UNIVERSITY OF SAO PAULO
RIBEIRAO PRETO MEDICAL SCHOOL
BASIC AND APPLIED IMMUNOLOGY PROGRAM

Mèdéton Mahoussi Michaël Boko

Role of S100A9 in the comorbidity asthma and acute pneumonia

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Thesis presented to Ribeirao Preto Medical School, University of Sao Paulo for the degree of PhD in Sciences

Area of concentration: Basic and Applied Immunology

Advisor : Prof. Dr. Vânia Luiza Deperon Bonato, PhD

Co-advisor : Dr. Thais Fernanda de Campos Fraga da Silva, PhD

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DEDICATIONS

To

Almighty **God**

My father **Ignace BOKO**

My mother **Suzanne HOUNSA**

My sisters **Rachelle and Janvienne BOKO**

My wife **Marcelline HOUNMENOU**

My children **Maël and Marion BOKO**

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Boko MMM, 2023.

O mais importante da vida não é a situação em que estamos, mas a direção para a qual nos movemos.

RESUMO

BOKO, M. M. M. Papel da S100A9 na comorbidade asma e pneumonia aguda. 2023. Tese (Doutorado) – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2023.

A pneumonia causada por *Streptococcus pneumoniae* pode melhorar ou exacerbar a asma. A exacerbação na comorbidade asma e pneumonia pode ser decorrente da indução de inflamação neutrofílica, que é caracterizada como asma mais grave e de difícil tratamento. S100A9 é uma alarmina que atua como sinal de perigo endógeno e promove o recrutamento de neutrófilos. Essa alarmina está envolvida na imunopatologia de diferentes doenças que afetam os pulmões, como a asma e a pneumonia.

Nossa hipótese é de que a pneumonia aguda induzida por *S. pneumoniae* exacerba a inflamação pulmonar na asma por meio da indução de S100A9, que contribui para a inflamação neutrofílica e a produção de armadilhas extracelulares de neutrófilos (NET). Camundongos C57BL/6 Wild Type (WT) e deficientes para a expressão de S100A9 ($S100A9^{-/-}$) foram sensibilizados e desafiados com ovalbumina e infectados ou não com *S. pneumoniae* durante o desafio. Em camundongos WT, a infecção pneumocócica concomitante à exposição ao alérgeno resultou em aumento significativo no recrutamento de neutrófilos no fluido do lavado broncoalveolar (BALF), acompanhado por aumento do infiltrado de leucócitos perivasculares e peribronquiais, secreção de S100A9 e produção de NET nos pulmões em comparação com os camundongos WT expostos apenas ao alérgeno. Durante a comorbidade, camundongos $S100A9^{-/-}$ exibiram redução significativa nas concentrações de CXCL1, uma quimiocina que atrai neutrófilos, e do influxo de neutrófilos no BALF. Além disso, durante a comorbidade, camundongos $S100A9^{-/-}$ apresentaram aumento na morte de neutrófilos. A intervenção farmacológica com tasquinimod ou azeliragon, inibidores da sinalização S100A9, ou com BB Cl-amidina, um inibidor de NET, protegeu os camundongos da inflamação neutrofílica e da produção de NET durante a comorbidade asma e pneumonia aguda. Nossos resultados forneceram evidências de que S100A9 e NET podem ser alvos terapêuticos na asma grave que cursa com inflamação neutrofílica decorrente de pneumonia.

Palavras-chave: Asma experimental, pneumonia pneumocócica aguda, neutrófilos, S100A9, NET.

ABSTRACT

BOKO, M. M. M. Role of S100A9 in the comorbidity asthma and acute pneumonia. 2023. Thesis (Doctorate) – Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto, 2023.

Pneumonia caused by *Streptococcus pneumoniae* can improve or exacerbate asthma. The exacerbation of asthma and pneumonia comorbidity may be due to the induction of neutrophilic inflammation, which is characterized as more severe asthma and difficult-to-treat. S100A9 is an alarmin that acts as an endogenous danger signal and promotes neutrophil recruitment. This alarmin is involved in the immunopathology of different diseases that affect the lungs, such as asthma and pneumonia.

We hypothesize that acute pneumonia induced by *S. pneumoniae* exacerbates lung inflammation in asthma through the induction of S100A9, which contributes to neutrophilic inflammation and the production of Neutrophil Extracellular Traps (NET). C57BL/6 Wild Type (WT) animals and mice deficient for the expression of S100A9 ($S100A9^{-/-}$) were sensitized and challenged with ovalbumin (OVA) and infected or not with *S. pneumoniae* during the challenge. In WT mice, pneumococcal infection concomitant with allergen exposure resulted in a significant increase in neutrophil recruitment in the bronchoalveolar lavage fluid (BALF), accompanied by increased perivascular and peribronchial leukocyte infiltrate, S100A9 secretion, and NET production in the lungs compared to WT mice exposed only to the allergen. During the comorbidity, S100A9 deficient mice exhibited a significant reduction in the concentrations of CXCL1, a chemokine that attracts neutrophils, and in BALF neutrophil influx. Furthermore, during the comorbidity, S100A9 deficient mice showed increased neutrophil death. Pharmacological intervention with tasquinimod or azeliragon, inhibitors of S100A9 signaling, or BB Cl-amidine, a NET inhibitor, protected mice from neutrophilic inflammation and NET production during the comorbidity of asthma and acute pneumonia. Our results provided evidence that S100A9 and NET can be therapeutic targets in severe asthma that course with neutrophilic inflammation resulting from pneumonia.

Key words: Experimental asthma, acute pneumococcal pneumonia, neutrophils, S100A9, NET.

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ABBREVIATIONS

ACK	:	Ammonium-Chloride-Potassium
AEC	:	Airway epithelial cells
AHR	:	Airway hyperreactivity
AIM2	:	Absent in melanoma 2
ATP	:	Adenosine triphosphate
BALF	:	Bronchoalveolar lavage fluid
BCG	:	Bacillus Calmette–Guérin
CAA	:	Complete *Aspergillus* Allergen
CCL2	:	C-C Motif Chemokine Ligand 2
CCL20	:	C-C Motif Chemokine Ligand 20
CFA	:	Complete Freund Adjuvant
CFU	:	Colony-Forming Unit
CO_2	:	Carbon dioxide
COPD	:	Chronic obstructive pulmonary disease
COVID-19	:	Coronavirus disease 2019
CXCL1/2	:	C-X-C motif chemokine ligand 1/2
CXCL10	:	C-X-C motif chemokine ligand 10
CXCL8	:	C-X-C motif chemokine ligand 8
CXCR2	:	CXC chemokine receptor 2
DAMP	:	Damage-associated molecular pattern
DAPI	:	4',6-diamidino-2-phénylindole
DC	:	Dendritic cells
DNA	:	Deoxyribonucleic acid
ECP	:	Eosinophil cationic protein
EDN	:	Eosinophil-derived neurotoxin:
EDTA	:	Ethylenediaminetetraacetic acid
ELISA	:	Enzyme-linked immunosorbent assay
EPO	:	Eosinophil peroxidase
FACS	:	Fluorescence-activated Cell Sorting
FcεRI	:	High affinity receptor for immunoglobulin E
FLAP	:	5- lipoxygenase-activating protein
GM-CSF	:	Granulocyte macrophage-colony stimulating factor
H&E	:	Hematoxylin and Eosin
H_2O_2	:	Hydrogen peroxide

H3Cit	:	Citrullinated Histone
HDM	:	House Dust Mite
IFN-γ	:	Interferon-gamma
IgE	:	Immunoglobulin E
IgG	:	Immunoglobulin G
IL-1β	:	Interleukin-1 beta
IL-10	:	Interleukin 10
IL-13	:	Interleukin 13
IL-17	:	Interleukin 17
IL-22	:	Interleukin 22
IL-23	:	Interleukin 23
IL-25	:	Interleukin 25
IL-33	:	Interleukin 33
IL-4	:	Interleukin 4
IL-5	:	Interleukin 5
IL-6	:	Interleukin 6
IL-8	:	Interleukin 8
IL-9	:	Interleukin 9
ILC2	:	Innate lymphoid cells 2
ILC3	:	Innate lymphoid cells 3
IRF3	:	Interferon regulatory factor 3
IRF3/7	:	Interferon regulatory factor 3/7
LPA	:	Lysophosphatidic acid
LPS	:	Lipopolysaccharide
mAb	:	Monoclonal antibody
Mac-1	:	Macrophage-1 antigen
MBP	:	Major basic protein
MFI	:	Mean fluorescence intensity
MMP9	:	Matrix metallopeptidase 9
MOI	:	Multiplicity of infection
MPO	:	Myeloperoxidase
MRP14	:	Myeloid-Related Protein-14
MRP8	:	Myeloid-Related Protein-8
MyD88	:	Myeloid differentiation primary response 88
NET	:	Neutrphil Extracellular Traps

NF-kB	:	Nuclear factor kappa B
NLR	:	NOD-like receptor
NLRP3	:	NLR family pyrin domain containing 3
OD	:	Optic Density
OVA	:	Ovalbumin
PAD4	:	Protein arginine deiminase 4
PAMP	:	Pathogens Associated Molecular Pattern
PBS	:	Phosphate Buffered Saline
PDE4	:	Phosphodiesterase 4
PLY	:	Exotoxin Pneumolysin
PMA	:	Phorbol myristate acetate
PPARγ	:	Proliferator-Activated Receptor γ
PRR	:	Pattern Recognition Receptors
RAGE	:	Receptor for Advanced Glycation End products
ROS	:	Reative Oxygen Species
RT	:	Room Temperature
S100A8	:	S100 calcium binding protein A8
S100A9	:	S100 calcium binding protein A9
Sp	:	*Streptococcus pneumoniae*
STING	:	Stimulator of interferon genes
TBS-T	:	Tris-buffered saline with Tween
TGF-β	:	Transforming growth factor-beta
Th1	:	Type 1 T helper
Th17	:	Type 17 T helper
Th2	:	Type 2 T helper
Th9	:	Type 9 T helper
TLR2	:	Toll-like receptor 2
TLR4	:	Toll-like receptor 4
TLR9	:	Toll-like receptor 9
TNF	:	Tumor necrosis factor
Treg	:	Regulatory T Cells
TSB	:	Tryptone soy broth
TSLP	:	Thymic stromal lymphopoietin
WHO	:	World Health Organization
WT	:	Wild type

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1. Introduction

1.1. Asthma

Asthma is a non-communicable disease that affects the lower airways (Global Initiative for Asthma, 2022). As a complex and heterogeneous disease, asthma is characterized by chronic inflammation, reversible airway obstruction, airway hyperreactivity (AHR) and tissue remodeling. The symptoms of the disease are coughing, wheezing, shortness of breath, and chest tightness (Network, 2022). Asthma affects people of all ages. The number of cases reported is approximately 300 millions people worldwide (Global Initiative for Asthma, 2022). The deaths related to asthma are higher in those low-income countries compared to high-income countries (Network, 2022). Asthma is more prevalent in boys than in girls. However, in adults, women are more affected than men. Despite the higher incidence and prevalence of asthma observed in children, high mortality and morbidity ratios are observed in adults (Dharmage et al., 2019).

Several risk factors are associated to asthma. Genetic predisposition and environmental factors are critical in the increase of the global incidence of asthma. The genetic factor associated to asthma is related to family history (Burke et al., 2003; Liu et al., 2009). Indeed, if an individual has a family history of asthma, he is more likely to develop asthma (Burke et al., 2003; Liu et al., 2009). Likewise, asthma status is more likely to be the same in identical twins than non-identical (Global Asthma Network, 2018).

