assays, bacteria were resuspended in sterile PBS and heat-inactivated at 95ºC for 15 min.

3.4.4 Purification of naïve neutrophils from bone-marrow cells.

Femurs and tibias were obtained from WT and/or IL-10$^{-/-}$ mice. Bones were cleaned from muscle and fat residues and bone marrow was flushed by perfusion with RPMI 1640 medium supplemented with 10% FBS and 2mM EDTA. Red cells were lysed with a hypotonic solution of NaCl 0.2% and lysis was stopped with an isotonic solution of NaCl 1.6%. Next, cells were counted and stained for negative selection by using a neutrophil purification kit (MACS, Miltenyi Biotech). After purification, neutrophils to be transferred were diluted in sterile PBS. For *in vitro* assays, neutrophils were placed in HBSS containing Ca^{2+} and Mg^{2+} supplemented with 0.1% gelatin (Sigma- Aldrich G7765).

3.4.5 Adoptive transfer assays and infections.

IL-10$^{-/-}$ mice received intranasally 50 □l of THYE containing 1x10^6 purified neutrophils obtained from WT mice. Non-transferred IL-10$^{-/-}$ mice, non-transferred WT mice and IL-10$^{-/-}$ mice transferred with IL-10$^{-/-}$ neutrophils were included as control groups. After 24 h, mice were intranasally infected with 3x10^7 CFU of *S. pneumoniae* D39. The survival rate, weight loss and clinical score were daily monitored until 10 days post infection (dpi). Additionally, adoptive transfer assays were also performed to evaluate disease severity parameters such as bacterial loads, lung infiltration by proinflammatory cells and tissue damage, at 48 h post infection (hpi). At least three independent assays were performed.

3.4.6 Tissue preparation.

Total blood was obtained by cardiac puncture in mice under deep terminal anesthesia. Then, dissection of the trachea was performed in euthanized mice, using sterile surgical material. A cannula was inserted through the trachea and serial washes were performed with 1,500 µl of sterile PBS to obtain bronchoalveolar lavage. Lungs, spleen and brain were extracted with sterile surgical instruments. Lungs were cut into small pieces with sterile scissors and digested in 7 ml of Collagenase IV (1mg/ml) for 1 h at 37ºC with agitation. After the incubation period, 7 ml of 5 mM EDTA was added to each tube and mixed gently (this process was exclusively applied for lungs). Then, lungs and the remaining organs were homogenized and passed through cell strainer (70 □m pore) and recovered in their original tube containing PBS. For bacterial load determination, serial dilutions of each organ were plated in blood agar plates at 37ºC with 5% CO_2. Bacterial loads were calculated as CFU per organ. Lungs and BALFs pellets were separated by centrifugation during 5 min at 0.4 g. Each pellet was treated with a sterile solution of 1X ammonium chloride-potassium (ACK) for 5 min and then washed with PBS. Cells obtained after this process were stained with antibodies to characterize the inflammatory cell infiltrate by flow cytometry. Cytokine protein levels (IL-6, TNF, IFN-γ, IL-10, IL-12p40) were determined in BALF supernatants by ELISA (BD OptEIA).

3.4.7 Lung histopathological analyses.

Histopathological analyses were performed in mice of each group at 48 hpi; lungs were removed and fixed in 4% paraformaldehyde for 48 h. After, lungs were

embedded in paraffin wax blocks and sliced with a microtome in 5 µm sections. Then, slices were incubated at 60°C for 1 h and stained with Hematoxylin and Eosin (H&E staining). Images from each slide were obtained by using a Leica SCN400F slide scanner and analyzed with the Aperio ImageScope Software. Pathological changes in lungs were evaluated by a pathologist and quantified following the parameters detailed in Table S2.

3.4.8 Flow cytometry.

Analyses of myeloid cells were performed by flow cytometry using the following markers: anti-CD45 (clone 30-F11, BD), anti-CD11b (clone M1/70, BD), anti-CD11c (clone HL3, BD), anti-Ly6C (clone AL-21, BD), anti-Ly6G (clone 1A8, Biolegend), anti-MHC-II (clone M5/114.15.2, BD), anti-Siglec-F (clone E50-2440, BD), anti-CD103 (clone M290, BD), anti-CD64 (clone 10.1, Biolegend). Dead cells were excluded from analyses using the LIVE/DEAD Fixable Viability Stain 510 (BD). Data were acquired on Fortessa X-20 flow cytometer (BD Bioscience, Oxford, UK) and analyzed using FACSDiva (BD Bioscience, UK) or FlowJo software (Tree Star, Ashland, Oregon). Cell type identification was performed according to the gating strategy showed in the supplementary material (Fig.S1). IL-10 expression data were calculated according to eGFP expression represented by fluorescence in the FITC channel.

3.4.9 Neutrophil characterization by flow cytometry and Transmission Electron Microscopy.

IL-10::eGFP mice were intranasally instilled with vehicle (uninfected) or $3x10^7$ CFUs of *S. pneumoniae*. Lungs and bronchoalveolar lavage (BALF) were obtained from both groups after 12, 24 and 48 hpi. Lungs were processed and stained for flow cytometry. Cells obtained from 24 hpi BALFs were stained and sorted by using a BD FACS Aria II (BD Biosciences, San Jose, USA). $CD11b^+CD11c^-Ly6G^+$ cells were selected and subsequently separated by size (Forward scatter). FACS was assessed at the Flow Cytometry Facility of the Pontificia Universidad Católica de Chile. Purified neutrophil subtypes obtained by the sorting strategy were fixed by immersion in 2.5% glutaraldehyde and subsequently treated and prepared by experts at the Advanced Microscopy Facility of the Pontificia Universidad Católica de Chile. Sections of 80 nm of diameter were obtained using an ultramicrotome Leica Ultracut R. Grids were visualized and photographed at 8200X in a Phillips Tecnai 12 electron microscope at 80 kV. TEM microphotographs were analyzed with Image-J software. Morphological features evaluated for each neutrophil subtype included size, euchromatin fraction, quantification of cytoplasmic structures and cytoplasm electrodensity. Each cell characteristic was evaluated according to previously reported parameters (Borenstein et al., 2018). This assay was repeated twice.

3.4.10 *In vitro* assays for the evaluation of IL-10 production by neutrophils.

Bone marrow-derived neutrophils were incubated with different stimuli during 1 h in constant agitation at 37ºC in 5% CO_2 and subsequently incubated for 23 h. Cells

were recovered for counting and viability evaluation by flow cytometry. Supernatants were stored at -80ºC and then evaluated for IL-10 detection by ELISA. Bacteria used in these assays were inactivated by heat at 90ºC for 15 min. Heat-killed bacteria were seeded in blood agar to confirm bacterial inactivation. The assays were performed adding three different multiplicity of Infection (MOIs) of heat-killed *S. pneumoniae* (MOI 25, 50, 100) to neutrophils. The experiments were also performed with BALFs obtained from mice intranasally infected or instilled with vehicle. BALFs were obtained 24 h after the instillation. Subsequently, BALFs were centrifugated and separated, and supernatants were stored at -80ºC until use. Additionally, neutrophils were also exposed to DNA obtained from *S. pneumoniae* D39 (MOI 25 and MOI 50). At least three interdependent assays were performed.

3.4.11 Statistical analyses

T-tests with Mann-Whitney post-tests were performed to analyze the IL-10 producing cells at 24 and 48 hpi and to compare the phenotypical characteristics of N1 and N2 subsets, evaluated by TEM. T-tests with Mann-Whitney post-tests were also performed in adoptive transfer assays, in order to compare all the experimental groups with each other in analyses related to lung infiltration, bacterial loads in organs and cytokine determination in BALFs. Survival curves were compared using a log rank test. A one-way ANOVA followed by a Kruskal-Wallis test was performed to analyze the IL-10 expression and production in the *in vitro* assays. Two-way ANOVA and Sidak's multiple comparisons post-test were performed to analyze the clinical score and the expression of IL-10 and surface markers by neutrophils. In all

cases, a P value of <0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism 5 for Mac, version 5.01. All data were expressed as means plus standard error (SEM).

3.5 RESULTS

3.5.2 Neutrophils contribute to IL-10 production at early times during pneumococcal pneumonia.

To assess which cells are involved in IL-10 production in the lungs during the acute phase of pneumococcal pneumonia, two experimental groups of IL-10::eGFP mice were infected: the first group was infected during 24 h and the second group remained infected for 48 h. Mice supervision was assessed according to Table S1 and was recorded daily before mice processing (**Not shown**). Lung cells were stained for flow cytometry characterization with a FACS panel designed to identify dendritic cells, alveolar macrophages, monocytes, interstitial macrophages, M-MDSC, and eosinophils. Further, quantification of IL-10-producing cells was performed by detection of $eGFP^+$ cells (**Fig. 1**). Alveolar macrophages, which displayed the highest basal eGFP MFI previous infection, showed a decrease in their number and MFI over time after *S. pneumoniae* infection (**Fig. 1A**). Interstitial macrophages showed a tendency to increase in eGFP numbers and MFI at 24 hpi, however this increase was not significant (**Fig. 1B**). M-MDSC and eosinophils also showed eGFP signal at basal levels in non-infected mice, however the numbers of $eGFP^+$ eosinophils were similar in non-infected mice vs. 24 hpi mice, and even decreased at 48 hpi (**Fig. 1C and 1D**). Conversely, the number of $eGFP^+$ cells as well as the eGFP MFI increased significantly in neutrophils at 24 and 48 hpi (**Fig. 1E**). These results suggest that IL-10 expression is significantly increased only in neutrophils in response to *S. pneumoniae* infection.

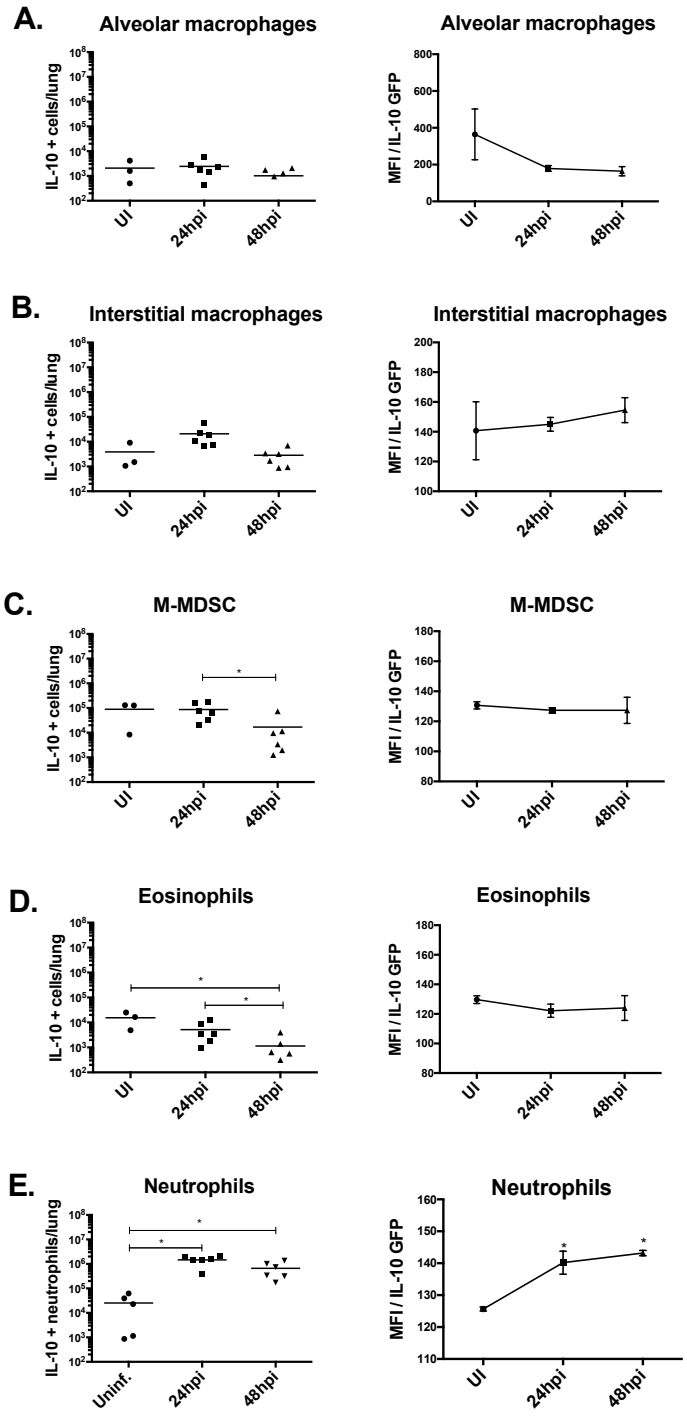


Figure 1. Neutrophils are the main source of IL-10 during the early phase of *S. pneumoniae* infection. A-E. Number of cells expressing IL-10 in lungs in uninfected mice (n = 5), and mice infected after 24 (n = 6) and 48 hpi (n = 6) and their corresponding MFI measures. Statistics: T-test with Mann-Whitney post-test. $P<0.05$. Comparisons of the mean of infected and uninfected mice, 24 and 48 hpi. hpi: h post-infection; MFI: Median Fluorescence Intensity.