The environmental factors associated with asthma are indoor and outdoor allergens, tobacco smoke, chemical irritants, air pollution, climate change and infections (Dharmage et al., 2019; WHO, 2022). Anger or fear, stress, physical exercise and certain medicines also predispose to asthma. Of note, in addition to gender, race and age, diet and food allergy have also been found to be potential risk factors for the development of asthma (Alsharairi, 2019; Dharmage et al., 2019). Moreover, obesity is related to severe asthma (Sutherland, 2014).

Asthma phenotypes were described as allergic or eosinophilic, non-allergic or non-eosinophilic, or mixed granulocytic inflammation (Russell and Brightling, 2017). Asthma may be classified as Type 2 or non-Type 2 asthma ; the last one is characterized by difficult-to-treat asthma (Carr and Peters, 2022; Fraga-Silva et al., 2023).

Type 2 asthma is mediated by allergen-specific IgE synthesis, Th2 immune response and type 2 inflammation, which are the main readouts of the disease. Epithelial and dendritic cells play a critical role in generating Th2 immune response (Lambrecht and Hammad, 2012; Deckers et al., 2013; Vroman et al., 2015). A recent review published by our group highlights the immunopathology of asthma. The following **Figure 1** (Fraga-Silva et al., 2023) depicts the immune and inflammatory events that characterize allergic asthma as follows. Pattern Recognition Receptors (PRR), including TLR4 expressed on airway epithelial cells (AEC), recognize Pathogens Associated Molecular Patterns (PAMP) derived from the allergen and therefore induce the activation and recruitment of dendritic cells (DC) and innate lymphoid cells 2 (ILC2) through the secretion of IL-25, IL-33, TSLP (Thymic stromal lymphopoetin), GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor), IL-1, chemokine (C-C motif) ligand (CCL)2/CCL2 (Hammad et al., 2009; Nathan et al., 2009; Lambrecht and Hammad, 2012). Activated epithelial cells also produce danger signals such as ATP (Adenosine triphosphate), uric acid, LPA (Lysophosphatidic acid) and ROS (Reactive oxygen species). These alarmins participate to the activation of DC (Idzko et al., 2007; Ckless et al., 2011; Kool et al., 2011; Lambrecht and Hammad, 2012). In the very early phase of immune response, ILC2 secrete IL-5, IL-9, IL-13. Activated DC migrate to the mediastinal lymph node and prime naive T cells that differenciate into Th2 cells, which produce IL-4, IL-5 and IL-13. Mucus production is induced by IL-4 and IL-13. B cell activation is also induced by IL-4 and IL-13. Eosinophil recruitment is induced by IL-5 (Harper and Zeki, 2014; Lambrecht and Hammad, 2015). Activated B cells secrete IgE that binds on FcεRI expressed on mast cells. In subsequent contacts, allergen binds on FcεRI and triggers the release of pre-formed histamine, and production of leukotrienes, TNF, IL-9 and enzymes (Hall and Agrawal, 2014). MBP, ECP (Eosinophil cationic protein), EPO and EDN (Eosinophil-derived neurotoxin) produced by eosinophils induce AEC damage during asthma (Acharya and Ackerman, 2014). IL-9, produced by ILC2 and Th9 cells, was reported to be involved in the immunopathogenesis of asthma since allergic patients have higher circulating numbers of this cell population compared with nonallergic control subjects (Jones et al., 2012).

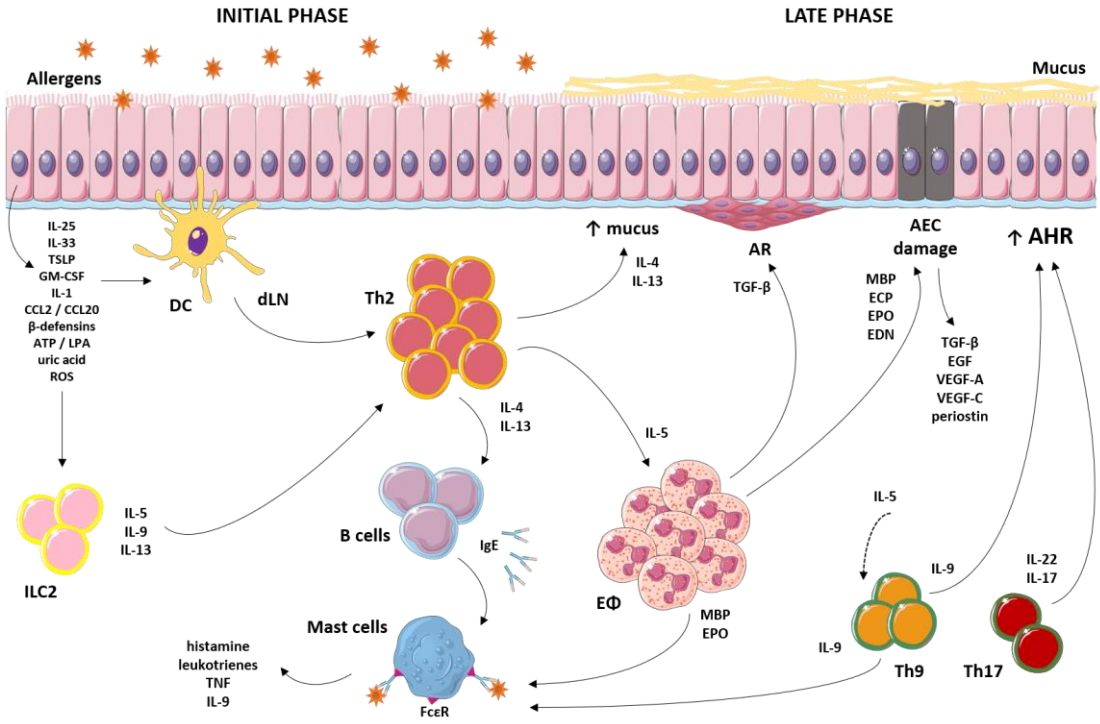


Figure 1 : Immunopathology of allergic asthma (Fraga-Silva et al., 2023).

Non-Type 2 asthma is described as difficult-to-treat asthma, generally refractory to treatment with corticosteroids. It is characterized by neutrophilic or mixed eosinophilic/neutrophilic inflammation and elevated IL-17 production in bronchoalveolar lavage fluid and lung biopsies (Molet et al., 2001; Chakir et al., 2003; Al-Ramli et al., 2009; Wilson et al., 2009; Vroman et al., 2015; Hammad and Lambrecht, 2021). Episodes of severe asthma generally are associated to non-type 2 asthma. The hallmark of severe asthma is the mixed recruitment of granulocytes including eosinopils and neutrophils. Our review, recently published, as depicted in the **Figure 2** (Fraga-Silva et al., 2023) also outlined the immunopathology of severe asthma. Exposure to allergens, diesel particles and infection with virus or bacteria activate AEC to produce CXCL8, CCL2 and CCL20 (Sozzani et al., 1997). These chemokines induce the recruitment and activation of DC that secrete IL-1β, TGF-β, IL-6 and IL-23 (Vroman et al., 2015a). These cytokines induce the recruitment of ILC3 and the differenciation of Th1, Th2 and Th17 lymphocytes. ILC3 and Th17 cells produce IL-17 that induces neutrophil recruitment. IFN-γ-producing Th1 cells also promote neutrophil recruitment (Harper and Zeki, 2014). Besides, Th17 cells affect the proliferation of airway smooth muscle cells, which contributes to AHR (Aujla and Alcorn, 2011; Vroman et al., 2015). Th17 cells might stimulate type II AEC (AEC-II)

to produce S100A9, CXCL1/2 and CXCL8 that induce neutrophil recruitment (Sonnenberg et al., 2011; Vroman et al., 2015). During neutrophilic inflammation in severe asthma, activated neutrophil may produce Neutrophil Extracellular Traps (NET) that might cause tissue damage through their components including DNA, histones, neutrophil elastase, myeloperoxidase, and cathepsin G, ROS and MMP-9 (Matrix metalloproteinase-9) (Liu et al., 2017).

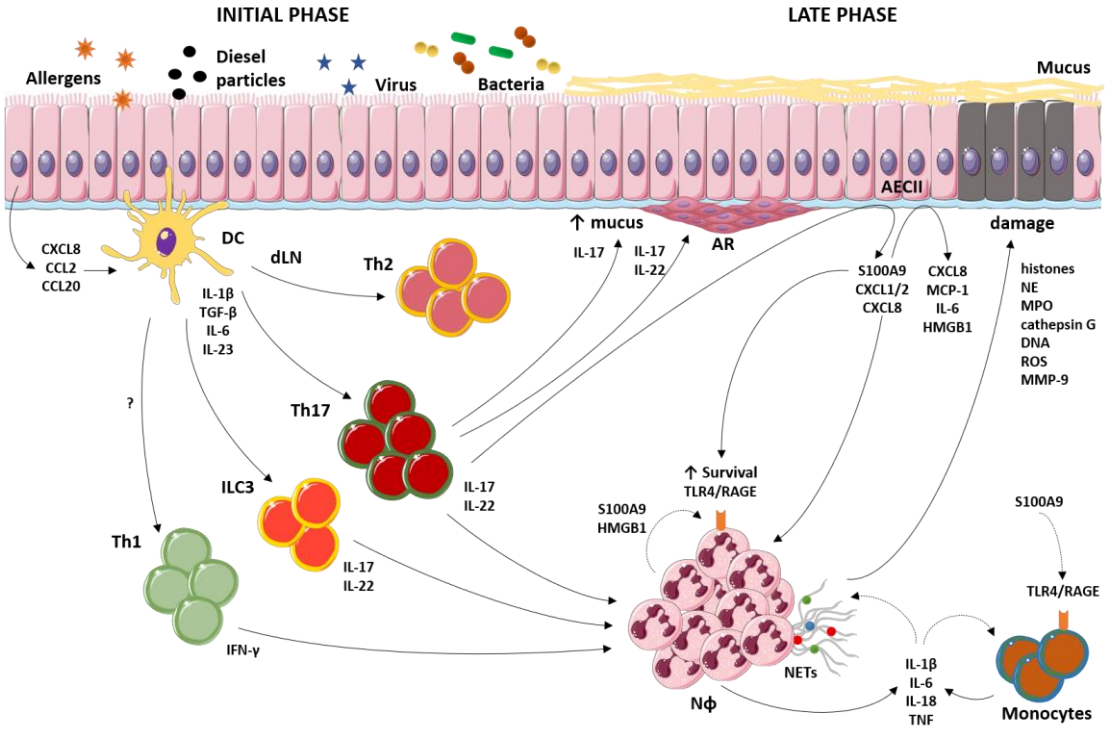


Figure 2 : Immunopathology of severe asthma (Fraga-Silva et al., 2023).

1.2. Streptococcus pneumoniae

Streptococcus pneumoniae, also known as pneumococcus, is a Gram-positive, non-motile, diplococcus bacterium that do not form spores. It has a polysaccharide capsule, which is an essential factor in its virulence, and it is the major bacterial specie that causes pneumonia (WHO, 2023). Pneumococcus belongs to the family of Streptococcaceae and is the common cause of community-acquired pneumonia (Michelow IC et al., 2004), responsible for million of deaths worlwide (WHO, 2023). Pneumococcus commonly causes invasive diseases including acute pneumonia, sepsis, and meningitis in infants, elderly, as well as immunocompromised individuals (Bogaert et al., 2004; Peng et al., 2019). Recent data published by the WHO revealed that about one million children die from pneumococcal disease every year (WHO, 2023). Healthy

people might also carry the bacteria in the upper respiratory tract (Weiser et al., 2018). Through respiratory secretions, the bacteria could be transmitted from patients and healthy carriers person-to-person (WHO, 2023).