3.5.3 Neutrophil subsets detected in the lungs display differential features that suggest specific roles during the early phase of pneumonia.

Our previous studies described the basal presence of two neutrophil populations in the lungs displaying differences in size and granularity, and that both expand during pneumococcal infection (Peñaloza et al., 2018). During the characterization of neutrophils in the present study, we confirmed the presence of these two subsets by flow cytometry and maintained the nomenclature initially designated: N1 (small size neutrophils) and N2 (large size neutrophils) (**Fig. 2A**). To determine whether these populations are playing different roles during infection, the expression of maturity and activation surface markers was evaluated during the first 48 hpi. Results obtained from these analyses showed no significant differences in the expression of surface markers in both populations. However, an increasing trend was observed for the expression of MHC-II on the surface of N1 cells at 24 hpi (**Fig. S1**). Further, based on the results obtained in the experiments described above, $eGFP^{+}$ N1 and N2 neutrophil populations were identified in the lungs of IL-10::eGFP infected mice and quantified by flow cytometry. IL-10 expression was observed in both populations and increased significantly at 24 and 48 hpi (**Fig. 2B)**. Our results also show that the contribution of N2 neutrophils to the increased IL-10 expression levels observed 48 hpi is higher than the observed for the N1 population (**Fig. 2C**). To expand the knowledge of these two populations, additional characterization was performed by TEM. These analyses were performed in neutrophils separated by size using Fluorescence Activated Cell Sorting (FACS). Results showed that purified neutrophil populations exhibited important changes in the shape of the nucleus and chromatin condensation (**Fig. 2D**). The N2 population exhibited a nucleus with decondensed

chromatin, while the N1 population had a nucleus predominantly constituted by heterochromatin. Moreover, we also observed differences related to the amount of primary and secondary granules present in the cytoplasm as well as the presence of autophagy-related structures, particularly in the N2 population **(Fig. 2E).** These results suggest that neutrophil subsets might be playing different roles during the first 48 hpi of *S. pneumoniae* infection.

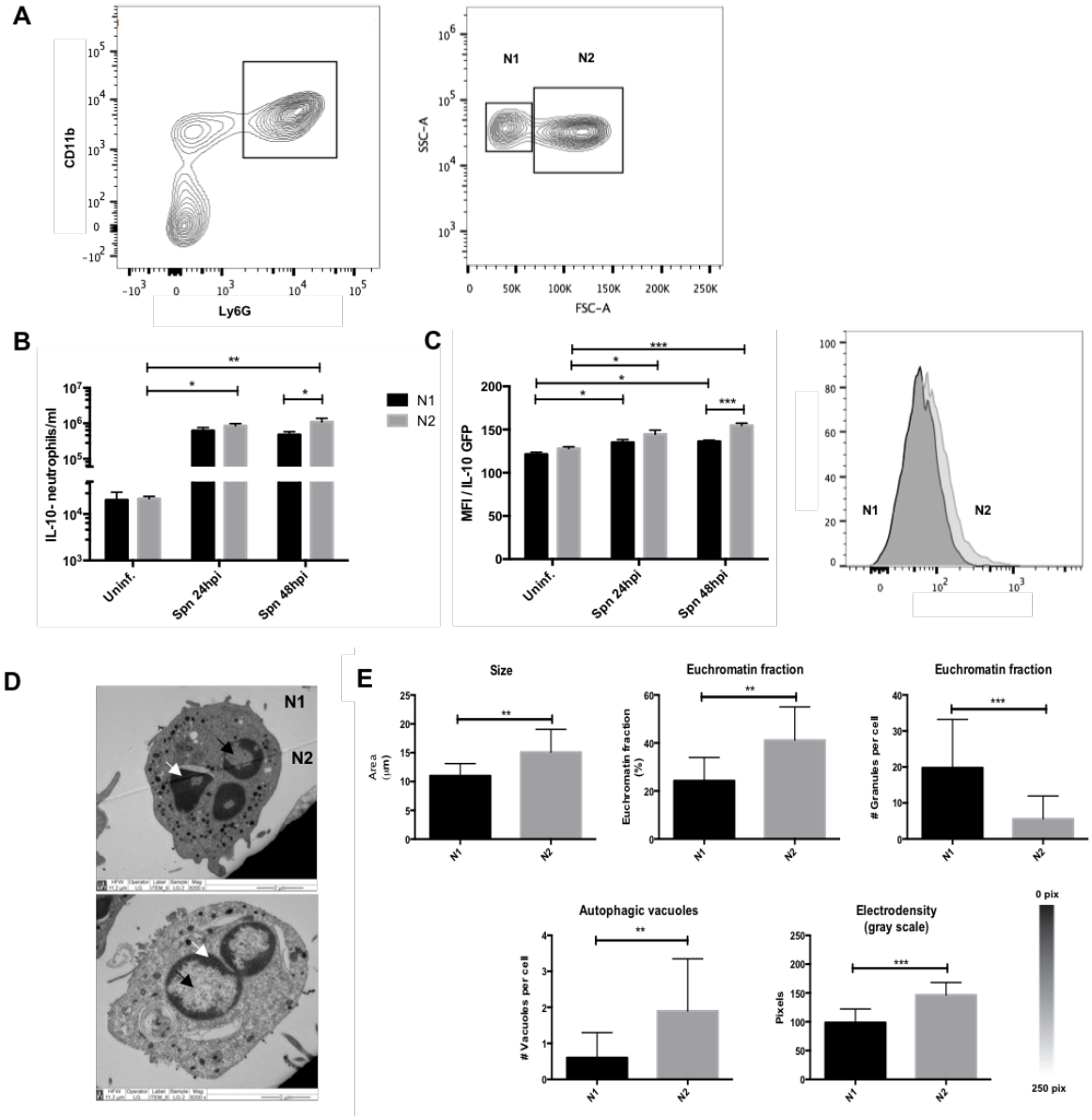


Figure 2. Neutrophil subsets exhibit structural and functional differences after 24hpi. A. Representative flow cytometry plots of lung cells shows neutrophils gated as $CD11b^{+}Ly6G^{+}$ cells. Neutrophil subsets were gated according to FSC parameters as N1 (small population) and N2 (large population). **B.** Number of N1(black bar) and N2 (gray bar) neutrophils expressing IL-10 in uninfected mice together with the observed neutrophils at 24 and 48hpi. **C.** IL-10 expression of neutrophils (from IL-10::eGFP mice subsets in lungs at 24 and 48hpi. **D.** Representative TEM images of N1 and N2 subsets obtained from 24h infected lungs. The white arrows point to the heterochromatin and the black arrows point to the euchromatin fraction. **E.** Phenotypic parameters measured in N1 and N2 populations with ImageJ. Statistics: Tukey's test following two-way ANOVA and T-test following Mann-Whitney. *, $P<0.05$; **, $P<0.05$; **, $P<0.05$. TEM: Transmission Electron Microscopy. hpi: h post-infection; Uninf: Uninfected.

3.5.4 *S. pneumoniae* recognition by neutrophils induces IL-10 production.

In order to elucidate which are the stimuli modulating IL-10 production in neutrophils, we performed *in vitro* assays with bone-marrow neutrophils obtained from IL-10::eGFP mice. These neutrophils were incubated with heat-killed bacteria (MOI 25, 50 and 100) and BALFs obtained from infected and non-infected mice. We included in these experiments two *S. pneumoniae* strains: encapsulated and non-capsulated. Additionally, BALFs mixed with heat-killed bacteria were included as a treatment. After 6, 12 and 24 h, eGFP fluorescence was measured in cells by flow cytometry and IL-10 production was measured by ELISA in the supernatants from cultured neutrophils. Results showed that only heat-killed *S. pneumoniae,* but not BALF, stimulated eGFP production in neutrophils at 12 hpi (**Fig. 3A**) Observations obtained from these assays were confirmed by ELISA, where IL-10 levels were detected only in culture supernatants from neutrophils stimulated with the capsulated and non-capsulated bacteria (**Fig. 3B**). However, the increase of IL-10 levels observed in the supernatants from neutrophils co-cultured with the non-capsulated strain was not significant. Additionally, we observed a reduced IL-10 production at 6

and 12 post-treatment in neutrophils infected with higher MOI of the non-capsulated strain. At 24 hpi, no IL-10 production was observed in neutrophils infected with the non-capsulated strain (**Fig. 3B**). When neutrophils were treated with BALF from infected mice and heat-killed bacteria (MOI 25), an increase in IL-10 production was observed, which may be attributed to *S. pneumoniae* PAMPs. In addition we stimulated naïve neutrophils with DNA and purified cell wall extract obtained from *S. pneumoniae* D39 (data not shown). IL-10 production was evaluated in those supernatants by ELISA at 24 hpi and no IL-10 was detected in neutrophils stimulated with bacterial DNA (data not shown) and very low levels of IL-10 were observed in neutrophils treated with cell wall extract (**Fig. 3B**). These in vitro results suggest that IL-10 expression in neutrophils observed in the figure 1E could be induced by the *S. pneumoniae* PAMPs.

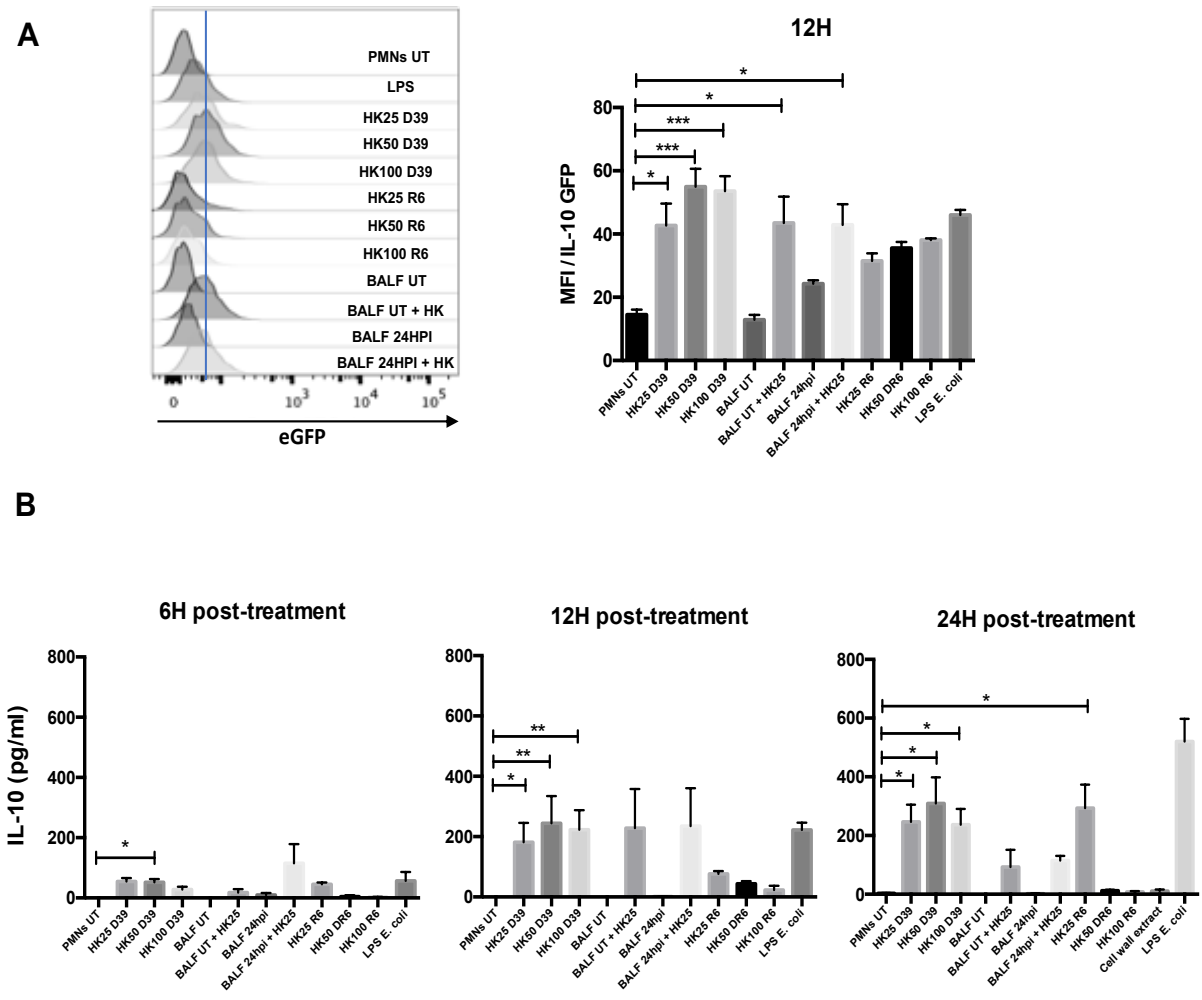


Figure 3. *S. pneumoniae* induces IL-10 production in neutrophils in vitro after 24 hpi. Bone marrow-derived neutrophils were incubated with different MOIs of heat-killed *S. pneumoniae* (D39 and R6 strains) to determine IL-10 production in response to stimulus. **A.** IL-10 expression was evaluated by flow cytometry at 12hpi. **B.** Supernatants were recovered and evaluated for IL-10 detection by ELISA at 6, 12 and 24hpi. One-way ANOVA and Kruskal-Wallis post-tests were performed for statistical analyses. *, P <0.05 in each treatment comparison with the untreated cells. Three independent experiments.

3.5.5 Adoptive neutrophil transfer improves survival and clinical signs of IL-10$^{-/-}$ mice independently of IL-10 production by transferred cells.

In order to determine the role of IL-10–producing neutrophils during pneumococcal pneumonia, we performed neutrophil adoptive transfer assays into C57BL/6 wild type (WT) and IL-10$^{-/-}$ mice (the latter highly susceptible to *S. pneumoniae* infection) (Peñaloza et al., 2015). We transferred WT and IL-10$^{-/-}$ neutrophils, purified by MACS, to each group and then infected the mice with *S. pneumoniae*. Survival rates and clinical parameters were evaluated over the course of ten days after infection. Data obtained in this experiment showed that all non-transferred WT mice lived until the end of the experiment and had a low clinical score, while non-transferred IL-10$^{-/-}$ had the highest clinical score and 100% of mortality at day 4. Neutrophil transfer from either WT or IL-10$^{-/-}$ mice improved IL-10$^{-/-}$ mice survival, increasing their survival rate up to 60% **(Fig. 4)**. The increased survival of individuals at the endpoint of the curve was similar in the two groups of IL-10$^{-/-}$ transferred mice and significant differences were not observed at any point in survival or score curves between these groups. However, the group of WT mice transferred with WT neutrophils showed a decrease in the survival rate and an increase in disease parameters **(Fig. 4)**.

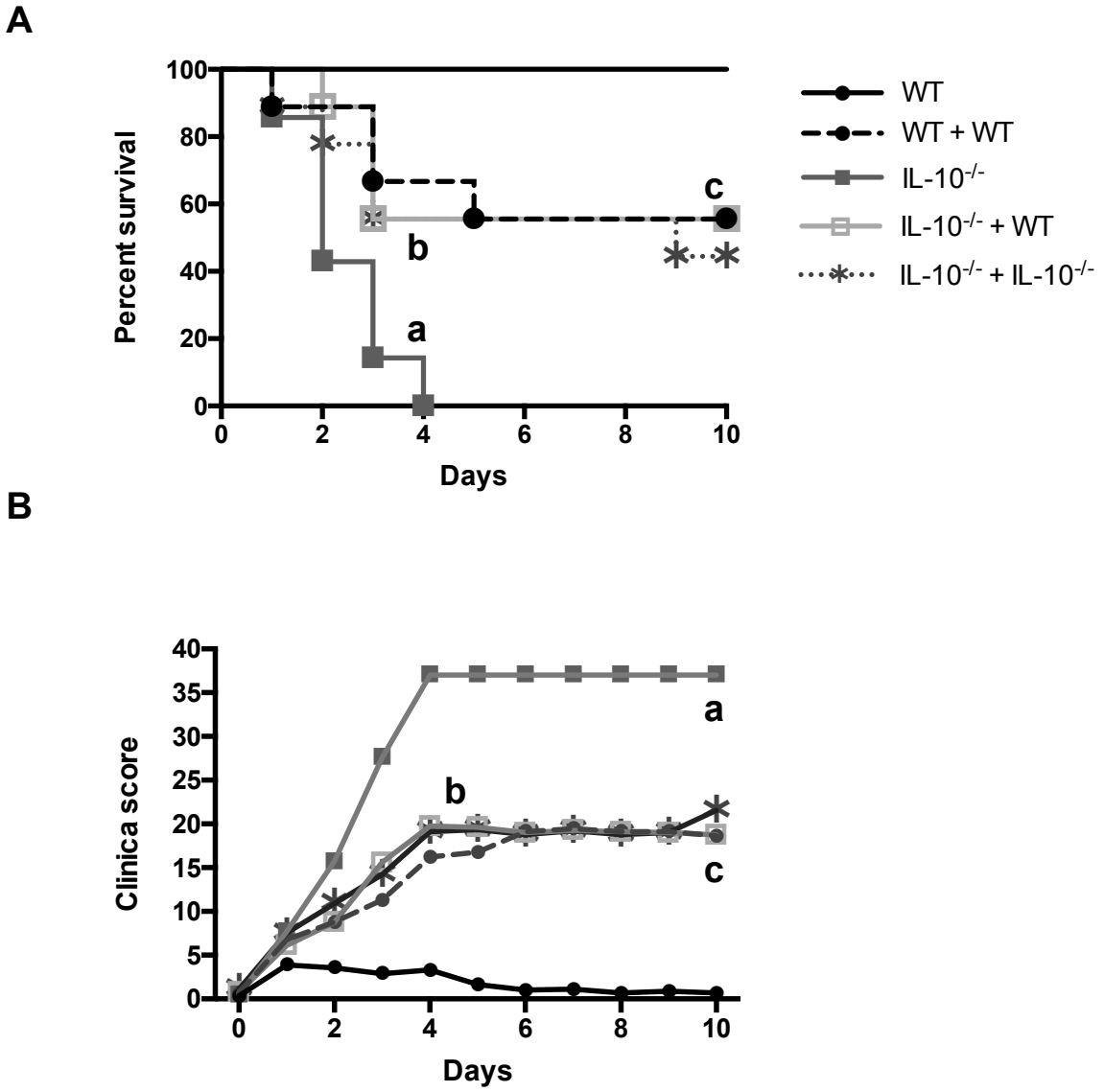


Figure 4. Neutrophil adoptive transfer improves IL-10-/- mice survival and clinical score independently of IL-10 production. Neutrophils obtained from WT and IL-$10^{-/-}$ mice were intranasally transferred to WT and IL-$10^{-/-}$ mice and 24 h later all mice were infected with $3x10^7$ cfu of *S. pneumoniae* (n = 7 to 9 for each group). This experimental design was performed to evaluate the survival and clinical parameters of mice in the different groups during 10 days. **A.** Survival curve including WT and IL-10-/- mice transferred and non-transferred. **B.** Disease parameters were evaluated according to a clinical score guideline including activity, weight loss, body posture and general appearance. Statistics: Log-rank (Mantel-Cox) test and Two-way Anova with Sidak's multiple comparisons post-test were performed. (a) ****, $P<0.0001$ between WT and IL-$10^{-/-}$, (b) *, $P<0.05$ between IL-$10^{-/-}$ and IL-$10^{-/-}$+ WT, (c) **, $P<0.05$ between WT and WT + WT.

3.5.6 Adoptive transfer of IL-10-producing neutrophils reduced lung damage, but impaired bacterial clearance and promoted *S. pneumoniae* dissemination.

Adoptive transfer assays were also performed to evaluate other parameters, as lung infiltration with inflammatory cells, cytokine profiles, bacterial burden in organs and histopathological changes in transferred mice. Results obtained from the analyses of lung infiltration showed no significant differences among the experimental groups, regarding the presence of live neutrophils and monocytes in lungs at 48 hpi (**Fig. 5A**). Likewise, the levels of the proinflammatory cytokines IL-12, IFN-γ and TNF-α measured in BALFs did not show significant differences among the experimental groups (**Fig. 5B, 5C and 5D**). In contrast, increased levels of IL-10 were observed in IL-10$^{-/-}$ mice transferred with WT cells, as compared with the null levels observed in the other two IL-10$^{-/-}$ control groups. Additionally, WT mice transferred with WT cells showed an increase of more than a 100% of IL-10 production as compared with the levels observed in non-transferred WT mice, indicating that the transferred neutrophils contributed to IL-10 increase in BALFs at 48 hpi (**Fig. 5E**).

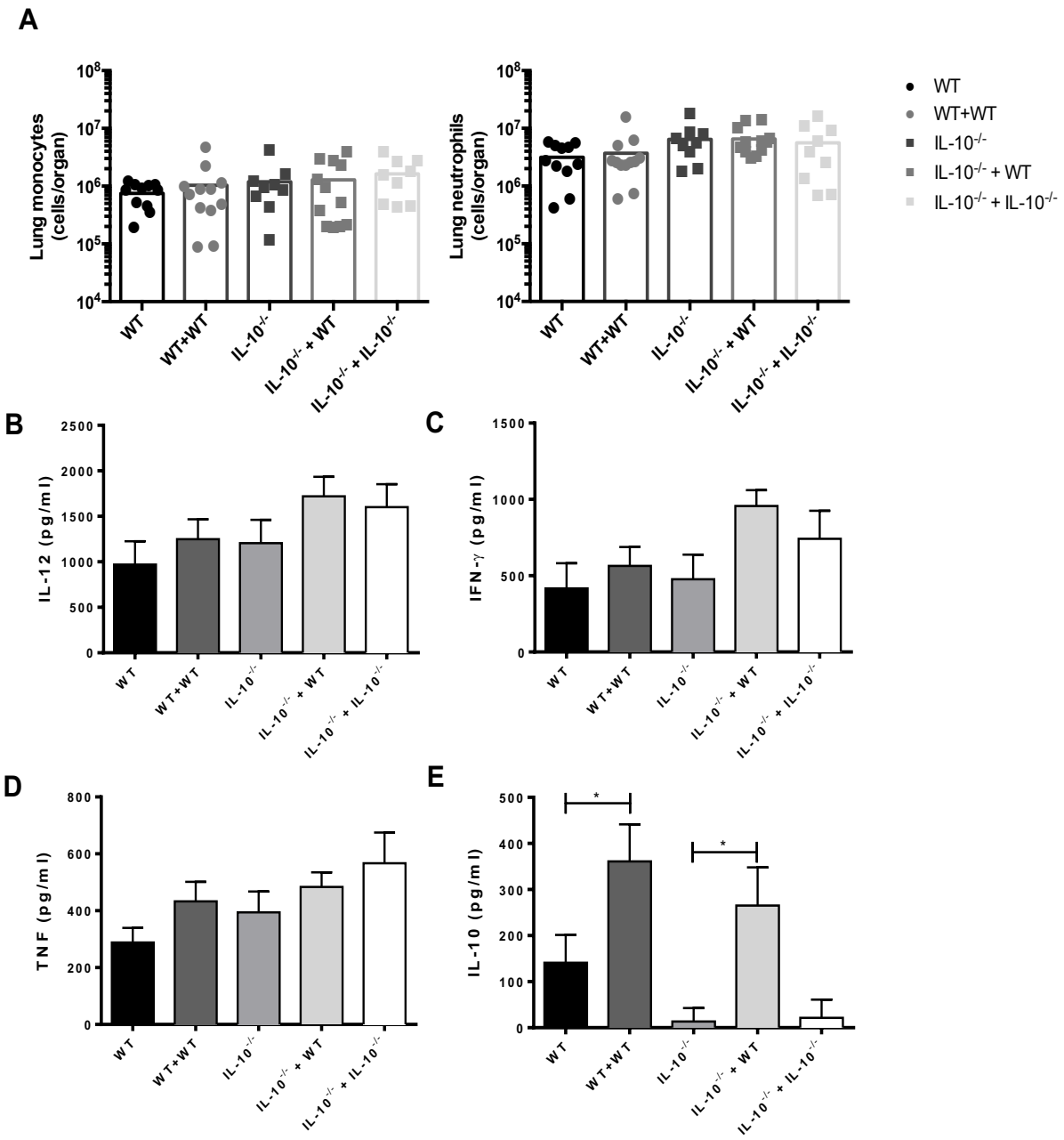


Figure 5. IL-10 produced by WT neutrophils does not have impact in reduction of pro-inflammatory cells infiltration in IL-10-/- mice. A. Monocytes and neutrophils infiltration in mice lungs of each group, was measured at 48hpi by flow cytometry. Pro-inflammatory **(B, C, D)** and anti-inflammatory **(E)** cytokines obtained from BALFs at 48hpi were determined by ELISA in each control and transferred group. Statistics: T-test with Mann-Whitney post-test. $P<0.05$. Comparisons of the mean of each group with the mean of every other groups. n = 9 to 12 for each group. BALFs: Bronchoalveolar Lavages; hpi: h post infection.