It was described that asthmatic patients have a higher risk of developing pneumonia following infection with *S. pneumoniae* (Jounio *et al.*, 2010; Talbot *et al.*, 2005). Asthma patients have higher *S. pneumoniae* colonization than healthy individuals and a higher risk of severe pneumococcal disease (Li et al., 2020). However, *S. pneumoniae* infection may attenuate allergic lung inflammation by negatively regulating the Th2 immune response (Hartmann et al., 2015). On the contrary, *S. pneumoniae* infection may drive severe asthma by promoting Th17 responses, neutrophil infiltration, lung inflammation, and airway hyperresponsiveness (Yang et al., 2015).

Currently, reports reveal about 90 distinct pneumococcal serotypes worldwide (WHO, 2023). Virulence factor including polysaccharide capsule and exotoxin pneumolysin (PLY) are determinant in the pathogenicity of pneumococcal strains. Polysaccharide capsule inhibit phagocytosis, complement factor binding and entrapment by NET (Abeyta et al., 2003; Wartha et al., 2007; Hyams et al., 2010), whereas PLY, a member of the cholesterol-dependent cytolysins, causes cell lysis by binding to cholesterol-containing membranes and by forming large pores into membranes upon oligomerization (Mitchell and Mitchell, 2010).

The host response against *S. pneumoniae* is mediated by both leukocytes of innate and adaptive immunity. The presence of invasive bacteria into the lungs lead to the activation of innate leukocytes including alveolar macrophages and dendritic cells, and in addition, epithelial cells that recognize the bacteria through TLR (Kadioglu and Andrew, 2004). In response to infection with *S. pneumoniae*, bacterial cell wall extracellular components such as lipopeptides and lipoteichoic acid and possibly PLY are recognized by TLR2 and TLR4, respectively at the cellular surface (Aliprantis et al., 1999; Schwandner et al., 1999; Malley et al., 2003). Phagocytes internalize the bacteria, and its degradation into phagolysome induces the release of bacterial peptidoglycan and nucleic acids. Unmethylated CpG-containing DNA is recognized by TLR9 in endosome as well as by a cytosolic sensor that signals through the adapter molecule STING and IRF3 (Heeg et al., 1998; Hiroaki Hemmi et al., 2000; Albiger et al., 2007; Koppe et al., 2012a). Besides, pneumococcal DNA is also recognized by

AIM2 (Koppe et al., 2012b). Moreover PLY-mediated membrane disruption and consequently, other bacterial components enter the cytosol. Pneumococcal peptidoglycan fragments are recognized by the cytosolic NOD-like receptor (NLR) NOD2. Activated receptors including TLR, NOD2, and the cytosolic DNA sensor induce the production of proinflammatory cytokines such as TNF, IL-1β, IL-6, chemokines and type I interferons through the NF-kB and/or IRF3/7 pathways (Koppe et al., 2012b). TNF cooperates with the chemoattractants CXCL1 and CXCL2 for the recruitment of neutrophils to the lungs (Greenberger et al., 1996; Van Der Poll et al., 1997; O'Brien et al., 1999). PLY also activates the NLRP3 inflammasomes. This process requires K^+ efflux. NLRP3 and AIM2 mediate the activation of caspase-1 that cleave pro-IL-1β into mature IL-1β (Rabes et al., 2016).

The protective immune response against pneumococcus depends on the differentiation of IFN-γ- and IL-17-producing cells (Ivanov et al., 2012; Marqués et al., 2012). This adaptive immune response requires the activation of TLR2, TLR4 and TLR9, and the participation of MyD88, which induce the production of IL-1β, IL-6, IL-8 (Knapp et al., 2004; Wang et al., 2017). In addition to IL-17 and IFN-γ, IL-22 is also associated with protection after infection with *S. pneumoniae* (Ivanov et al., 2013; Trevejo-Nunez et al., 2016), and ILC3 (Innate Lymphoid Cells 3) is the major IL-22-producing leukocyte during infection (Van Maele et al., 2014). In experimental pneumonia, there is an increase in the influx of neutrophils in the bronchoalveolar lavage and in the lungs. However, neutrophil depletion was not determinant for infection control (Wilson et al., 2015). Protective function against infection was also attributed to epithelial cells dependent on TLR agonists and MyD88 (Cleaver et al., 2014; Dudek et al., 2016).

1.3. S100A9

S100 proteins are named in this way because of their solubility in 100% saturated ammonium sulfate (Moore, 1965). They have approximately 10 kilodalton and are Ca^{2+} binding proteins that often exist in the form of heterodimer. They contain a helix–loop–helix structural domain and have high affinity to bind other divalent ions including Zn^{2+} as well as Cu^{2+} and Mn^{2+} (Moroz and Antson, 2003). Human S100A8 consist of 93 amino acid residues whereas S100A9 is composed by 113 amino acid residues. S100A9 has a truncated isoform with 110 amino acids and is produced by many cells including macrophages, monocytes, activated neutrophils, endothelial and

epithelial cells (Lusitani et al., 2003; Angel et al., 2006). During hidradenitis suppurativa infection, keratinocytes were also able to produce S100 proteins (Lima et al., 2016). In granulocytes, S100A9 interacts with TLR4 or RAGE (Receptor for Advanced Glycation End products) inducing its own production. Moreover, S100A9 binds to both receptors in monocytes and thereby induce the production of IL-1β, IL-6, IL-18 and TNF via the MyD88 and NF-kB pathways. In granulocytes and monocytes, S100A9 plays autocrine and panacrine functions (Kessel et al., 2013). In neutrophils, S100A8 and S100A9 proteins comprise approximately 40% of the cytoplasmic proteins (Edgeworth et al., 1989; Niki et al., 1996). In contrast, low levels of S100A8/A9 are expressed by monocytes (Wang et al., 2018). S100A9 increases neutrophilic inflammation and blocks neutrophil apoptosis (Ryckman et al., 2003; Raquil et al., 2008). The absence of S100A9 in neutrophil impaired Mac-1 (CD11b) expression that compromised neutrophil migration (Croce et al., 2009).

After being released in the extracellular environment, S100A9 is recognized as a damage-associated molecular pattern (DAMP) (Nacken et al., 2003). During inflammation, S100A9 is released and plays actions in infectious and non-infectious diseases. S100A9 modulates the immune response and induces leukocyte recruitment and cytokine production (Wang et al., 2018). Elevated concentration of S100A9 was detected in the spleens of BALB/c mice infected with *Plasmodium berghei* ANKA and in this murine malaria model, S100A9 was found to be expressed by mononuclear cells in red pulp (Mizobuchi et al., 2014). Infection with Influenza A virus also triggered the release of S100A9 (Tsai et al., 2014). In the early stage of *Klebsiella pneumoniae* infection-induced sepsis, a significant up regulation of S100A9 was described (Swathi Raju et al., 2016). During chronic tuberculosis, S100A9 promoted neutrophil recruitment by regulating CD11b expression in those cells. In an arthritis mouse model, transendothelial migration of neutrophils was enhanced when neutrophils were treated with S100A9. However treatment with monoclonal antibody anti-S100A9 inhibited leukocyte infiltration in the joints (Chen et al., 2015). In this study, the authors also found an inhibition of granulocytes, lymphocytes and monocytes in vitro when RAGE signaling was blocked, suggesting that the leukocyte migration was dependent on RAGE (Chen et al., 2015). Likewise, impaired production of cytokines was observed in S100A9-deficient neutrophils following stimulation by TLR4 (Cesaro et al., 2012). By investigating the molecular mechanisms regulating the chronicity of inflammation

and psoriatic lesions, it was found that S100A9 plays a critical role in these process by driving IL-23/Type 3 immunity and keratinocytes were found as the main source of S100A9 by the authors (Silva de Melo et al., 2023).

S100A9$^{-/-}$ mice infected with *S. pneumoniae* showed a significant reduction of neutrophilic inflammation in the lungs accompanied by a significant reduction of cytokines and chemokines associated with neutrophil recruitment, including CXCL1, CXCL2, CXCL10, IL-6 and GM-CSF (Granulocyte macrophage-colony stimulating factor) (Filippo et al., 2014).

S100A9 also plays suppressive roles. It inhibits the expression of B7 molecules, resulting in a reduced capacity of DC to present antigens and prime T cells (Shimizu K et al., 2011). S100A9 also mediates macrophage suppression after those cells take up neutrophils in apoptosis (De Lorenzo et al., 2010).

Excessive production of S100A9 might be deleterious leading to tissue damage due to the excessive inflammatory response. It was found that elevated levels of S100A9 activates RAGE signaling and induce inflammatory damage in septic shock patients (Lorey et al., 2017). Severe chronic obstructive pulmonary disease (COPD) also triggered excessive production of S100A9 that resulted in uncontrolled immune response (Pouwels et al., 2015). On the opposite, an insufficient production of S100A9 induced by infection in patients with COPD exacerbated the illness, suggesting that S100A9 acts as a rheostat for controlling host immune response and regulates homeostasis during inflammation (Pouwels et al., 2015). Otherwise, S100A9 serve as potential therapeutic target for disease control and can be used as diagnostic marker or to predict disease progress, such as in type I diabetes, multiple sclerosis, systemic lupus erythematosus, Alzheimer, influenza, cancer, psoriasis, tuberculosis and asthma (Coutant et al., 1998; Polman et al., 2005; Bengtsson et al., 2012; Chang et al., 2012; Kummer et al., 2012; Tsai et al., 2014; Wang et al., 2014, 2018; Raymond et al., 2014; Chimenti et al., 2016; Lee et al., 2017; Nyalwidhe et al., 2017; Scott et al., 2020).

1.4. S100A9, asthma and *S. pneumoniae*

Studies have reported that S100A9 participates in the immunopathology of asthma (Lee et al., 2017; Palmer et al., 2019; Quoc, 2021). S100A8 and S100A9 were reported to prevent neutrophil apoptosis in asthma (Kim et al., 2015). Indeed, neutrophil from peripheral blood stimulated with *Dermatophagoides pteronissinus*, a prevalent

allergen of the airways, produced S100A8 and S100A9 that inhibited the induction of apoptosis in these cells (Kim et al., 2015). However, the role of S100A9 is still unclear in asthma.

Transcripts of S100A9 were significantly up regulated in peripheral blood mononuclear cells of children with asthma compared to healthy controls (Aoki et al., 2009). S100A9 has also been detected in the sputum of patients with severe uncontrolled asthma compared to individuals with controlled asthma (Lee et al., 2013). S100A9 levels in asthma may depend on the severity of the disease. Elevated levels of S100A9 were found in sputum from patients with neutrophil-dominant severe asthma compared to the sputum from eosinophil-dominant and paucigranulocytic groups. A positive correlation was found between S100A9 levels and the percentage of neutrophils in the sputum, but not eosinophils (Lee et al., 2017). In the serum of individuals with neutrophilic asthma, higher levels of S100A9 were found compared to serum of patients with non-neutrophilic asthma.