We also observed that IL-10$^{-/-}$ mice transferred with WT neutrophils showed increased bacterial loads in the lungs as compared with the non-transferred IL-10$^{-/-}$ group (**Fig. 6A**). Moreover, bacterial loads in the blood and spleen were significantly higher in IL-10$^{-/-}$ mice transferred with WT neutrophils than non-transferred IL-10$^{-/-}$ group (**Fig. 6B and 6C**). There were no differences among bacterial loads in the brains in mice of each group (**Fig. 6D**).

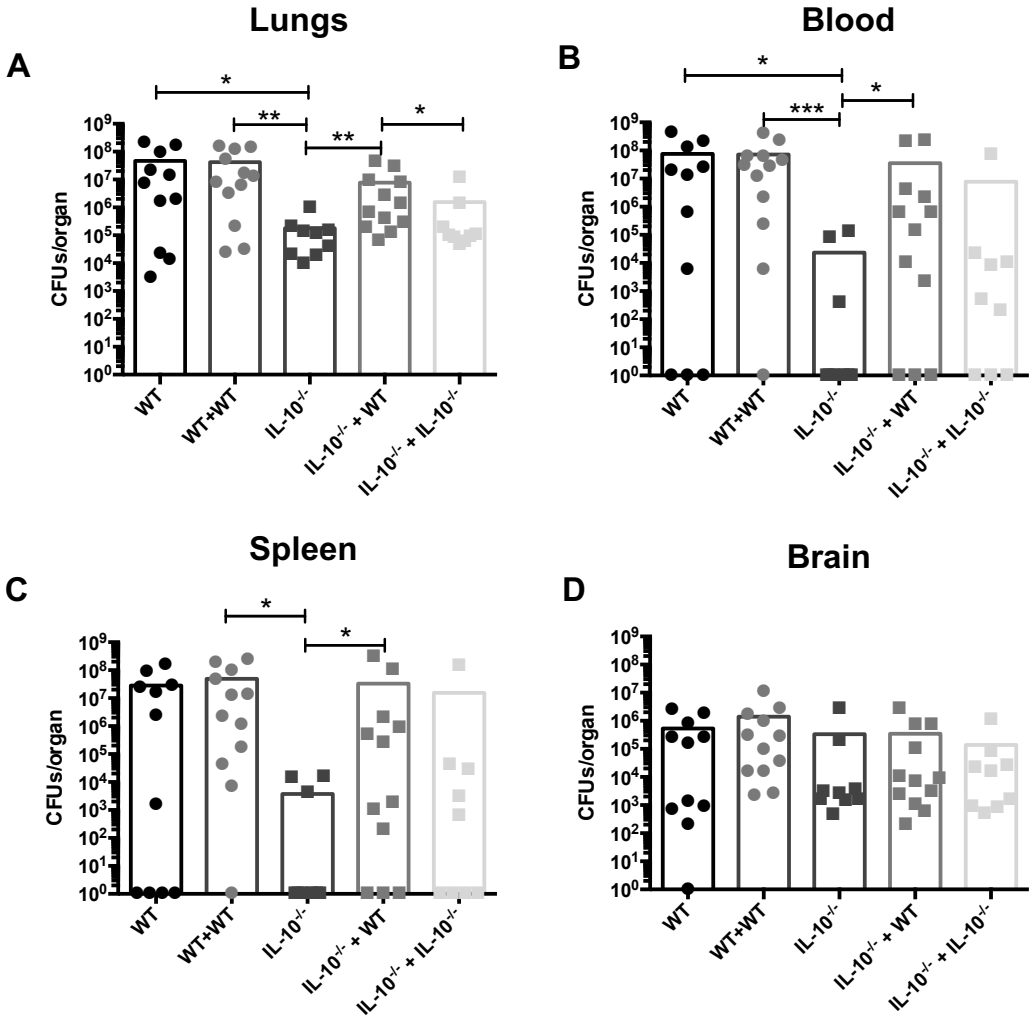
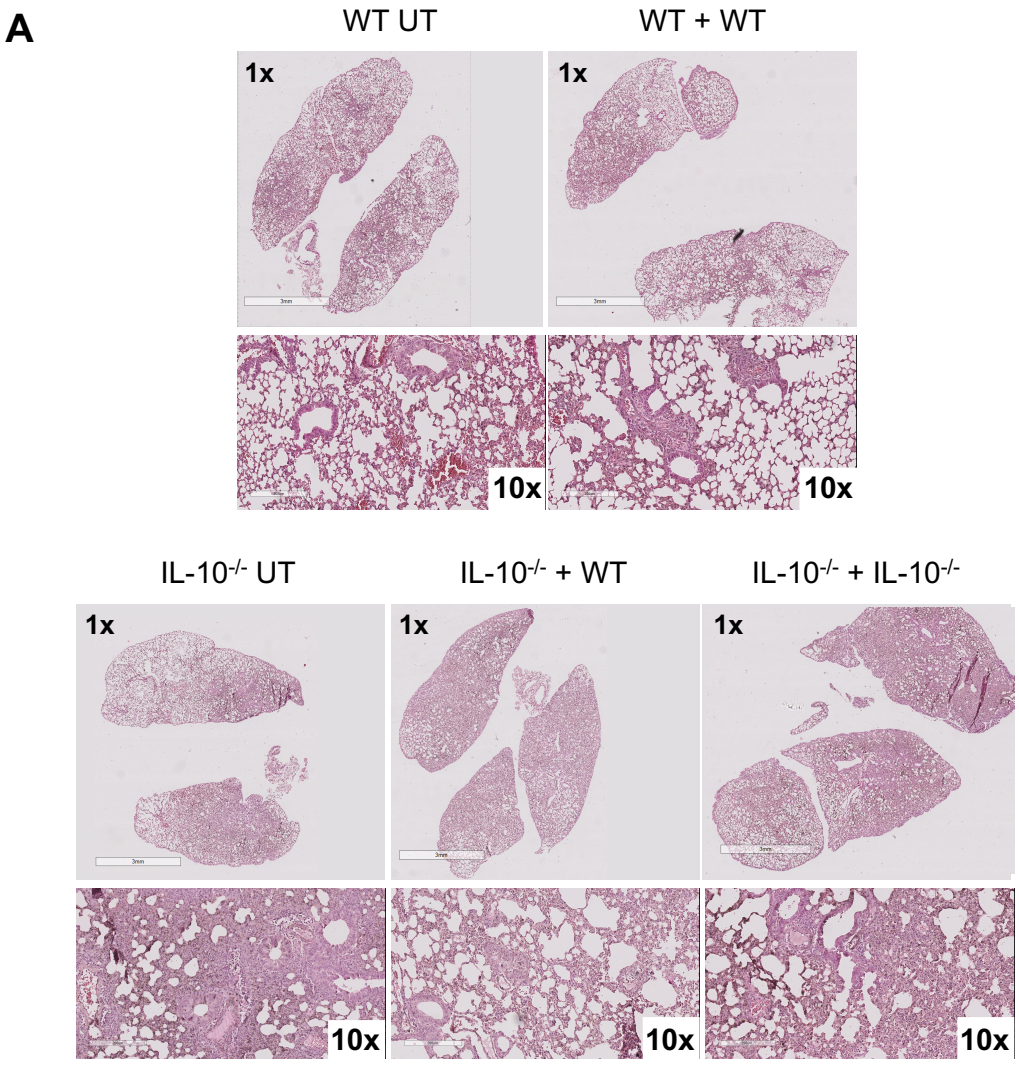


Figure 6. WT neutrophils transfer impair bacterial clearance. Bacterial loads were measured in lungs, blood, spleen and brain, and were expressed as CFU per organ at 48hpi. Statistics: T-test with Mann-Whitney post-test. P<0.05. Comparisons of the mean of each group with the mean of every other groups. n = 9 to 12 for each group. hpi: h post infection; CFU: Colony Forming Units.

Regarding histopathological analyses, images obtained from WT and IL-10$^{-/-}$ mice confirmed our previous findings (Peñaloza et al., 2015), where at 48 hpi WT mice show slight lung damage in contrast to the substantial loss of lung architecture observed in IL-10$^{-/-}$ mice (**Fig. 7**). Interestingly, we also observed that IL-10$^{-/-}$ mice transferred with wild-type neutrophils, showed less lung damage with more conserved alveolar structures and low hemorrhagic spots throughout the lungs than the other IL-10$^{-/-}$ groups (**Fig. 7A**). Histopathological observations were scored according to the level of pro-inflammatory cell infiltration, hemorrhage incidence, loss of lung architecture and alveolar structure, and the extension of damage in both lungs (Supplementary data, Table 2). Results obtained from this analysis showed that IL-10$^{-/-}$ mice transferred with WT neutrophils (able to produce IL-10), had significantly lesser total score in comparison to the other IL-10$^{-/-}$ mouse groups (**Fig. 7B**). Statistical analysis applied individually per parameter did not show significant differences among groups, however it is possible to observe that items concerning alveolar wall swelling and lung damage in IL-10$^{-/-}$ mice transferred with WT neutrophils, were similar to the observed in non-transferred WT mice (**Fig. S2**).


A
WT UT
WT + WT
1x
1x
10x
10x
IL-10-/- UT
IL-10-/- + WT
IL-10-/- + IL-10-/-
1x
1x
1x
10x
10x
10x
B
Histopathological Score (0-14)
14
12
10
8
6
4
2
0
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ns
Total Score
WT UT
WT + WT
IL-10-/- UT
IL-10-/- + WT
IL-10-/- + IL-10-/-

Figure 7. Adoptive neutrophil transfer from WT to IL-10-/- mice induce a decrease in the histopathological score. A. Representative lung tissue sections stained with H&E at 1X and 10X magnification. These images were used to evaluate histopathological changes after neutrophils adoptive transfer. **B.** Histopathological score for lung damage. This score was used to quantify lung damage after neutrophils adoptive transfer. To capture photographs and analyze them, the Aperio ImageScope Software and GraphPad Prism Software were used. Statistics: Two-way Anova with Tukey's multiple comparisons post-test were performed. ***, $P<0.05$; ****, $P<0.05$.

3.6 DISCUSSION.

Neutrophils have been recently described as important actors of both the innate and adaptive immune response due to their plasticity and immune-modulation properties as the ability to produce IL-10 (Zhang et al., 2009; Kasten et al., 2010; Gabryšová et al., 2014). This role for neutrophils as important suppressor cells has been previously described in sepsis models (Ocuin et al., 2011; Bouabe et al., 2011) and in chronic infectious diseases, such as tuberculosis and Chagas (Boari et al., 2012; Moreira-Teixeira et al., 2017). In agreement with the literature, we report a heterogeneous population of neutrophils that produce IL-10 in response to *S. pneumoniae* infection. We observed that among the innate immune cells able to produce IL-10, only neutrophils showed a significant increase in IL-10 expression (**Fig. 1E**). The high number of IL-10 producing neutrophils found at 24 hpi allowed us to conclude that these cells are key components to prevent exacerbated inflammatory response during the early phase of *S. pneumoniae* lung infection. It is important to note that the involvement of neutrophils in IL-10 production is different among the broad range of pathogens able to cause pneumonia. For example, in viral pneumonia and tuberculosis, neutrophils are suppliers of IL-10, however the substantial production of this cytokine is produced by interstitial macrophages and T cells (Sun et al., 2009; Sun et al., 2011; Doz et al., 2013). In the case of lung infection caused by *Klebsiella pneumoniae*, MDSCs have the leading role in regulation mediated by IL-10 (Peñaloza et al., 2019). These results contrast with our findings and makes us presume that the role of neutrophils, as the dominant source of IL-10 production, may be a distinctive feature of pneumococcal pneumonia.