In a murine model of allergic asthma induced by *Alternaria alternata*, $S100A9^{-/-}$ mice showed increased airway eosinophilic inflammation, increased bronchial hyperreactivity to methacholine, perivascular and peribronchial leukocyte infiltration, a higher production of IL-4, IL-5, and IL-13 and elevated levels of serum IgE compared to WT animals exposed to the allergen. The percentage of regulatory T cells significantly reduced in the lung of $S100A9^{-/-}$ mice compared to WT mice, all exposed to the allergen. Mechanistically, asthma induction by *A. alternata* induced the production of S100A9 that activated $CD4^{+}$ regulatory T cell to suppress the effector fuction of Th2 cells resulting in the protection during allergic airway inflammation (Palmer et al., 2019). In a similar way, the administration of recombinant S100A9 in an OVA-induced asthma model in rats significantly decreased pulmonary resistance (Yin et al., 2010). These fndings show that S100A9 protects against allergic airway inflammation in rodents models.

In a murine asthma model induced by complete *Aspergillus* allergen (CAA), the blockage of S100A9 using monoclonal antibody (mAb) anti-S100A9 significantly reduced total number of cells in the airway and eosinophils compared to control group treated with saline or IgG isotype control (Greenlee et al., 2008). Furthermore, the pro or anti-inflammatory role of S100A9 in allergic asthma might depend on the inhaled

allergen that triggers the inflammation (Greenlee et al., 2008; Yin et al., 2010; Manni and Alcorn, 2019; Palmer et al., 2019). Using mouse model of neutrophilic asthma induced by OVA/CFA (Complete Freund Adjuvant), the authors found increased neutrophilic inflammation, increased airway resistance and higher levels of IL-1β, IL-17 and IFN-γ in recombinant S100A9 tread mice. Evaluated parameters were restored following intranasal administration of mAb anti-S100A9, suggesting that S100A9 may initiate and amplify neutrophil recruitment in severe asthma (Lee et al., 2017). The induction of severe asthma model using OVA/LPS showed higher levels of S100A9 in BALF, serum and lung tissues compared to mice with eosinophilic and mixed granulocytic asthma (Quoc, 2021). Notably, in a murine severe asthma model, S100A9 inhibition by paquinimod in mice sensitized and challenged with OVA/complete Freund's adjuvant (CFA) and OVA, respectively, resulted in the improvement of experimental disease symptoms (Lee et al., 2021). Furthermore, S100A9 might be a potential biomarker and therapeutic target for neutrophilic asthma (Lee et al., 2017, 2021; Quoc, 2021).

As previously mentioned, asthma severity depend on various factors, including infection with *S. pneumoniae* (Talbot et al., 2005; Jounio et al., 2010; Ray and Kolls, 2017). In pneumococcal pneumonia, there is an increase in the influx of neutrophils in the bronchoalveolar lavage and in the lungs (Filippo et al., 2014; Wilson et al., 2015).

As previously reported in this section, $S100A9^{-/-}$ mice infected with *S. pneumoniae* showed a significant reduction of neutrophilic inflammation in the lungs accompanied by a significant reduction of cytokines and chemokines associated with neutrophil recruitment (Filippo et al., 2014). Upon infection with *S. pneumoniae*, the interplay between AEC-II and leukocytes, including macrophage and DC might lead to the release of CXCL1, CXCL2 and TNF through the synergistic action of TLR2, TLR4 and TLR9 (Lee et al., 2007; Fraga-Silva et al., 2023). During the early phase of infection, S100A9 might be released increasing neutrophil recruitment. In pneumococcal pneumonia, the presence of bacteria induces the production of NET (Beiter et al., 2006). Furthermore, during severe asthma activated neutrophils might induce NET production that could exacerbate asthma.

However, there is also evidence that infection with *S. pneumoniae* is beneficial in asthma. In mice, it was found that allergic airway inflammation was reduced upon

administration of live or killed *S. pneumoniae* before, during or after OVA sensitization (Hartmann C et al, 2015 ; Preston JA et al, 2011). Epidemiological studies revealed that the frequence of asthma attack was reduced when children with asthma were treated with sulfisoxazole and pneumococcal vacine (Schuller DE et al, 1983). In additon, vaccination with a 23-valent pneumococcal polysaccharide vaccine was associated to a decreased risk of hospital admission for asthma compared with nonvaccinated individuals (Ansaldi F et al, 2005).

S. pneumoniae infection during OVA sensitization induced regulatory T cells (T reg) and increased IL-10 production (Preston JA et al, 2011). Moreover, allergic immune response in the draining lymph nodes, lung and spleens were suppressed and an increase of Treg cells was found in mice that receive pneumococcal vaccine (Thorburn AN et al, 2010). Further investigations revealed that pneumococcal component, 3 polysaccharide and pneumolysoid were responsible for Treg cell induction by *S. pneumoniae* infection or vaccination. These components drove the suppression of OVA-induced inflammation by inducing the differenciation of Treg cells that inhibit Th2 immune reponse and prevent Th17 immune response (Thorburn AN et al, 2013). However, studies with experimental models have shown that previous infection with *S. pneumoniae* in neonatal mice followed by induction of experimental asthma increase neutrophil recruitment, Th17 cell differencition and AHR (Yang B et al, 2015). It is likely that the effect of *S. pneumoniae* in allergic asthma needs further investigation.

1.5. Severe asthma treatment

Two main treatments are currently used for the treatment of asthma: bronchodilators and corticosteroids. The treatment depends on the age and the severity of illness. Corticosteroids play an anti-apoptotic role that stimulate neutrophil survival in peripheral blood and sputum (S. Saffar et al., 2011). Moreover, the recurrent use of corticosteroids can promote resistance and increased susceptibility to infections, mainly rhinovirus (Lambrecht and Hammad, 2015). In addition, corticosteroids can negatively affect the child’s physical development, resulting in increased susceptibility to development of obesity (De Leonibus et al., 2016). Therefore, new strategies to treat severe or neutrophilic or low-Th2 asthma are required. Antagonists of CXCR2 receptor (SCH527123 and AZD5069) have been investigated. Severe asthmatic patients subjected to SCH527123 treatment exhibited a significant reduction in neutrophil

recruitment compared to the group treated with placebo (Nair et al., 2012). In a random, double-blind, placebo-controlled trial, administration of AZD5069 did not reduce the frequency of severe exacerbations in patients with uncontrolled severe asthma (O'Byrne et al., 2016). Nevertheless, in both studies, lung function and quality of life remain unchanged or limited (Nair et al., 2012; O'Byrne et al., 2016).

Clinical trials blocking IL-17 and TNF receptors in neutrophilic asthma were disappointing (Wenzel et al., 2009; Holgate et al., 2011; Busse et al., 2013; Thomson, 2016). Targeting IL-6 and IL-1β might be of benefit in neutrophilic asthma (Chu et al., 2015; Hernandez et al., 2015); however, no clinical studies about both molecules are registered in asthma to have data regarding its effectiveness and safety (Thomson, 2016). Of note, kinase, phosphodiesterase 4 (PDE4) and 5-lipoxygenase-activating protein (FLAP) inhibitors may have beneficial effects in neutrophilic inflammation (Chaudhuri et al., 2014; Thomson, 2016). Moreover, macrolides including clarithromycin and azithromycin, statins, Peroxisome Proliferator-Activated Receptor γ (PPARγ) agonist and theophylline may also benefit neutrophilic asthmatic patients (Simpson et al., 2008; Maneechotesuwan et al., 2013; Essilfie et al., 2015; Thomson, 2016; Gibson et al., 2017). Otherwise, bronchial thermoplasty has led to the reduction of exacerbations in uncontrolled severe asthma and the improvement of symptoms in those patients (Laxmanan and Kyle Hogarth, 2015; Thomson, 2019).

Given that an improvement of experimental neutrophilic asthma symptoms was observed after treatment with paquinimod and anti-S100A9 (Lee et al., 2017, 2021) and looking for the beneficial effect of tasquinimod and paquinimod respectively in a guinea pig granuloma model induced by the Bacillus Calmette–Guérin (BCG) (Yoshioka, 2016) and COVID-19 pathogenesis (Guo et al., 2021), it is possible that S100A9 blockers may be good targets in severe asthma.

2. Justification

Both asthma and pneumococcal pneumonia are diseases that affect the lungs and are mediated by inflammation triggered by exacerbated immune response. Asthma exacerbation depends on several factors including pathogens, smoking, and comorbidities. Among enviormental factors that may exacerbate the immunopathology of asthma, viral, bacterial and fungi infections are the most common (Ray and Kolls, 2017). The major bacterial specie that causes pneumonia is *S. pneumoniae* that causes million of deaths worldwide (WHO, 2023). Asthma patients have higher *S. pneumoniae* colonization than healthy individuals and a higher risk of severe pneumococcal disease (Jounio *et al.*, 2010; Talbot *et al.*, 2005; Li et al., 2020). Inhaled corticosteroids in asthma increase the risk of pneumonia (Kim et al., 2019). Although *S. pneumoniae* infection may attenuate allergic lung inflammation by negatively regulating the Th2 immune response (Hartmann et al., 2015), pneumococcus infection may drive severe asthma by promoting Th17 responses, neutrophil infiltration, lung inflammation, and airway hyperresponsiveness (Yang et al., 2015; Ray and Kolls, 2017; Fraga-Silva et al., 2023). Nevertheless, how *S. pneumoniae* infection could aggravate asthma pathology remains unclear.

Considering that *S. pneumoniae* induces NET production and S100A9 induces neutrophil recruitment and neutrophil survival, is it possible that acute pneumonia induced by *S. pneumoniae* exacerbates asthma.

Our hypothesis is that S100A9 contributes to increase neutrophilic inflammation during the comorbidity of asthma and acute pneumonia and induces neutrophil survival. Neutrophils produce NET that aggravate asthma causing exacerbation of pulmonary inflammation.

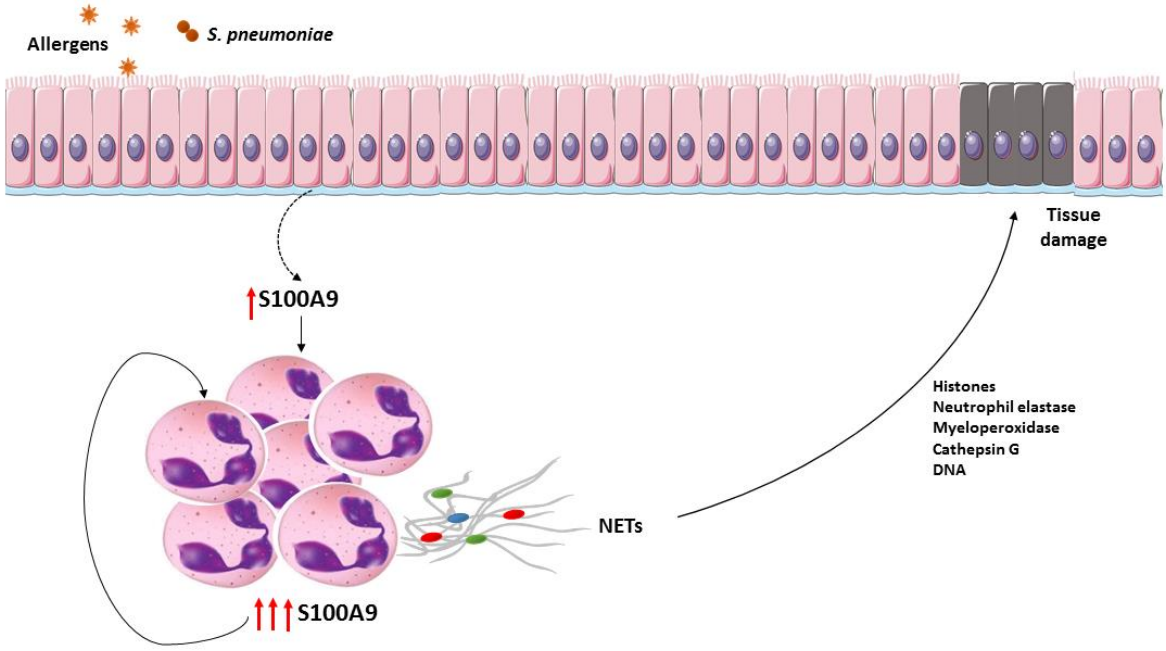


Figure 3 : Hypothesis of the study. During the acute infection with S. *pneumoniae*, S100A9 is produced by epithelial cells and neutrophils, and contributes for the infiltration of neutrophils in the BALF and into the lungs, along with Th17 cells. S100A9 also induces neutrophil survival. Neutrophils produce NET that aggravates asthma causing exacerbation of pulmonary inflammation.