Since *S. pneumoniae* induces a high influx of neutrophils into the lungs that subsequently produce IL-10, this could be a host mechanism to prevent inflammatory damage activated by a bacterial infection (Moreira et al., 2008; Duell et al., 2012; Griss et al., 2016). Considering this information, we wanted to determine whether IL-10 production is induced directly by *S. pneumoniae* or whether host factors are required to induce this regulatory phenotype in neutrophils. It has been reported that components of the cell wall of *S. pneumoniae,* such as peptidoglycan, lipoteichoic acid, and lipoproteins, are recognized by Toll-like receptors (TLRs) in macrophages and monocytes, inducing IL-10 production (Moreira et al., 2008; Nguyen y Götz, 2016). The recognition of these patterns is usually associated with TLR2 and TLR4, and these receptors have been significantly involved in IL-10 production by neutrophils (Zhang et al., 2009; Balderramas et al., 2014). Here we collected evidence to reveal that neutrophils only produce IL-10 in response to *S. pneumoniae* antigens. We first observed that neutrophils increase their IL-10 expression significantly during *S. pneumoniae* lung infection, and then we confirmed by flow cytometry and ELISA that IL-10 levels were significantly higher when neutrophils were co-cultured with the capsulated strain. On the other hand, the non-capsulated strain induced low and non-significant increase in the levels of IL-10 produced by neutrophils. These results could be explained by the exposure of several molecules on the surface of non-capsulated strain that can be recognized by neutrophils, inducing transcriptional pathways that promote a pro-inflammatory profile, while the capsule of the D39 strain coats some of those molecules, allowing a differential exposure of bacterial PAMPs and favoring the activation of the transcriptional pathways involved in IL-10 production. Furthermore, polysaccharides

present in the pneumococcus capsule could activate the pathways required to induce the expression of IL-10 genes, as reported for polysaccharide A from *Bacteroides fragilis* (Johnson et al., 2018; Ramakrishna et al., 2019). Next, to rule out the participation of other bacterial molecules in IL-10 production, we added DNA and cell wall extract (peptidoglycan) obtained from *S. pneumoniae* to the assays. These results showed that bacterial DNA did not induce IL-10. Similarly, low or null levels of IL-10 were found in the supernatants of neutrophils stimulated with peptidoglycan at 24h post-culture. Further studies are required to clarify which bacterial components are mostly required in the induction of IL-10. However, based on our results, we suggest that molecular components that stand out the capsule and even capsule polysaccharides are potential inductors of IL-10 in neutrophils.

In the last years, the discovery of novel roles for neutrophils has been accompanied by the description of changes in phenotypic and functional features, which has led to the appearance of new subtypes of neutrophils (Perobelli et al., 2015; Perobelli et al., 2017). These subsets have been described mainly in cancer and autoimmune diseases (Perobelli et al., 2015). However, data about neutrophils characterization during *S. pneumoniae* infection is still an ongoing research field. After the evaluation of the contribution of neutrophils to IL-10-production during pneumococcal pneumonia, we characterized the two neutrophil subsets present in the lungs previously described as N1 and N2 (Peñaloza et al., 2018). These subsets observed in non-infected and infected lungs were initially identified based on their size (N1: small; N2: large). Further, flow cytometry analyses performed to evaluate differences in IL-10 production showed a significant difference in IL-10 expression

between N1 and N2 at 48 hpi only in infected mice. This result is in line with a recent study, where different neutrophils subsets were described in blood and BALFs obtained from children with viral pneumonia (Cortiens et al., 2017).

The characterization of neutrophil subtypes has been established based on differences in surface markers or cytokine and chemokine profiles (Kruger et al., 2015). However, neutrophil plasticity can also be supported by other phenotypical characteristics. In this context, we performed analyses based on images obtained by TEM. First, N1 cells are small cells with a considerably high number of cytoplasmic granules, a small number of vacuoles, and a nucleus mainly constituted by heterochromatin. On the other hand, N2 neutrophils are characterized by a larger size, low presence or absence of granules, a marked presence of vacuoles, and an increase in the euchromatin fraction. According to the morphological findings, N1 and N2 subtypes display characteristics very similar to those observed in neutrophil subtypes reported in a systemic inflammatory syndrome in response to intravenous *Staphylococcus aureus* infection (Tsuda et al., 2004). Tsuda and colleagues reported the presence of two neutrophil subtypes, the first defined as PMN-I which appeared as small cells with marked pro-inflammatory and cytotoxic properties, and the second named PMN-II, which looked larger than the other subtype and displayed anti-inflammatory properties marked by the expression of IL-10 (Tsuda et al., 2004; Savig et al., 2015). Neutrophil subtypes similar to N1 and N2 have also been described in cancer with anti- and pro-tumor properties, respectively (Chen et al., 2019). Furthermore, N2 neutrophils present a significant euchromatin fraction, suggesting important changes in gene expression explaining their IL-10 production

changes, as previously reported in macrophages (Chen et al., 2019). Our data is consistent with this classification of N1 and N2 subsets, with potentially differential functions associated with their phenotypic characteristics. However, further transcriptomic studies or complementary analyses based on single-cell sequencing are required to confirm that N1 and N2 are two subtypes of neutrophils that play differential roles during pneumococcal lung infection.

Next, we performed adoptive transfer of neutrophils to determine the impact of neutrophils able to produce IL-10 in a murine model of pneumonia. Neutrophil adoptive assays have previously been described in other infectious processes (Boari et al., 2012; Hosoki et al., 2019). However, this work is the first implementing this assay in pneumococcal pneumonia, based on a protocol previously carried out in our laboratory (Peñaloza et al., 2019). First, we conclude that transferred neutrophils improve IL-10$^{-/-}$ mice survival, independently of their ability to produce IL-10, while in WT mice, this transfer decreases mice survival rate by 30%. These results are the consequence of our experimental design, where mice had more neutrophils than they should before infection. This implies a faster and more effective bacterial killing in the IL-10$^{-/-}$ mice but also implies the amplification in the IL-10 response in WT mice. This last statement was confirmed by the increased levels of IL-10 observed in BALFs from the WT transferred mice at 48 hpi (**Fig. 5E**) and had an impact on bacterial loads, which apparently was enough to break the balance between infection and inflammation required to guarantee the survival of WT mice. These results reinforce the widely described dual role of IL-10 in infections (Peñaloza et al., 2016). Additionally, our results indicate that WT neutrophils play a role in preventing

excessive tissue injury. Although flow cytometry analyses did not show a clear impact in lung infiltration with pro-inflammatory cells, histopathological changes were evident in IL-10$^{-/-}$ mice after WT neutrophil transfer, which exhibited less damage and better lung architecture when compared to the other IL-10$^{-/-}$ experimental groups. Noteworthy, this result indicates, along with other findings in this study, that the regulatory capacities of neutrophils related to IL-10 production could be determinant in the course of pneumococcal infection both at a local and systemic level.

In conclusion, our findings contribute to a better understanding of one of the abilities attributed to neutrophils, reflecting significant plasticity and their relevance as modulators of the immune response in pneumonia caused by *S. pneumoniae.* Our findings contribute to increased knowledge on neutrophils, describing the basal presence of two populations of neutrophils in mouse lungs that change their proportions and IL-10 expression exclusively in response to pneumococcal stimuli and, as an important source of this anti-inflammatory cytokine, have a key role in the outcome of the pathological process. Moreover, the *in vitro* and *in vivo* assays aimed at better understanding the unrecognized role of IL-10-producing neutrophils in pneumococcal pneumonia with results that could be generalized to pneumonia in humans, eventually translating into novel strategies in the management of patients and the potential development of treatment approaches based on immune-modulation therapies directed to control neutrophil recruitment and/or function during the critical first 48 hpi.

4.6 GRANTS SUPPORT

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4.7 SUPPLEMENTARY MATERIAL

Parameter	Value	Weighted score	Maximum score
Weight loss	5%	0	4
	10%	1	
	15%	2	
	20%	3	
	25%	4	
Activity	Normal	0	4
	Slightly decreased	1	
	Decreased	2	
	Severely decreased	3	
	Coma	4	
Time to return to upright position	Normal	0	6
	< 5 seconds	2	
	< 30 seconds	4	
	No return is observed	6	
Fur appearance	Normal	0	3
	Slightly dirty	1	
	Dirty	1	
	Piloerection	1	
Posture	Normal	0	2
	Slightly hunched over	1	
	Extremely hunched over	2	
Eyes appearance	Normal	0	4
	Protruding eyes	1	
	Sunken eyes	1	
	Closed eyelids	1	
	Ocular secretion	1	
Breathing problems	Irregular breathing	2	4
	Dificulty breathing	2	
Neurological issues	Normal	0	10
	Ataxia	2	
	Paralysis	2	
	Epileptic episodes	2	
	Status epilepticus	6	
Total			37

Table S1. Monitoring chart for infection caused by *Streptococcus pneumoniae.*

Score	Cell infiltrate	Hemorrhage	Swelling of alveolar walls	Lung damage	Damage extension
0	Normal amounts of alveolar macrophages; no presense of leukocytes in alveoles or bronchioles.	No presence of erithrocytes in alveoli	Absent	Normal lung architecture	No lessions are observed
1	Moderate increase of leukocytes in alveoli and/or bronchioles	Limited presence of erithrocytes in alveoli	Moderate	Minimal change in lung architecture	Focal unilateral lession
2	Severe increase of leukocytes in alveoli and/or brochioles	Elevated presence of erithrocytes in alveoli	Severe	Moderate change in lung architecture with alveoles and brochioles structures distinguishable	Focal and bilateral lessions/diffuse unilateral lession
3	Cellular infiltrate does not allow to recognize bronchioli structure	_____	Indistinguisible structure	Any lung structure can be recognized	Diffuse lessions in both lungs

Table S2. Score of histopathological changes in lungs. The parameters included in the table were evaluated in representative photographs from complete lungs with the Aperio ImageScope Software. The score of microscopic lesions was determined at 20X magnification and the damage extension was evaluated at 4X.

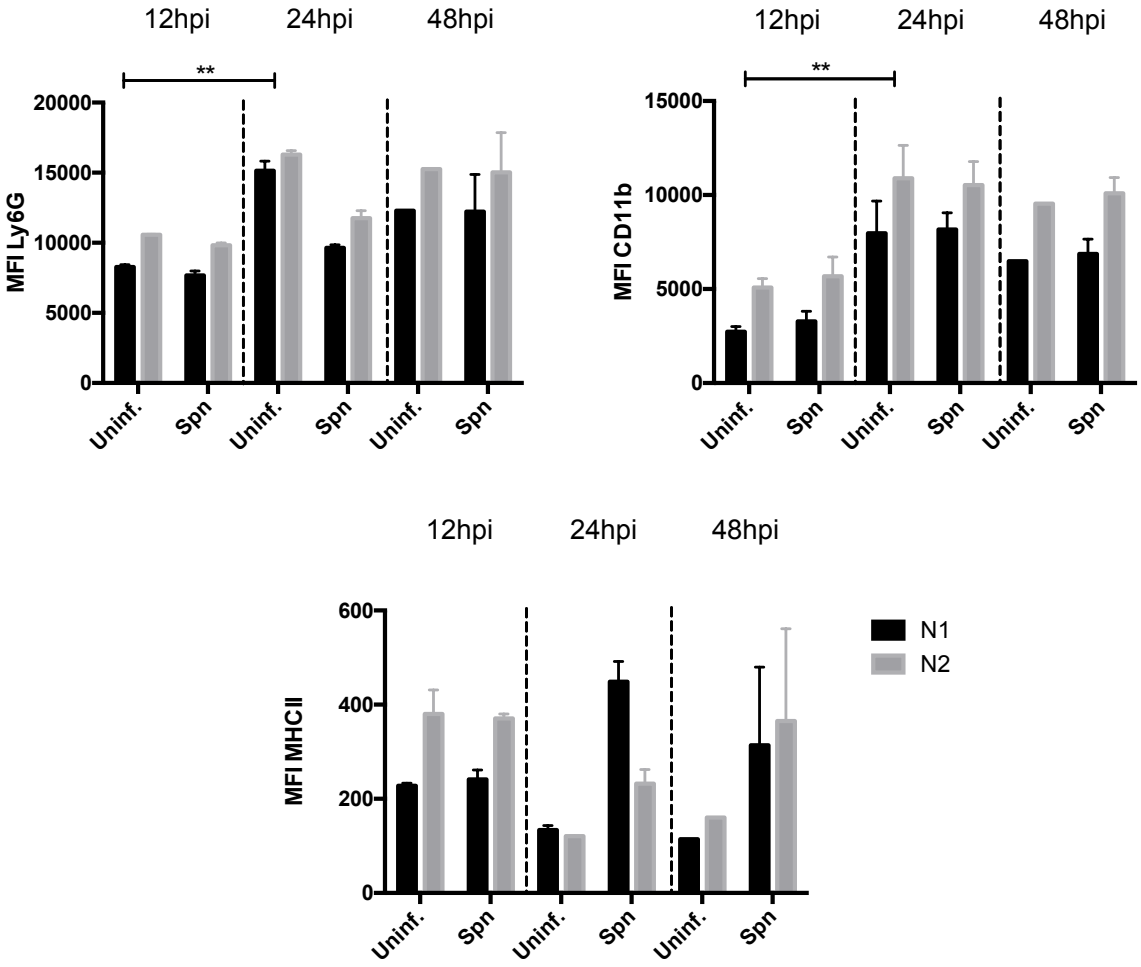

Figure S1. Neutrophil subtypes N1 and N2 express similar activation and maturity surface markers. Ly6G, CD11b and MHCII expression was evaluated by flow cytometry and was measured by median fluorescence intensity (MFI). Statistics: Holm-Sidak test following two-way ANOVA. $p < 0.05$.

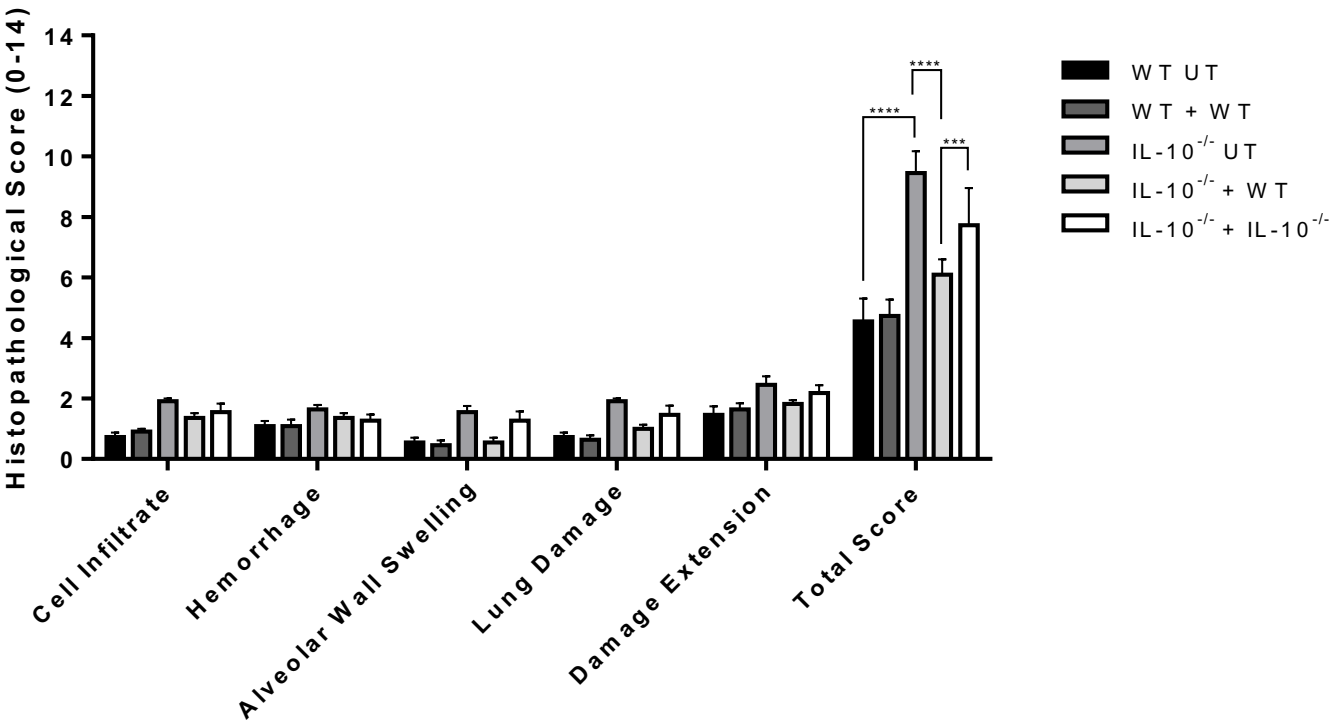

Figure S2. Complete histopathological score for lung damage. Five lung damage parameters were evaluated in representative photographs of complete lungs, to determine a complete score for severity of pneumonia. Statistics: Two-way Anova with Tukey's multiple comparisons post-test were performed. $P<0.05$.

5. GENERAL DISCUSSION

IL-10 is a cytokine with modulatory properties, aimed to reduce tissue injury and reestablish the homeostasis in inflammatory processes (Ouyang, et al., 2011). In infectious diseases, this cytokine exhibits a paradoxical performance influenced by intrinsic characteristics of the pathogen, as well as aspects related to the infected tissue (Peñaloza et al., 2016). A clear example of the dual behavior of IL-10 is observed in pneumococcal pneumonia, where a rapid induction of this cytokine is associated with the reduction in lung injury at the expense of an impairment in bacterial clearance (Peñaloza et al., 2015). Considering the relevance of pneumonia as one of the deadliest infections worldwide, it becomes imperative to understand all the mechanisms involved in disease development and to identify the main actors involved in the process, for example, cells producing IL-10. In response to this requirement, we aimed to determine which cells are involved in IL-10 production during *S. pneumoniae* lung infection in a murine model. To address this question, we evaluated the IL-10 expression in innate immune cells present in lungs during the first 48 hpi. These experiments showed that all myeloid cell populations in the lungs express IL-10 at basal levels. However, neutrophils are the only group of cells producing significative amounts of IL-10 in response to *S. pneumoniae* infection. Our results support the concept of neutrophils plasticity by adding a novel role to their functions aimed to modulate the immune response during pneumonia.

Neutrophil's plasticity denotes the changes in phenotypic and functional characteristics that explain the versatility attributed to these cells, and this plasticity

is the basis for the emergence of new classifications of neutrophils and the dissection of this heterogeneous population in different subtypes (Perobelli et al., 2017). According to this, different neutrophil subsets have been described in cancer and autoimmune diseases, as well as in infectious diseases (Perobelli et al., 2017). In fact, in a recent study, Peñaloza and colleagues described the presence of different infiltrating neutrophils in mice lungs during pneumococcal pneumonia (Peñaloza et al., 2018). In this work, authors reported the presence of two neutrophil subsets in infected and non-infected mice, identified by size as N1 (small) and N2 (large) (Peñaloza et al., 2018). In this line and to complement the characterization of these two subsets, we performed infection assays in mice, to evaluate differences in IL-10 production between N1 and N2, and results from these assays showed a slight difference in IL-10 expression between N1 and N2 at 24hpi, that becomes significant at 48hpi, exclusively in mice infected with *S. pneumoniae*.

Furthermore, we performed complementary analyses in N1 and N2 populations, based on images obtained by TEM, and we found that N1 are small cells with a cytoplasm full of granules and a nucleus mainly constituted by heterochromatin; while N2 are larger cells, with low granularity and a nucleus characterized by an increased euchromatin fraction. These results confirm the previous findings of the presence of at least two neutrophils populations in lungs and add new characteristics to each one of these subtypes. According to our morphological findings, N1 and N2 subtypes appear to be similar to PMN-I and PMN-II subtypes described during sepsis caused by *S. aureus* (Tsuda et al., 2004). Similar descriptions of N1 and N2 type of neutrophils also exist in cancer, where anti-tumor neutrophils are small and

pro-inflammatory (phenotypically similar to N1) while pro-tumor cells are mature neutrophils with higher size that confers an anti-inflammatory environment proper for tumor growth and metastasis (Sagiv et al., 2015). Another significant observation from our experiments is the difference in chromatin conformation between N1 and N2. This finding suggests important modifications at transcriptional and translational level between both subtypes, as it has been previously described in macrophages, where epigenetic mechanisms induce changes in chromatin condensation and induce a switch from a homeostatic profile to a pro-inflammatory profile and vice-versa (Chen et al., 2019). All the information obtained in this part of the study leads to a better recognition of each subtype and the assumption of possible specific functions of these N1 and N2 cells associated to their phenotypic characteristics.

Although in this study we describe neutrophil subtypes in mice lungs, it is relevant to highlight similarities of N1 and N2 neutrophils to human neutrophils populations described so far. For instance, Cortiens and colleagues have reported that children infected with respiratory viruses, presented regulatory subpopulations of neutrophils that became more evident with the subsequent co-infection with bacterial pathogens (Cortiens et al., 2017). Remarkably, authors describe circulating neutrophils with similar characteristics to the ones described in this thesis, opening the possibility to extrapolate data found in mice to humans and, in some way, contribute to the development of treatment strategies aimed at modulating neutrophils response. Now, it is important to mention that further studies involving proteomics and complementary analyses based on single-cell sequencing, are required to confirm

metabolic and functional differences between N1 and N2 in order to fully develop their differential roles during pneumococcal lung infection in mice and humans.

Nowadays, the emergence of this “new” ability of neutrophils to change its phenotype and play different roles, raises a new scenario in pneumonia, where the aim of their recruitment into the lungs goes further than killing, and in turn, could become in a strategy for a rapid immune modulation, resulting in the decrease of tissue damage. However, it is relevant to remark that the impact of IL-10 producing neutrophils is different among the broad pathogens able to cause pneumonia. The literature shows that although neutrophils produce IL-10 in viral pneumonia, tuberculosis, and pneumonia caused by *K. pneumoniae*, other cells like macrophages and MDSCs, are in charge of the establishment of the anti-inflammatory response in these diseases (Bouabe et al., 2011; Sun et al., 2011; Peñaloza et al., 2019). This fact contrasts with our findings and makes us presume that the role of neutrophils as the dominant source of IL-10 production may be a hallmark of pneumonia caused by *S. pneumoniae*. Though, to validate this assumption is necessary to verify that recognition of pneumococcal PAMPs is mandatory and enough to induce the expression of IL-10 in neutrophils. This hypothesis has been previously confirmed in macrophages and monocytes, were *S. pneumoniae* molecules such as PGN, LTA and lipoproteins directly induce the production of IL-10, mainly through TLR2 and TLR4 pathways (Moreira et al., 2008; Zhang et al., 2009; Nguyen et al., 2016; Balderramans et al., 2014). However, studies connecting IL-10-producing neutrophils with *S. pneumoniae* were not available. Therefore, one of the main goals of this thesis was to determine whether

IL-10 production in pneumonia comes from the recognition of pneumococcal patterns or whether host molecules are required to induce the regulatory phenotype in neutrophils.

To test this hypothesis, we performed *in vitro* assays to evaluate IL-10 expression in neutrophils by flow cytometry and confirmed our findings with ELISA. The stimulus used to induce IL-10 were heat-killed capsulated (D39) and non-capsulated (R6) *S. pneumoniae*, as well as BALF from infected mice. Results obtained by flow cytometry showed that a significant expression of IL-10 was only obtained in neutrophils stimulated with D39 heat-killed strain. Similarly, ELISA assays showed that IL-10 was only found in wells bearing neutrophils stimulated with heat-killed *S. pneumoniae* and, in contrast, BALFs from infected mice did not induced a significant increase in IL-10 expression. According to these results the first conclusion is that molecules present in S*. pneumoniae* are enough to generate significant levels of IL-10 in neutrophils, and that host molecules present in BALF are not involved in this cytokine production in neutrophils. Interestingly, we also observed that the non-capsulated strain failed to induce a significant IL-10 production. In fact, the increase of R6 MOI, affected negatively the IL-10 levels observed in neutrophils supernatants. These last findings could be explained by one of the following points: 1) the non-capsulated strain has several surface proteins exposed over the cell wall and this could be inducing the synergic activation of transcriptional pathways that privilege the establishment of a pro-inflammatory profile. On the contrary, the capsulated strain keeps hidden most of those molecules, allowing a differential exposition of bacterial PAMPs to neutrophils and favoring the activation of the transcriptional

pathways involved in IL-10 production. 2) The second fact is that polysaccharides (present in the capsule) could be the factor triggering the expression of IL-10 genes in neutrophils. This last assumption was the reason to include the R6 strain in the assays, because we wanted to confirm or discard the capsule participation in induction of IL-10 in neutrophils. Thus, according to our results we could postulate capsule as potent IL-10 inductor and this could be supported by previous findings where the polysaccharide A from *Bacteroides fragilis* capsule, induced IL-10 production in T regulatory cells (Johnson et al., 2018; Ramakrishna et al., 2019).