3. Objectives

3.1. Main objective

This study aimed to evaluate the role of the protein S100A9 in the comorbidity asthma and acute pneumococcal pneumonia.

3.2. Specific objectives

- To investigate the neutrophilic inflammation in asthma and acute pneumonia comorbidity induced by OVA and *S. pneumoniae* infection in mouse model;
- To investigate whether NET is involved in asthma and acute pneumonia pathogenesis and the effect of NET pharmacological inhibition;
- To investigate whether S100A9 contributes to the development of neutrophilic asthma induced by acute *S. pneumoniae* infection;
- To investigate the therapeutic effect of tasquinimod and azeliragon during the comorbidity asthma and acute pneumonia.

4. Materials and methods

4.1. Mice

Female C57BL/6 WT and S100A9$^{-/-}$ mice (6-8 weeks old) were obtained from the breeding facility of Ribeirao Preto Medical School, University of Sao Paulo (Ribeirao Preto, SP, Brazil). Animals were maintained in specific-pathogen-free (SPF) conditions by microisolator cages in a ventiled rack (Alesco, Monte Mor, SP, Brazil) receiving food and water *ad libitum*. All experiments were performed according to the local Ethics Committee on Animal Experimentation (Protocol Number 245/2019).

4.2. OVA-induced asthma model

Mice were submitted to OVA-induced asthma as previously described (Martins et al., 2021). Briefly, mice were sensitized weekly three times in seven days intervals with 10 µg of OVA grade VI plus 2 mg of aluminium hydroxide (Sigma-Aldrich, St Louis, MO, USA) by intraperitoneal route (200 µL / animal). Seven days after the last sensitization, mice were challenged three consecutive days intranasally with 30 µg (30 µL / animal) of OVA grade V (Sigma-Aldrich, St Louis, MO, USA). Animals were euthanaized 24 hours after the last OVA challenge, by intraperitoneal inoculation of 100 µL of anesthetic solution containing 20% ketamine hydrochloride (Agener, Embu-Guaçu, São Paulo, Brazil) and 10% xilasin (Laboratorios Calier SA, Barcelona, Spain), as approved by local Ethics Committee on Animal Experimentation.

4.3. Culture and infection with *S. pneumoniae*

TIGR4 strain of *S. pneumoniae* (ATCC BAA-334) frozen at -80°C in tryptone soy broth (TSB) (BD, cat. 211825) containing 10% glycerol was thawed, plated on blood agar and incubated overnight at 37°C, 5% CO_2. After growth, colonies were inoculated in TSB medium, when growth were checked at OD600 = 0.06 and incubating for 4 to 5 hours. After incubation, the bacteria were adjusted to OD600 = 0.3, plated on blood agar and incubated overnight. Growth bacteria was counted and the concentration of bacteria was adjusted to 50 x 10^8 /mL of colony-forming unit (CFU) in sterile Phosphate Buffered Saline (PBS). Mice were infected with 1 x 10^8 CFU of *S. pneumoniae* by intranasal route (20 uL / animal) during the second challenge with OVA. Animals were euthanaized 48 hours after the infection.

4.4. Treatments

WT OVA-sensitized mice were treated with three doses (5 mg / kg each dose) of BB-Cl-amidine (SML2250 ; Sigma-Aldrich), tasquinimod (SML2489 ; Sigma-Aldrich) or azeliragon (Cat. No. T2507 ; TargetMol) by intraperitoneal route 1.5, 3 and 1 hours, respectively before each OVA challenge and *S. pneumoniae* infection. Non-treated mice were inoculated with the drug dilution vehicle, PBS - 22,5 % DMSO (200 µL / animal), by intraperitoneal route.

4.5. Bronchoalveolar lavage

Bronchoalveolar lavage fluid (BALF) were obtained by trachea cannulation with subsequent injection of 1 mL of iced PBS and three washes. Samples are collected and centrifuged at 450x g by 10 minutes. Supernatant was stored at -20ºC for cytokine measurement. BALF cells was resuspended in 100 µL of Ammonium-Chloride-Potassium (ACK) red cells lysis buffer for 2 minutes. Lysis reaction is stopped by addition of 1 mL of PBS. BALF cells were centrifuged at 450x g by 10 minutes, ressuspended in 250 µL of PBS and centrifuged in cytocentrifuge (Thermo Fisher Scientific, Waltham, MA, EUA) at 18x g for 3 min. The cells were stained with rapid panoptic (Labor Clin, Sao José do Rio Preto, SP, Brazil) for differential cell count.

4.6. Enzyme Linked Immunosorbent Assay (ELISA)

CXCL1, S100A9 and MPO levels are measured in BALF using ELISA kits following the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The limit of detection was 15.6 pg/mL for CXCL1, 31.3 pg/mL for S100A9 and 250 pg/mL for MPO.

4.7. Flow cytometry

After euthanasia, lungs were perfused with 5 ml of PBS through the right ventricule. Perfused lower right lobes lung were cut into small pieces and digested for 50 minutes at 37°C, 5% CO2 with collagenase (2.2 mg/mL) (Sigma-Aldrich, St Louis, MO, USA) and DNAse (0.055 mg/mL) (Roche, Basel, Switzerland). The digestion was stoped using EDTA. Cell concentration were adjusted to $1x10^6$ and stained with FVS780 viability dye (BD Pharmingen, San Diego, CA, USA) for 15 minutes at room temperature, followed by incubation with Fc block (anti-CD16/32, BD) for 10 minutes at 4ºC. Then, cells were stained for 30 min at 4°C with specific antibodies to

characterization of lymphocytes ($CD3^+CD4^+$), neutrophils ($CD11b^+Ly6G^+$), eosinophils ($CD11b^+SiglecF^+$), alveolar macrophages ($CD64^+$), myeloides $CD11b^+$) and epithelial cells ($CD45/31^-EpCAM^+$) according to manufacturer's instructions (BD Pharmingen, San Diego, CA, USA and Thermo Fisher Scientific, Waltham, MA, EUA). To evaluate neutrophils and eosinophils death, lung cells were stained with AnnexinV dye (BD Pharmingen, San Diego, CA, USA) after antibodies staining. For cytokine evaluation and characterization of $IL\text{-}17^+$ and $IL\text{-}22^+$ lymphocytes, lung cell suspensions were stimulated with PMA (Phorbol 12-myristate 13-acetate, 100 ηg/mL, Sigma-Aldrich, St. Louis, MO, USA), ionomycin (500 ηg/mL, Sigma-Aldrich, St. Louis, MO, USA) monensin (4µL in 6 mL, BD Biosciences, San Jose, CA, USA) and *GolgiPlug* (1µL/mL, BD Biosciences, Franklin Lakes, New Jersey, EUA) for 5 hours. The monoclonal antibodies used for immunophenotyping are described in the **Table 1**. Samples were fixed with paraformaldehyde 1% (Labsynth, Diadema, SP, Brazil) and acquired in FACS Canto (BD Biosciences, San Jose, CA). Analyses were performed in FlowJo software (Becton Dickinson and Company, Franklin Lakes, NJ, USA).

Table 1. Monoclonal antibodies for immunophenotyping.

Cell Type	Antibody	Fluorochrome	Clone	Source
Eosinophils death	CD11b (+)	PE	M1/70	BD
	SiglecF (+)	BB515	E50-2440	BD
	FVS (+)	APC-Cy7		BD
	AnnexinV (+)	PE-Cy7		Invitrogen
Neutrophils death	CD11b (+)	PE	M1/70	BD
	Ly6G (+)	APC	1A8	BD
	FVS (+)	APC-Cy7		BD
	AnnexinV (+)	PE-Cy7		Invitrogen
Th17	FVS (-)	APC-Cy7		BD
	CD45 (+)	PE-Cy7	30-F11	BD
	CD3 (+)	FITC	145-2C11	BD
	CD4 (+)	APC	RM4-5	BD
	IL-17 (+)	PerCP Cy5.5	PC11-18H10	BD
	IL-22 (+)	PE	1H8PWSR	BD

Epithelial cells and S100A9	Live/Dead (-)	BV421		Invitrogen
	CD45 (-)	PECy7	30-F11	BD
	CD31 (-)	PECy7	390	BD
	EpCAM (+)	FITC	G8.8	BD
	S100A9 (+)	APC	2B10	BD
Neutrophils and S100A9	Live/Dead (-)	BV421		BD
	CD45 (+)	PE-Cy7	30-F11	BD
	Ly6G (+)	FITC	1A8	BD
	CD11b (+)	BV711	M1/70	BD
	S100A9 (+)	APC	2B10	BD
Eosinophils and S100A9	Live/Dead (-)	BV421		BD
	CD45 (+)	PE-Cy7	30-F11	BD
	Ly6G (-)	FITC	1A8	BD
	CD11b (+)	BV711	M1/70	BD
	Siglec-F (+)	PE	E50-2440	Biolegend
	CD64 (-)	APC F780	X54-5/7.1	e-Biosciences
	S100A9 (+)	APC	2B10	BD
Alveolar macrophages and S100A9	Live/Dead (-)	BV421		BD
	CD45 (+)	PE-Cy7	30-F11	BD
	Ly6G (-)	FITC	1A8	BD
	CD11b (+)	BV711	M1/70	BD
	Siglec-F (+)	PE	E50-2440	Biolegend
	CD64 (+)	APC F780	X54-5/7.1	e-Biosciences
	S100A9 (+)	APC	2B10	BD
Uncharacterized myeloid cells	Live/Dead (-)	BV421		BD
	CD45 (+)	PE-Cy7	30-F11	BD
	Ly6G (-)	FITC	1A8	BD
	CD11b (+)	BV711	M1/70	BD
	Siglec-F (-)	PE	E50-2440	Biolegend
	S100A9 (+)	APC	2B10	BD

For *t*SNE realisation, FlowJo software (Becton Dickinson and Company, Franklin Lakes, NJ, USA) was used. Downsample was done on CD45+ cells with 5000 events.

Then all groups were concatenated. 50 000 events were used for the entire *t*SNE, 25000 events in the OVA group and 25000 events in the OVA+Sp group. To construct the tSNE, the following markers were used : CD45, CD11b, Ly6G, SiglecF, CD64 and S100A9.

4.8. Histopathological analysis

To access inflammatory infiltrate and mucus production, the perfused upper right lobe of the lung was collected and fixed in buffered formalin (10% formalin, pH 7.0) for 24 hours. Lungs were washed in running water for 18 hours and storage in ethanol 70%. Then, samples were immerged in ethanol (Labsynth; cat. 00A1084.07.BJ), Xylene (Labsynth; cat. 00X1001.06.BJ) and embedded in paraffin. Slices were performed into 5 µm thick sections. Lung sections were stained with either hematoxylin-eosin (H&E). Pulmonary inflammation score was determined following the criteria: 0, no inflammation; 1, discrete number of inflammatory cells, mainly constituted by polymorphonuclear leukocytes, around vessels and bronchioles and discrete amount of mucus in bronchiolar epithelium; 2, moderate inflammatory infiltrate, mainly polymorphonuclear leukocytes, includes eosinophils, which are frequently found around vessels and bronchioles and a substantial amount of mucus in bronchiolar epithelium; and 3, intense inflammatory infiltrate, mainly polymorphonuclear leukocytes, includes eosinophils, which are frequently found around vessels and bronchioles and an increase amount of mucus in bronchiolar epithelium.