On the other hand, regarding to the pathways involved in IL-10 production, the literature has shown that TLR2, TLR4 and TLR9 receptors have a pivotal role in recognition of stimulus that induce this cytokine production, particularly in macrophages, B cells and dendritic cells (Moreira et al., 2008; Bamboat et al., 2010; Polari et al., 2019). Therefore, to evaluate which of these receptors are implicated in IL-10 production during *S. pneumoniae* infections, we performed additional *in vitro* assays adding DNA extracted from *S. pneumoniae* or cell wall extract (peptidoglycan) to neutrophils. Results from assays with pneumococcal DNA showed no IL-10 production in neutrophils supernatants (data not shown), indicating that TLR9 is not involved in IL-10 induced by *S. pneumoniae*. Similarly, low or null levels of IL-10 were found in supernatants of neutrophils stimulated with peptidoglycan at 24h post-culture. This last result indicates that the peptidoglycan is not involved in IL-10 production by *S. pneumoniae*, however it does not exclude the TLR2 participation.

TLR2 is a versatile receptor with the ability to form heterodimers with TLR2 and TLR6, which allows it to recognize multiple molecules and therefore activate different pathways (Li et al., 2013). Given the strong association of TLR2 - PI3Ks-Akt signaling pathway to IL-10 production, together with the fact that Gram-positive bacterial PAMPs are strong activators of this pathway, it is possible to consider that other pneumococcal molecules, such as lipoproteins and lipoteichoic acids, could be involved in the induction of the regulatory profile in neutrophils (Santos-Sierra et al., 2009; Fallah et al., 2011). In agreement with this, further studies are required to find the specific bacterial components that induce the IL-10 levels observed *in vitro,* as well as to confirm the role of capsular polysaccharides in the induction of IL-10 in neutrophils. Moreover, in this study we did not executed particular assays to evaluate the role of the TRL4 in IL-10 production. In consequence, more experiments to elucidate the signaling pathways involved in IL-10 production are required. We propose to perform *in vitro* experiments by using TLR2 and TLR4 inhibitors to find out which receptor is specifically involved in IL-10 production, or if a synergistic action of both receptors is required to induce the regulatory profile in neutrophils. Further, future *in vivo* experiments could also be performed, where different bacterial components would be administered intranasally in IL-10GFP mice to determine which of them is inducing similar IL-10 levels as those observed in neutrophils during a typical pneumococcal infection. Importantly, although with our experiments we did not obtain enough information to disclose the pathway involved in IL-10 production by neutrophils, we emphasize that in this work we are providing strong evidence about a direct induction of IL-10 mediated by *S. pneumoniae* PAMPs recognition, and our results open the discussion about whether this induction of IL-10 production

in neutrophils is one of the several strategies of *S. pneumoniae* to neutralize the immune response. This last assumption is supported by several studies indicating that pathogens use their virulence factors to downregulate the immune response through the induction of IL-10 production (Peñaloza et al., 2016; Schwartz et al., 2017). In fact, there is a recent study showing that a *S. pneumoniae* protein called Spr1875 induces IL-10 production in NK cells at 72hpi and this event is considered an immune evasion mechanism (Clark et al., 2020).

Confirmed the fact that neutrophils produce IL-10 in response to *S. pneumoniae*, the next goal of this work was to disclose whether these "regulatory neutrophils" have an impact during the progression of pneumococcal pneumonia. To achieve this goal, adoptive neutrophil transfer from WT mice to IL-10$^{-/-}$ mice was proposed to determine the impact of neutrophils able to produce IL-10 in a murine model that lacks this cytokine and, in consequence, represents a highly susceptible model to high loads of *S. pneumoniae* due to their disability to control inflammation and lung damage (Peñaloza et al., 2015; Clark et al., 2020). At first, mice survival was assessed and results showed that transferred neutrophils improved IL-10$^{-/-}$ mice survival, independently of their ability to produce IL-10. This result reflects the unlikely milieu proportioned by our experimental design, where mice had more neutrophils than they should before infection, which induced a rapid and more efficient bacterial killing and therefore reduced the damage associated with bacterial invasion. On the other hand, while WT mice showed a 100% of survival after 10 days of infection, WT transferred mice were more susceptible to pneumococcal infection. An explanation for this is the amplification of the IL-10 response obtained after the

addition an extra source of IL-10 to IL-10-competent mice. This assumption was confirmed by the increased levels of IL-10 observed in BALFs from the WT/WT mice at 48hpi (Fig. 5E). This finding suggests that the unusual increased IL-10 concentrations during lung infection could be detrimental for the host because it is related to the impairment of bacterial clearance (Peñaloza et al., 2016). Subsequently, in order to determine whether WT neutrophil transfer had an impact in the ability of the immune system to control bacterial growth, we evaluated bacterial loads at a local and systemic level. These results showed that WT mice transferred with WT neutrophils had a slight increase in bacterial loads in spleen and brain as compared to the non-transferred counterpart. This increase was not significant, indicating that minimal changes in bacterial loads in sterile organs like the spleen, can break the balance required to guarantee WT mice survival (Ercolli et al., 2018). Further, bacterial loads in lungs of IL-$10^{-/-}$ mice transferred with WT neutrophils, were significantly higher than bacterial loads found in lungs in the other IL-$10^{-/-}$ groups. These results were attributed to the IL-10 proportioned by WT neutrophils transference and showed a clear effect of this cytokine in the impairment of the immune response against the pathogen.

Neutrophil transfer assays also allowed us to elucidate how neutrophils might be influencing disease outcome. Considering this question, we evaluated lung infiltration (neutrophils and monocytes) and tissue damage (H&E staining). Results regarding lung infiltration were inconclusive because we found no significant differences in neutrophils or monocytes numbers among the experimental groups. In contrast, H&E analyses allowed to observe remarkable differences between the

histopathological score of IL-10$^{-/-}$ mice transferred with WT neutrophils, when compared to the other IL-10$^{-/-}$ experimental groups. In summary, the entire observations in this part of the study indicate that although neutrophils adoptive transfer did not have a clear effect in lung inflammation, IL-10 coming from neutrophils showed to had an important effect on bacterial growth and low tissue injury. Here becomes evident the widely described dual role of IL-10 in infections (Peñaloza et al., 2016). In summary, results obtained in this part of the study indicate that neutrophils expressing IL-10 could be determinant in the outcome of a pneumococcal infection, both at a local and systemic level. Now, this conclusion seems to be difficult to contrast with the role of neutrophils in pneumonia in humans, due to the scarce clinical studies mentioning IL-10-producing neutrophils. However, according to the study performed by Cortjens and colleagues, which describes the existence of a regulatory neutrophil subtype associated to bacterial pathogens, we propose that eventually our results could be extrapolated to humans, or at least establish a precedent in the characterization of neutrophils populations in lungs (Cortjens et al., 2017).

To get more comprehensive information about the role of each neutrophil subtype in pneumonia, the validation of our results against isolated neutrophils from people with pneumonia is required. To do this, we propose the development of studies that must include functional *in vitro* assays, as well as a complete proteomic and transcriptional profile of neutrophil subpopulations present in the infected lungs of both species. Results from these studies should provide a better knowledge of pneumonia pathology, increasing the probability of controlling the adverse effects of

the immune response, through therapies directed to regulate neutrophil's behavior. Additionally, another advantage of characterizing neutrophil's subpopulations, is the possibility of establishing the presence of regulatory neutrophils as biomarkers of infection, as it has been proposed for trauma-related inflammatory processes (Mortaz et al., 2019). In this way, a complete characterization of neutrophils would not only contribute to pneumonia treatment but also will allow to determine disease severity and orientate diagnostic towards a bacterial etiology (Cortjens et al., 2017).

6. CONCLUSIONS

Data obtained during the development of this thesis project, allow us to determine that neutrophils are important cells acting in the establishment of a regulatory response during the first 48hpi in a pneumococcal pneumonia disease. Also, we could characterize neutrophils population present in lungs during the first 48hpi and establish that *in vitro*, IL-10 production is exclusively induced by pneumococcal PAMPs. Finally, we demonstrated that neutrophils able to produce IL-10 have the potential of participate in the modulation of the immune response in a murine model, avoiding excessive lung damage in pneumococcal infection. Further is also relevant to mention that results obtained from WT/WT mice allowed to confirm that irregular levels of IL-10 can induce undesirable effects and that this could be strongly influenced by the magnitude of neutrophils recruitment to lungs in each individual. In conclusion, all the data showed in this study constitute the result of a detailed analysis of one of the many abilities of neutrophils, showing the impact of their plasticity and their relevance as modulators of the immune response in pneumonia caused by *S. pneumoniae.* A limitation of this study is the lack of functional and transcriptional assays to demonstrate the specific role of each neutrophil subtype, as well as complementary data regarding specific molecules inducing the regulatory profile in neutrophils. Though, we stand out the fact that our findings contribute to the actual knowledge about neutrophils describing the basal presence of two populations of neutrophils in mice lungs that change their proportions and IL-10 expression exclusively in response to pneumococcal stimulus and that as important source of an anti-inflammatory cytokine, have a significant role in the outcome of the

pathological process (**Fig. 8**). Moreover, the implementation of *in vitro* and *in vivo* assays aimed to clear up the unrecognized role of IL-10-producing neutrophils in pneumococcal pneumonia with results that could be generalized to pneumonia in humans. Our results, together with the complementary analyses suggested above, could provide orientation about management of patients and the potential development of treatment strategies based on combined therapies including immune-modulation directed to control neutrophils recruitment and/or function during the critical first 48hpi.

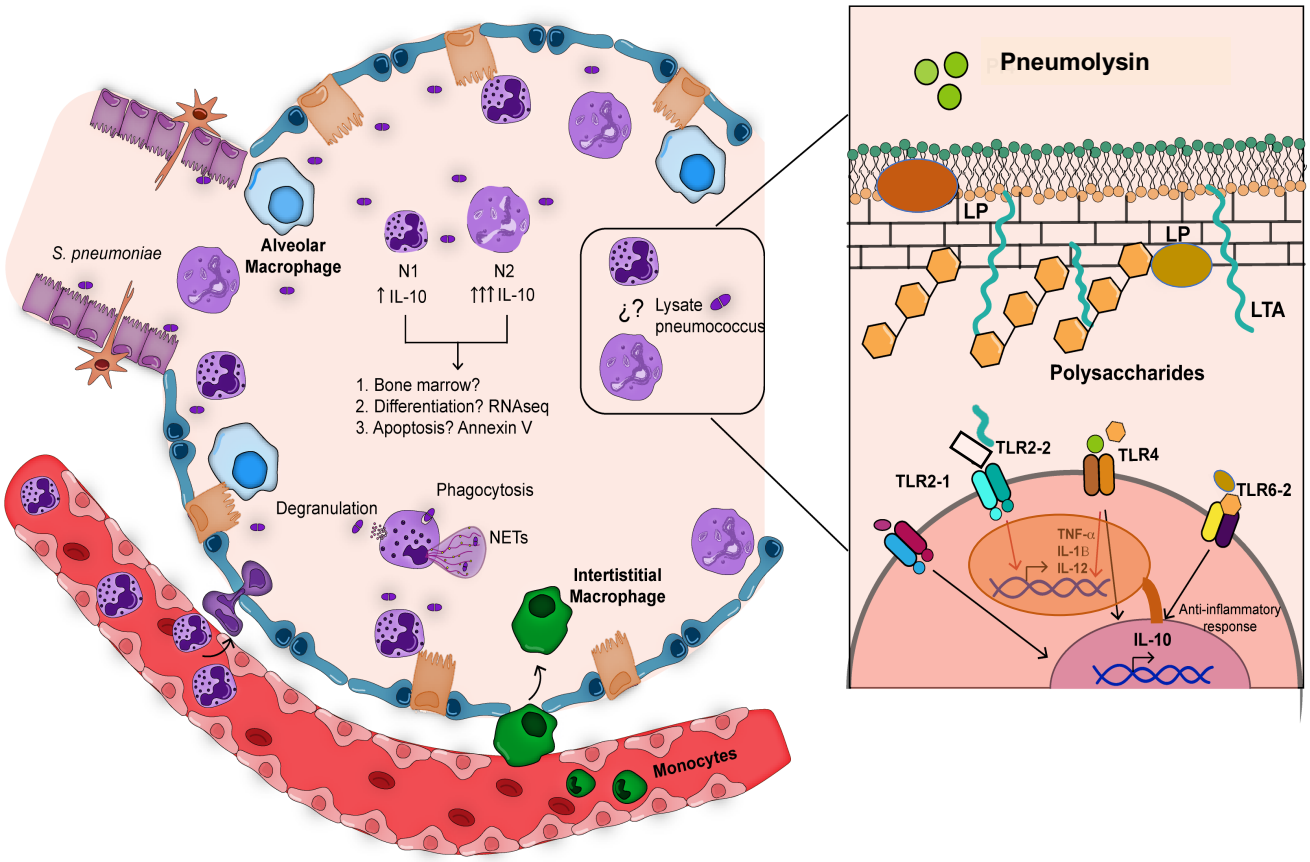

Figure 8. Neutrophils populations observed in pneumonia and their regulatory phenotype displayed in response to *S. pneumoniae* antigens. (Proposed model). Neutrophils are recruited to the lungs in response to *S. pneumoniae* infection to counteract the threat by exerting their effector functions. Additionally, one fifth of neutrophils produce IL-10 and through the release of this cytokine, neutrophils

contribute to the modulation of the immune response. Further, two neutrophil subtypes with significant differences in their size, granularity, chromatin organization, vacuoles are observed in lungs during bacterial pneumonia. The differences observed between the two populations suggest they are playing different roles, however further analyses are required to confirm or discard the theory that they are different cells or to determine whether N2 is a maturation state of N1. Additionally, although both subtypes of neutrophils produce IL-10, there is a marked difference between these two populations regarding their ability to produce IL-10, where N2 population seems to be contributing to the increased levels observed of this cytokine at 48hpi. This IL-10 production in neutrophils is related to the recognition of pneumococcal PAMPs like polysaccharides present in the capsule, or the lipoproteins anchored to the cell wall. This assumption is based on our results which showed that the capsulated *S. pneumoniae* induced IL-10 production in neutrophils directly, indicating that polysaccharides or the proteins exposed in bacterial surface, might be recognized by TLRs previously described to have an important role in the activation of the pathways required to start an anti-inflammatory response. Recognition of pneumococcal PAMPs and the subsequent IL-10 production, suggest that the pneumococcus is possibly inducing this regulatory response in neutrophils as a mechanism of immune evasion.