4.9. Immunofluorescence

Lungs were collected without performing BALF to prevent NET degradation and improved the identification of NET. Collected lungs were fixed with 4% paraformaldehyde in PBS and paraffin-embedded. Lung sections were cut (5 µm thick sections) for immunofluorescence staining.Slides were immerged in Xylene (Labsynth; cat. 00X1001.06.BJ) and 100% Ethanol (Labsynth; cat. 00A1084.07.BJ) for 15 minutes in each solution for deparaffinization and rehydration. Then antigen retrieval was performed using 1.0 mM Ethylene Tiamine Tetra acetic acid (EDTA; Labsynth; cat. 00E1005.06.AG) 10 mM Trizma-base (Sigma-Aldrich; cat. T1503), at 95°C for 30 minutes. Afterward, slides were incubated in 5% H_2O_2 in methanol (Millipore; cat. 106009) at RT for 20 minutes to quench endogenous peroxidase activity. After blocking

with IHC Select Blocking Reagent (Millipore, cat. 20773-M) at RT for 2 hours and stained in blocking buffer with rabbit anti-mouse antibodies directed against citrullinated histone H3 (1:100) (H3Cit; Abcam; cat. ab5103) and with goat anti-mouse antibodies directed against MPO (1:100) (anti-MPO, R&D Systems, cat. AF3667, 1:100) during 2 h at RT. Slides were washed with TBS-T (Tris-Buffered Saline with Tween 20) and incubated with secondary antibodies anti-mouse IgG Alexa Fluor 488 (1 :1000) (Invitrogen, Ref. A11055) and anti-rabbit IgG AlexaFluor 568 (1 :1000) (Invitrogen, Ref. A10042). Autofluorescence was quenched using the TrueVIEW Autofluorescence Quenching Kit (Vector Laboratories, cat. SP-8400-15). Images were acquired on a Zeiss LSM 880 Airyscan Elyra S.1. confocal microscope (Zeiss) and processed with the Image J software.

4.10. Statistical analysis

Data were analyzed and represented in graphs using GraphPad Prism Version 8.1 (GraphPad Software, Inc., San Diego, CA, USA). Outliers were identify by Grubbs test. Non-paired t-test were used to analysis of two-group comparisions while ANOVA one-way with Tukey's post test were applicated to calculate three or more groups's comparisions. Histological score was calculated by the Chi-square test. Data were represented as mean ± standard deviation (SD). Results were considered significant with a p-value less than 0.05.

5. Results

5.1. Acute pneumonia concomitant to allergen exposure induce neutrophilic inflammation

First, we characterized and confirmed that allergen exposure simultaneously to acute pneumonia induce a mixed granulocytic inflammation represented by eosinophil and neutrophil infiltration into the lungs. For that, C57BL/6 mice were sensitized weekly three times with ovalbumin (OVA) (grade VI) and alum by intraperitoneal route and challenged daily three times with OVA (grade V) by intranasal route. During the second challenge, animals were or not infected with 1 x 10^8 colony-forming units (CFU) of *S. pneumoniae*. Twenty four hours after the last challenge, bronchoalveolar lavage fluid (BALF) and the lungs were collected and assessed for immunological parameters **(Fig. 4 A)**. Animals exposed to OVA and infected with pneumococcus (OVA+Sp group) exibited a significant reduction of eosinophil influx and a significant increase of neutrophilic inflammation in the BALF compared to mice exposed only to OVA (OVA group) **(Fig. 4 B and C)**. Concentrations of CXCL1, a chemoattractant for neutrophils, was elevated in the BALF of OVA+Sp group compared to the OVA group (**Fig. 4 D**).

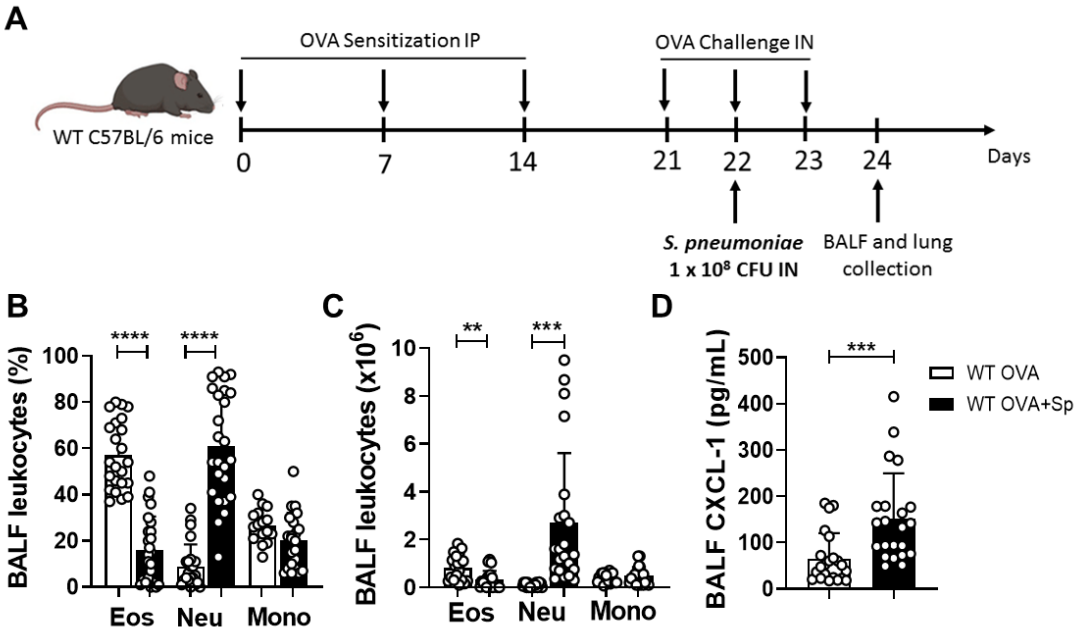


Figure 4. Characterization of the asthma and acute pneumonia comorbidity model. (A) C57BL/6 mice were sensitized and challenged with OVA and infected or not with *S. pneumoniae* during the challenge. **(B and C)** Differential and total count of BALF leukocytes. **(D)** concentrations of CXCL1 in BALF supernatants. Data in B, C, and D are representative of six independent experiments. Data are shown as mean ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **** $P < 0.0001$. IP = intraperitoneal; IN = intranasal.

Histological analysis in the lungs showed the presence of an increased inflammatory infiltrate, mainly polymorphonuclear leukocytes, including eosinophils, which were frequently found around vessels and bronchioles in the OVA+*S*p group compared to the OVA group (**Fig. 5 A and B**).

We also evaluated the percentage of eosinophils and neutrophils in the lungs using flow cytometry (**Supplementary Fig. 1**). We found a reduction of eosinophil influx and a significant increase of neutrophil influx in the lungs after infection of mice exposed to OVA (**Fig. 5 C, D and E**).

Given that neutrophilic asthma is generally associated to Th17 cells (Vroman, 2015), we evaluated the percentage of $CD4^+IL\text{-}17^+$ cells and $CD4^+IL\text{-}22^+$ cells in the lungs **(Supplementary Fig. 2)**. Flow cytometry analysis revealed an increased frequency of IL-17- and IL-22-producing $CD4^+$ cells in the lungs during the comorbidity compared to animals exposed only to OVA (**Fig. 5 F, G and H**).

These results confirm that the comorbidity asthma and acute pneumonia induced by *S. pneumoniae* promotes a pulmonary inflammation characterized predominatly by neutrophil recruitment.

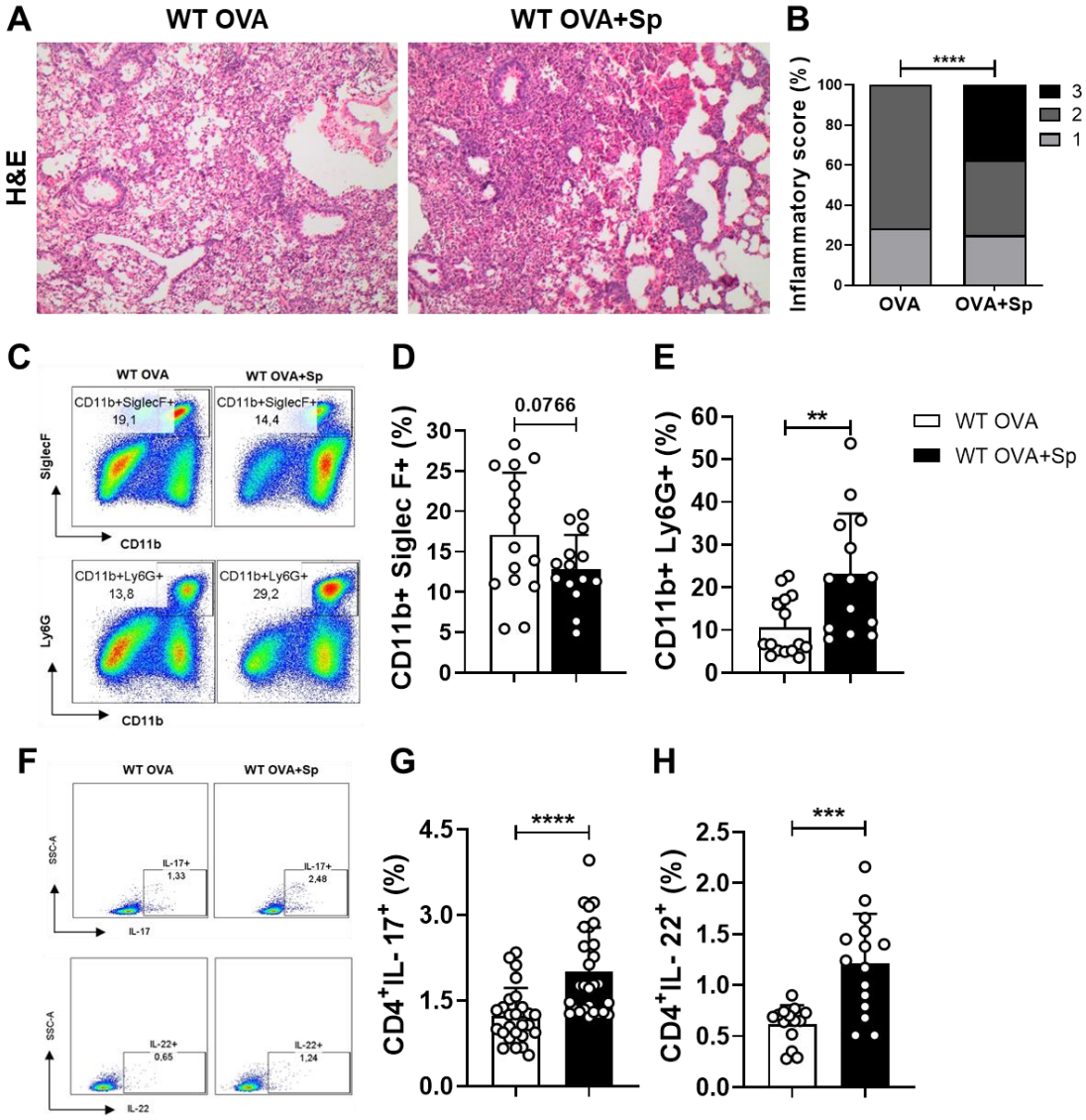


Figure 5. Histological analysis and lung Th17 cells during the comorbidity. (A) Histopathological analysis of the lungs. **(B)** Inflammation score. **(C, D and E)** Representative flow cytometry analysis and percentage of $CD11b^{+}SiglecF^{+}$ cells and $CD11b^{+}Ly6G^{+}$ cells in the lungs determined by flow cytometry. **(F, G and H)** Representative flow cytometry analysis and percentage of $CD4^{+}IL17^{+}$ cells and $CD4^{+}IL\text{-}22^{+}$ cells in the lungs. Data in G is representative of six independent experiments. Data in B, D, E and H are from three independent experiments. Data are shown as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ****, $P < 0.0001$.

5.2. Allergen exposure concomitant to acute pneumonia induced by *S. pneumoniae* increase NET production

After confirming an increased neutrophilic inflammation in the lungs during the comorbidity, we asked whether those neutrophils would exhibit a high NET production. For that, we quantified the concentration of myeloperoxidase (MPO), an enzyme required for NET formation (Metzler et al., 2011), in the BALF. Mice from OVA+Sp group exibited significant levels of MPO in the BALF compared to the OVA group, suggesting the involvement of NET in the asthma and acute pneumonia comorbidity (**Fig. 6 A**). To confirm that, we performed immunofluorescence analysis of lung tissue and found a significant expression of extracellular MPO and citrullinated histone (Cit-H3), a modified form of H3 histone involved in chromatin decondensation and NET production, during the comorbidity compared to mice exposed only to OVA (**Fig. 6 B and C**).

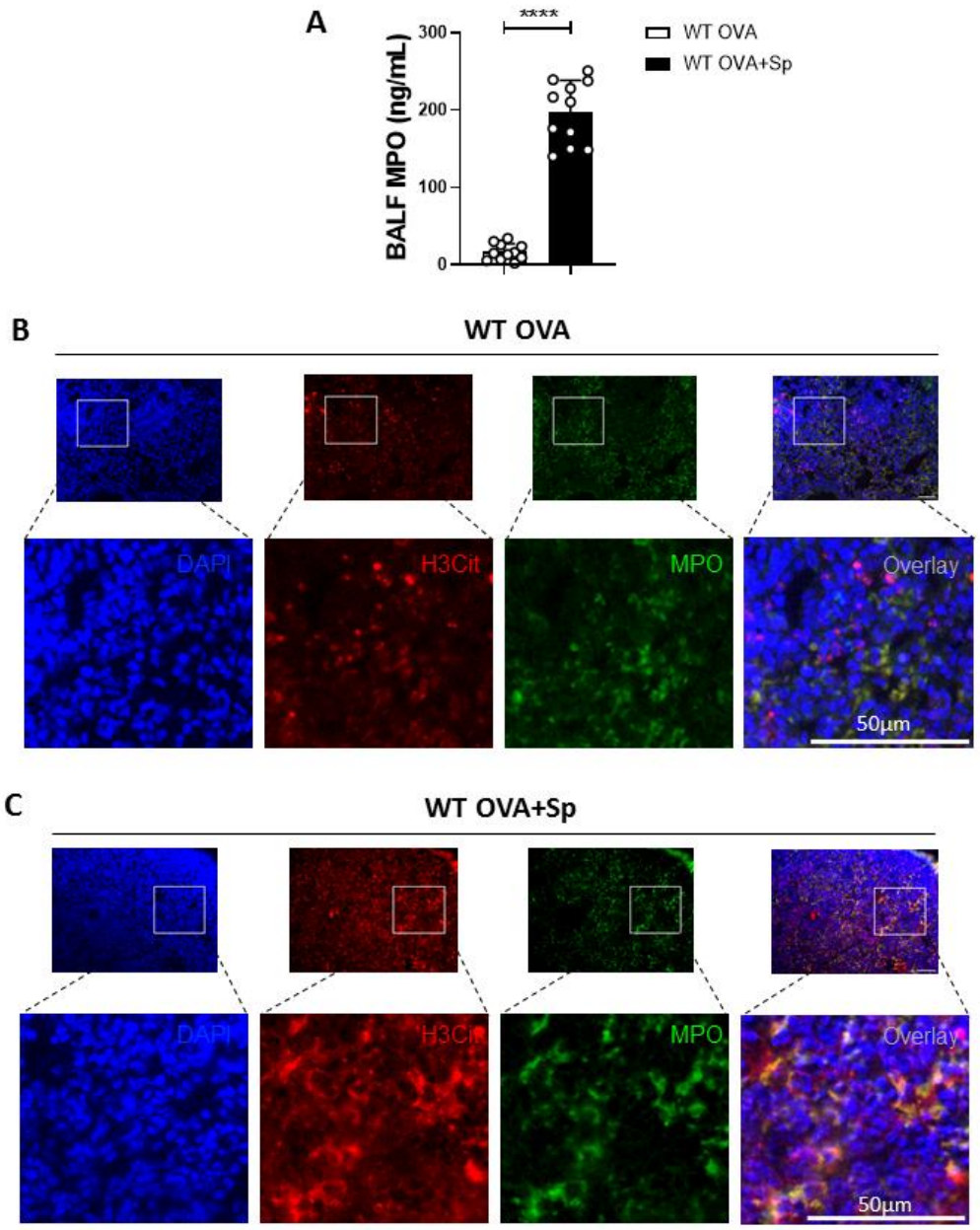


Figure 6. Evaluation of NET production in the lungs. (A) MPO concentrations in the BALF supernatants. **(B and C)** Representative immunofluorescence analysis of NET production by neutrophils (DAPI, blue = nuclei), MPO (green), and citrullinated histone (H3Cit, red). Scale bar: 50 µm. Data in A is from three independent experiments. Data are shown as mean ± SEM. $P < 0.001$. ****, $P < 0.0001$.

5.3. Treatment with BB Cl-amidine reduces neutrophilic inflammation in the BALF and lungs during the comorbidity

To investigate the contribution of NET in neutrophilic asthma induced by acute pneumonia, NET were targeted by three daily intraperitoneal administrations of BB Cl-amidine, an inhibitor of the arginine deiminase PAD4, which contributes to chromatin reorganization and NET formation (Papayannopoulos, 2018). BB Cl-amidine was administrated to OVA+Sp group 1 hour and 30 minutes before OVA challenge and infection with *S. pneumoniae* (**Fig. 7 A**). Treatment with BB Cl-amidine significantly reduced the percentage and total number of neutrophils in the BALF (**Fig. 7 B and C**). Histological analysis in the lung showed a significant reduction of leukocytes infiltration in the lungs of mice that received BB Cl-amidine compared to non-treated OVA+Sp group (**Fig. 7 D and E**).

MPO levels in the BALF as well as the expression of extracellular MPO and Cit-H3 were also significantly reduced in BB Cl-amidine treated mice compared to non-treated mice (**Fig. 8 A, B and C**).

Therefore, the treatment with BB Cl-amidine showed that targeting NET resulted also in reduction of neutrophils in the lungs and reduced pulmonary inflammation.

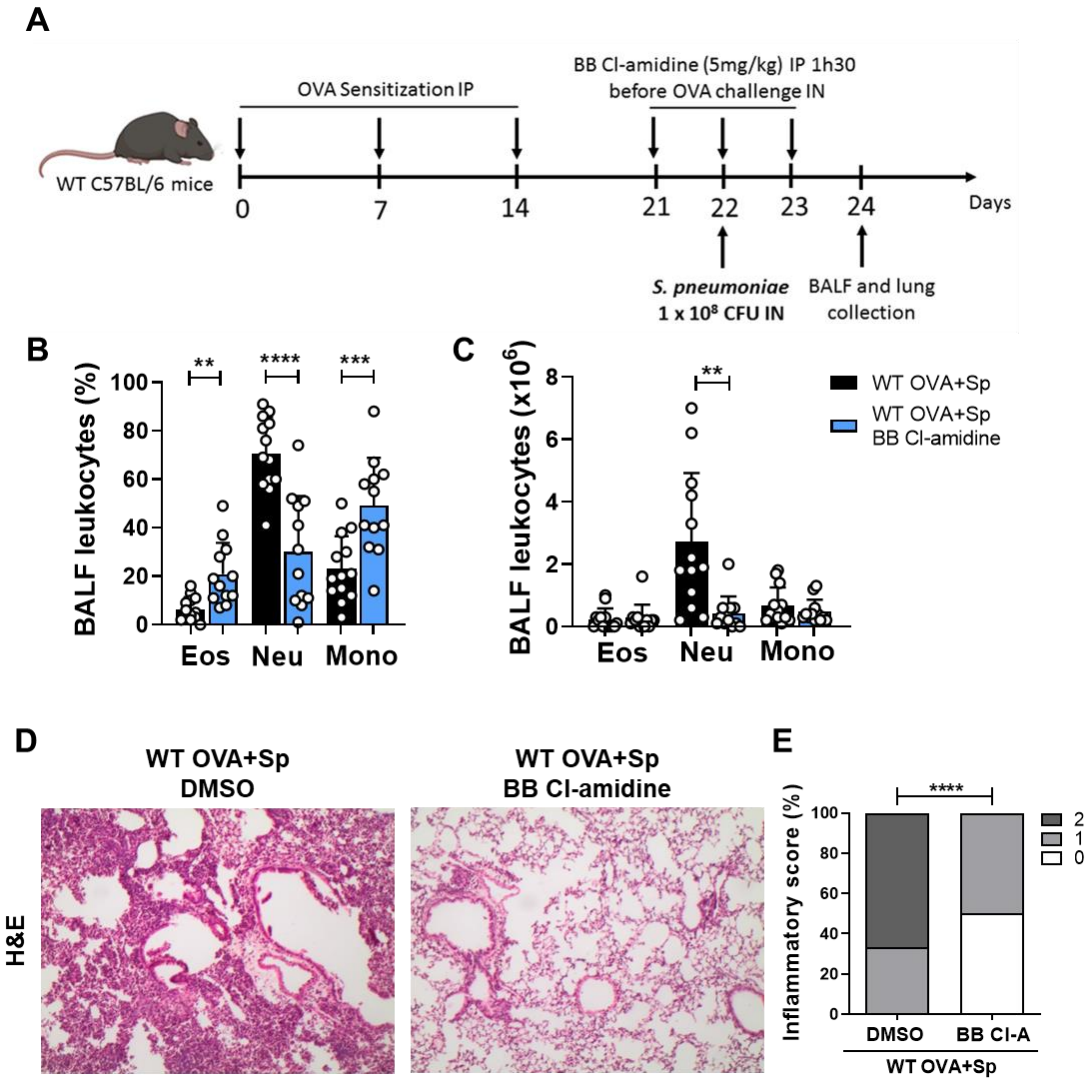


Figure 7. Evaluation of airway inflamation and histologial analysis in the lungs of animals treated with BB Cl-amidine. (A) C57BL/6 mice were sensitized with OVA and treated with BB Cl-amidine 1 hour and 30 minutes before each OVA challenge and infection with *S. pneumoniae*. **(B and C)** Differential and total cell count of BALF leukocytes. (**D)** Histopathological analysis of the lungs. **(E)** Inflammation score. Data in B, C and E are from three independent experiments. Data are shown as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$. ****, $P < 0.0001$.