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7. APPENDIX

7.1 SCIENTIFIC MEETINGS ATTENDED DURING THIS THESIS.

7.1.1 Liliana González, Loreani Noguera, Valentina Sebastián, Isidora Suazo, Bárbara Schultz, Omar Vallejos, Susan Bueno. IL-10-producing neutrophils: the unrecognized immune modulators in the *Streptococcus pneumoniae* lung infection. **8th Congress of European Microbiologists – FEMS 2019.** Glasgow, Scotland. 2019

7.1.2 Liliana González, Gerladyne Salazar, Hernán Peñaloza, Susan Bueno. *Streptococcus pneumoniae* recognition by neutrophils stimulates IL-10 production during pneumococcal pneumonia. **XXIV Congreso Latinoamericano de Microbiología – ALAM 2018.** Santiago, Chile. 2018

7.1.3 Liliana González, Yaneisi Vázquez, Jorge Mora, Christian Palavecino, Pablo Bertrand, Marcela Ferrés, Ana María Contreras, Andrea Beckhaus, Claudia Riedel, Alexis Kalergis, Susan Bueno. "Caracterización y evaluación de nuevos anticuerpos monoclonales para la detección de virus respiratorios en muestras clínicas". **IV Jornada de Investigación - Division of pediatrics.** Santiago, Chile. 2016.

7.2 SCIENTIFIC PUBLICATIONS GENERATED DURING THIS THESIS

7.2.1 González, L.A., Sebastián, V.P., Vallejos, O.P., Noguera, L.P., Melo-González, F., Suazo, I.C., Soto, J.A. Manosalva, A.H., Parker, D., Riedel, C.A., González, P.A., Kalergis, A.M., and Bueno, S.M. Characterization of the immune modulatory capacity of IL-10-producing neutrophils in Streptococcus pneumoniae lung infection. *Journal of Immunology* (submitted).

7.2.2 Peñaloza, H., González, L.A., Ahn, D., and Bueno, S.M., 2019. L-arginine enhances intracellular killing of carbapenem resistant *Klebsiella pneumoniae* ST258 by murine neutrophils. *Frontiers Cellular and Infection Microbiology* (submitted).

7.2.3 Vázquez, Y., González, L.A., Noguera, L., González, P.A., Riedel, C.A., Bertrand, P. and Bueno, S.M., 2019. Cytokines in the Respiratory Airway as Biomarkers of Severity and Prognosis for Respiratory Syncytial Virus Infection: An Update. *Frontiers in Immunology*, 10:1154.

7.2.4 Peñaloza, H., Noguera, L., Ahn, D., Vallejos O.P., Castellanos R.M., Vázquez, Y., Salazar-Echegarai, F.J., González, L.A., Suazo, I., Pardo-Roa, C., Salazar, G., and Bueno, S.M., 2019. Interleukin-10 Produced by Myeloid-Derived Suppressor Cells Provides Protection to Carbapenem-Resistant *Klebsiella pneumoniae* Sequence Type 258 by

Enhancing Its Clearance in the Airways. *Infection and Immunity*, 254, pp.51-64.

7.2.5 González, L.A., Vázquez, Y., Mora, J.E., Palavecino, C.E., Bertrand, P., Ferrés, M., Contreras, A.M., Beckhaus, A.A., Riedel, C.A. and Bueno, S.M., 2018. Evaluation of monoclonal antibodies that detect conserved proteins from Respiratory Syncytial Virus, Metapneumovirus and Adenovirus in human samples. *Journal of Virological Methods*, 254, pp.51-64.

7.3 SCIENTIFIC ARTICLES PUBLISHED DURING THIS THESIS

AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity®

HOST RESPONSE AND INFLAMMATION

Interleukin-10 Produced by Myeloid-Derived Suppressor Cells Provides Protection to Carbapenem-Resistant *Klebsiella pneumoniae* Sequence Type 258 by Enhancing Its Clearance in the Airways

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ABSTRACT Carbapenem-resistant *Klebsiella pneumoniae* sequence type 258 (CRKP-ST258) can cause chronic infections in lungs and airways, with repeated episodes of bacteremia. In this report we addressed whether the recruitment of myeloid cells producing the anti-inflammatory cytokine interleukin-10 (IL-10) modulates the clearance of CKRP-ST258 in the lungs and establishes bacterial persistence. Our data demonstrate that during pneumonia caused by a clinical isolate of CRKP-ST258 (KP35) there is an early recruitment of monocyte-myeloid-derived suppressor cells (M-MDSCs) and neutrophils that actively produce IL-10. However, M-MDSCs were the cells that sustained the production of IL-10 over the time of infection evaluated. Using mice unable to produce IL-10 (IL-10$^{-/-}$), we observed that the production of this cytokine during the infection caused by KP35 is important to control bacterial burden, to prevent lung damage, to modulate cytokine production, and to improve host survival. Importantly, intranasal transfer of bone marrow-derived M-MDSCs from mice able to produce IL-10 at 1 day prior to infection improved the ability of IL-10$^{-/-}$ mice to clear KP35 in the lungs, decreasing their mortality. Altogether, our data demonstrate that IL-10 produced by M-MDSCs is required for bacterial clearance, reduction of lung tissue damage, and host survival during KP35 pneumonia.

KEYWORDS interleukin-10, *Klebsiella pneumoniae* ST258, monocytic-myeloid-derived suppressor cells, neutrophils

One major cause of pneumonia in health care facilities worldwide is carbapenem-resistant *Klebsiella pneumoniae* sequence type 258 (CRKP-ST258). CRKP-ST258 strains encode KPC β-lactamases, which makes these bacteria resistant to different β-lactams, including carbapenems (1, 2). CRKP-ST258 isolates are the etiologic agent of different diseases, including pneumonia, sepsis, and urinary tract infections (3). CRKP-ST258 pneumonia in health care facilities is associated with high mortality rates (50%) although in some cases these bacteria can also establish chronic infections with frequent episodes of sepsis (4).

Similar to the well-studied laboratory reference strain *K. pneumoniae* ATCC 43816 (KPPR1), CRKP-ST258 expresses the typical pathogen-associated molecular patterns (PAMPs) of Gram-negative bacteria (3) that potently activate proinflammatory signaling on epithelial and immune cells through Toll-like receptor 4 (TLR4)/MyD88/NF-κB (4).

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Cytokines in the Respiratory Airway as Biomarkers of Severity and Prognosis for Respiratory Syncytial Virus Infection: An Update

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The human respiratory syncytial virus (hRSV) is one of the most important causes of upper and lower respiratory tract infections in children and the main cause of bronchiolitis worldwide. Disease manifestations caused by hRSV may vary from mild to severe, occasionally requiring admission and hospitalization in intensive care units. Despite the high morbidity rates associated to bronchiolitis, treatment options against hRSV are limited and there are no current vaccination strategies to prevent infection. Importantly, the early identification of high-risk patients can help improve disease management and prevent complications associated with hRSV infection. Recently, the characterization of pro- and anti-inflammatory cytokine patterns produced during hRSV-related inflammatory processes has allowed the identification of potential prognosis biomarkers. A suitable biomarker should allow predicting the severity of the infection in a simple and opportune manner and should ideally be obtained from non-invasive samples. Among the cytokines associated with hRSV disease severity, IL-8, interferon-alpha (IFN-alpha), and IL-6, as well as the Th2-type cytokines thymic stromal lymphopoietin (TSLP), IL-3, and IL-33 have been highlighted as molecules with prognostic value in hRSV infections. In this review, we discuss current studies that describe molecules produced by patients during hRSV infection and their potential as biomarkers to anticipate the severity of the disease caused by this virus.

Keywords: biomarker, cytokines, LRTI, hRSV, severity, prognosis

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INTRODUCTION

The human respiratory syncytial virus (hRSV) is a viral agent predominantly involved in acute lower respiratory tract infections (LRTIs), frequently associated to bronchiolitis and pneumonia in children and infants (1, 2). HRSV is responsible for approximately 60% of all LRTIs in children under 5 years old and causes more than 80% of the reported cases in infants (3, 4). At the age of 2 years, almost all children have been infected with hRSV at least once, and disease severity among these children may vary from mild to severe manifestations, sometimes requiring hospitalization with oxygen administration or admission into intensive care units (5, 6). Moreover, hRSV infection may cause exacerbated airway

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Evaluation of monoclonal antibodies that detect conserved proteins from Respiratory Syncytial Virus, Metapneumovirus and Adenovirus in human samples

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ABSTRACT

Human Respiratory Syncytial Virus (hRSV), human Metapneumovirus (hMPV) and Adenovirus (ADV), are three of the most prevalent viruses responsible for pneumonia and bronchiolitis in children and elderly worldwide, accounting for a high number of hospitalizations annually. Diagnosis of these viruses is required to take clinical actions that allow an appropriate patient management. Thereby, new strategies to design fast diagnostic methods are highly required. In the present work, six monoclonal antibodies (mAbs, two for each virus) specific for conserved proteins from hRSV, hMPV and ADV were generated and evaluated through different immunological techniques, based on detection of purified protein, viral particles and human samples. In vitro evaluation of these antibodies showed higher specificity and sensitivity than commercial antibodies tested in this study. These antibodies were used to design a sandwich ELISA tests that allowed the detection of hRSV, hMPV, and ADV in human nasopharyngeal swabs. We observed that hRSV and ADV were detected with sensitivity and specificity equivalent to a current Direct Fluorescence Assay (DFA) methodology. However, hMPV was detected with more sensitivity than DFA. Our data suggest that these new mAbs can efficiently identify infected samples and discriminate from patients infected with other respiratory pathogens.

1. Introduction

Respiratory tract infections are defined as the set of infections that affect the airways, including the oropharinx, bronchi and lungs (Antibiotic Expert Group, 2010). These infections are globally considered as a serious public health problem affecting all age people; however higher morbidity and mortality rates are more common in children, particularly when they evolve to its more severe manifestations: bronchiolitis and pneumonia (Lozano et al., 2012).

Pneumonia is the leading cause of mortality in children worldwide, even more than diarrhea, malaria and acquired immune deficiency syndrome together. Pneumonia accounts for about 17% of deaths of children under four years old (UNICEF Committing to Child Survival, 2013; Kahn, 2006). Laboratory diagnosis to identify the etiological agent causing a respiratory tract disease is applied to less than half infected patients and this problem becomes more complex in cases of lower respiratory tract infections in children (Ruiz et al., 1999; Zambon et al., 2001; Ampofo et al., 2008). Low respiratory tract damage may result from direct viral agent infection in children and adults, acting as a predisposition factor to bacterial pneumonia (Levin et al., 2010;

Abbreviations: hRSV, Human Respiratory Syncytial Virus; hMPV, human Metapneumovirus; ADV, Adenovirus; mAbs, monoclonal antibodies; ELISA, Enzime-Linked Immunosorbent Assay; DFA, direct immunofluorescence; RT-PCR, Reverse Transcriptase Polymerase Chain Reaction; IPTG, Isopropyl-β-D-1-thiogalactopyranoside

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