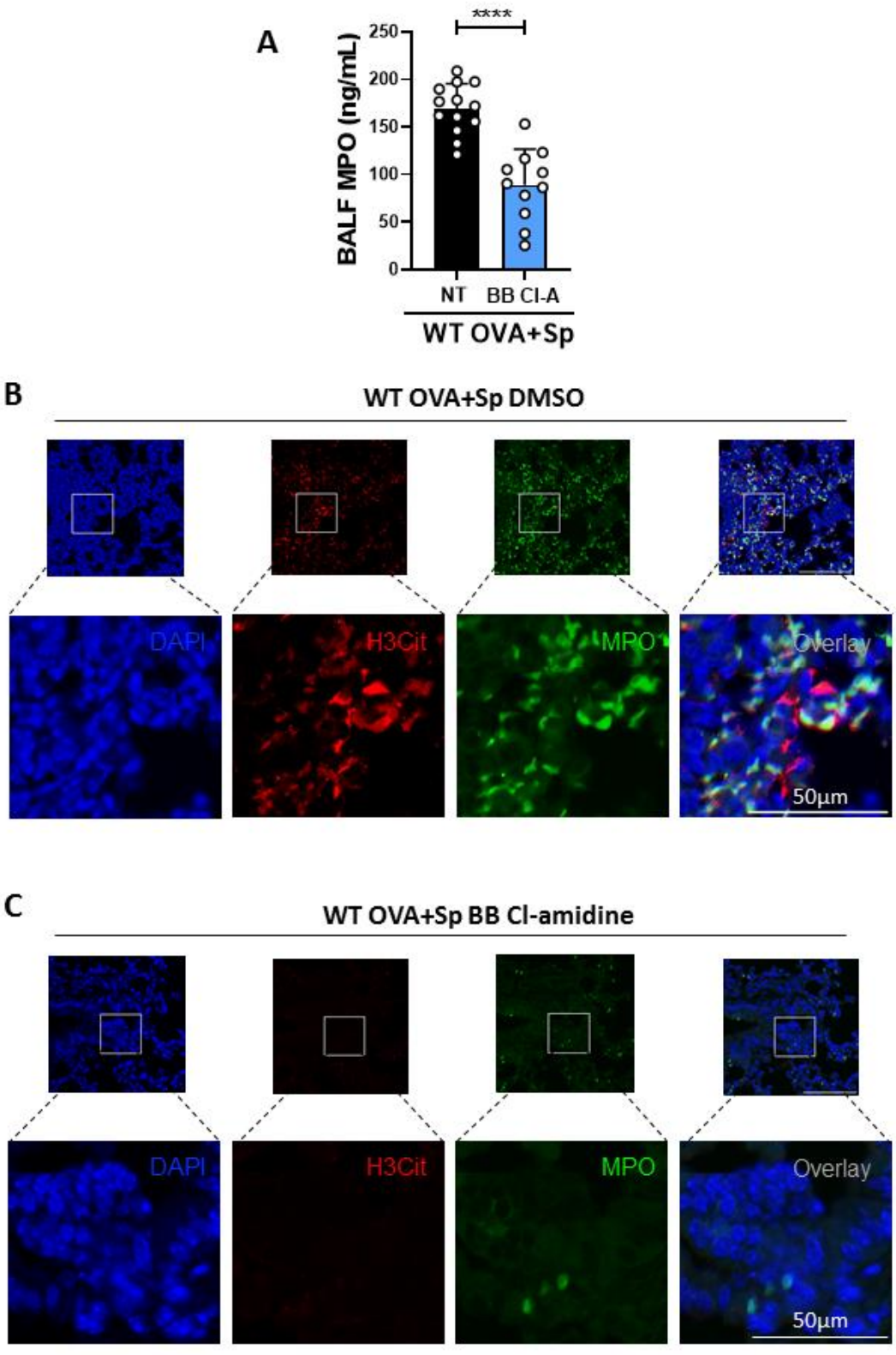


Figure 8. Evaluation of NET production in the lungs of animals treated with BB Cl-amidine. Mice were treated as depicted in the Figure 7 A. **(A)** MPO levels in the BALF supernatants. **(B and C)** Representative immunofluorescence analysis of NET production by neutrophils (DAPI, blue = nuclei), MPO (green), and citrullinated histone (H3Cit, red). Scale bar: 50 µm. Data in A is from three independent experiments. Data are shown as mean ± SEM. ****, $P < 0.0001$.

5.4. S100A9 production increased during asthma and acute pneumonia comorbidity

Our results show that asthma and acute pneumonia comorbidity increase lung neutrophil recruitment and NET production. Furthermore, we also found increased concentrations of S100A9 in the BALF of OVA+Sp group (**Fig. 9 A**). S100A9 induces neutrophil recruitment and survival (Ryckman et al., 2003; Raquil et al., 2008). S100A9 might be produced by macrophages, monocytes, activated neutrophils, endothelial and epithelial cells (Lusitani et al., 2003; Angel et al., 2006). To identify the cellular source of S100A9 in the context of asthma associated to the *S. pneumoniae* infection, lung cells from mice were stained and assessed for the percentage of neutrophils ($CD11b^+Ly6G^+$ cells), eosinophils ($CD11b^+SiglecF^+$ cells), alveolar macrophage ($CD11b^+CD64^+$ cells), epithelial ($EpCAM^+$ cells) and myeloid cells ($CD11b^+SiglecF^-$ cells). The percentage of S100A9-expressing cells was evaluated by flow cytometry **(Supplementary Fig. 3 and 4)**. No significant difference was found in the percentage of $S100A9^+$ neutrophils, alveolar macrophage and myeloid cells during the comorbidity compared to the OVA group (**Fig. 9 B - I**). $S100A9^+$ eosinophils decreased during the comorbidity compared to WT OVA mice (**Fig. 9 J and K**). However, animals exposed to OVA and infected with *S. pneumoniae* showed an increased percentage of $S100A9^+$ epithelial cells compared to animals only exposed to OVA (**Fig. 9 L, M and N**).

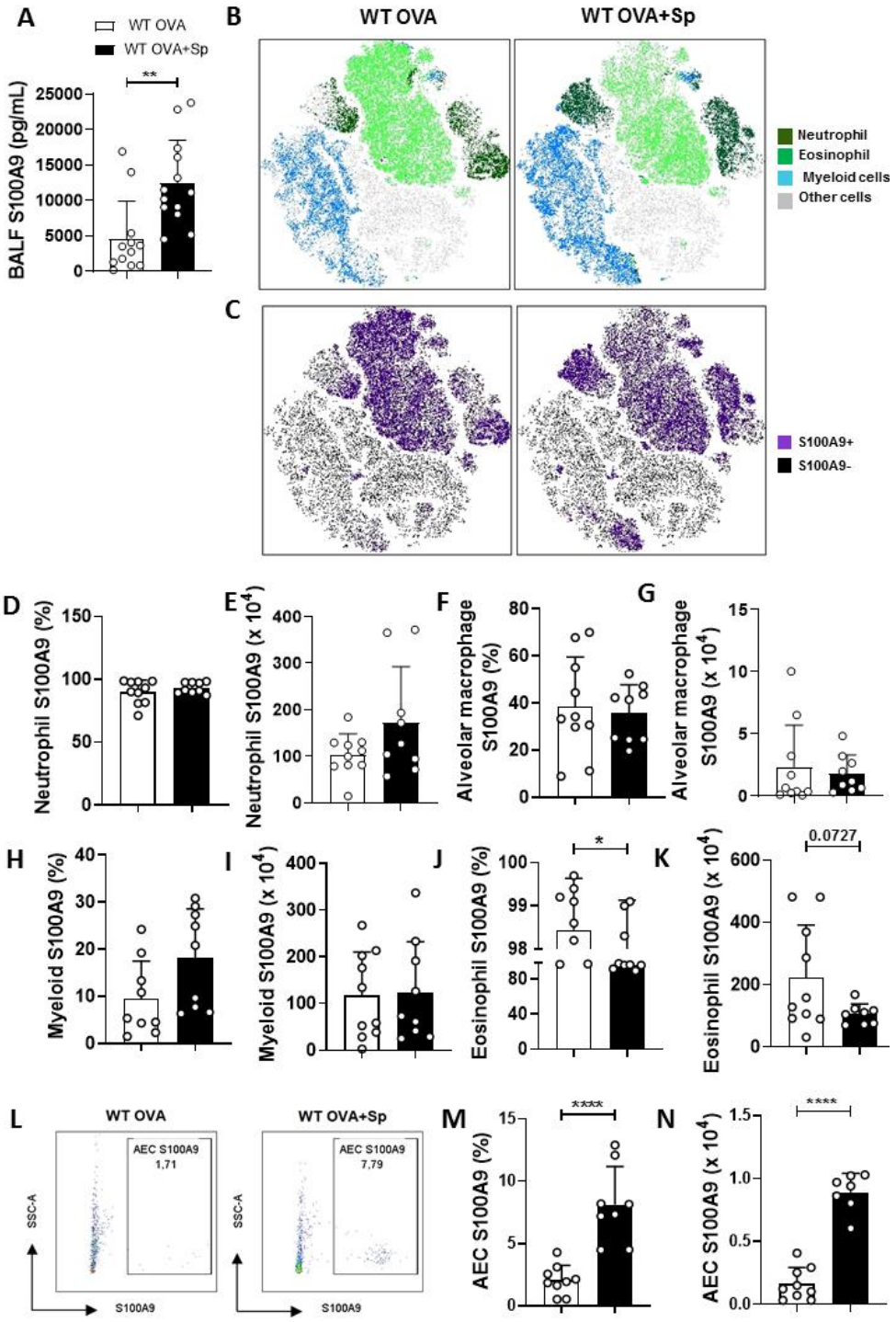


Figure 9. Frequency of S100A9⁺ cells in WT OVA and WT OVA+Sp groups. (A) S100A9 concentrations in the BALF supernatants. **(B and C)** *t*SNE plots depicting the percentage of lung leukocytes and S100A9^{+} cells in OVA and OVA+Sp groups. **(D, E, F, G, H, I, J and K).** Percentage and total number of lung leukocytes and S100A9^{+} cells. **(L, M and N)**. Representative flow cytometry analysis and percentage of S100A9⁺ epithelial cells in OVA and OVA+Sp groups. Data in A, D, E, F, G, H, I, J, K, M and N are from two independent experiments. Data are shown as mean ± SEM. *, $P < 0.05$, **, $P < 0.01$, ****, $P < 0.0001$.

We found that neutrophils along with other myeloid cells were the most abundant leukocytes in the lungs during the comorbidity (**Fig. 10 A, B and F**), whereas eosinophils and other myeloid cells were most abundant in the lungs of animals only exposed to OVA **(Supplementary Fig. 5 A, B and F)**. Of note, granulocytes including neutrophils and eosinophils were the most $S100A9^{+}$ cells in OVA and OVA+Sp groups (**Fig. 10 C, D, G and Supplementary Fig. 5 C, D, G**). In both group, neutrophils significantly expressed more S100A9 compared to other cells populations (**Fig. 10 E, H and Supplementary Fig. 5 E, H**). Despite this, S100A9 expression by neutrophils is higher in OVA than OVA+Sp group. However, no significant difference were found in S100A9 expression by other myeloid and epithelial cells in OVA compared to OVA+Sp mice **(Supplementary Fig. 6)**.

Immunofluorescence staining also confirmed a significant higher expression of S100A9 in $Ly6G^{+}$ cells during the comorbidity compared to animals only exposed to OVA (**Fig. 11 A and B**).

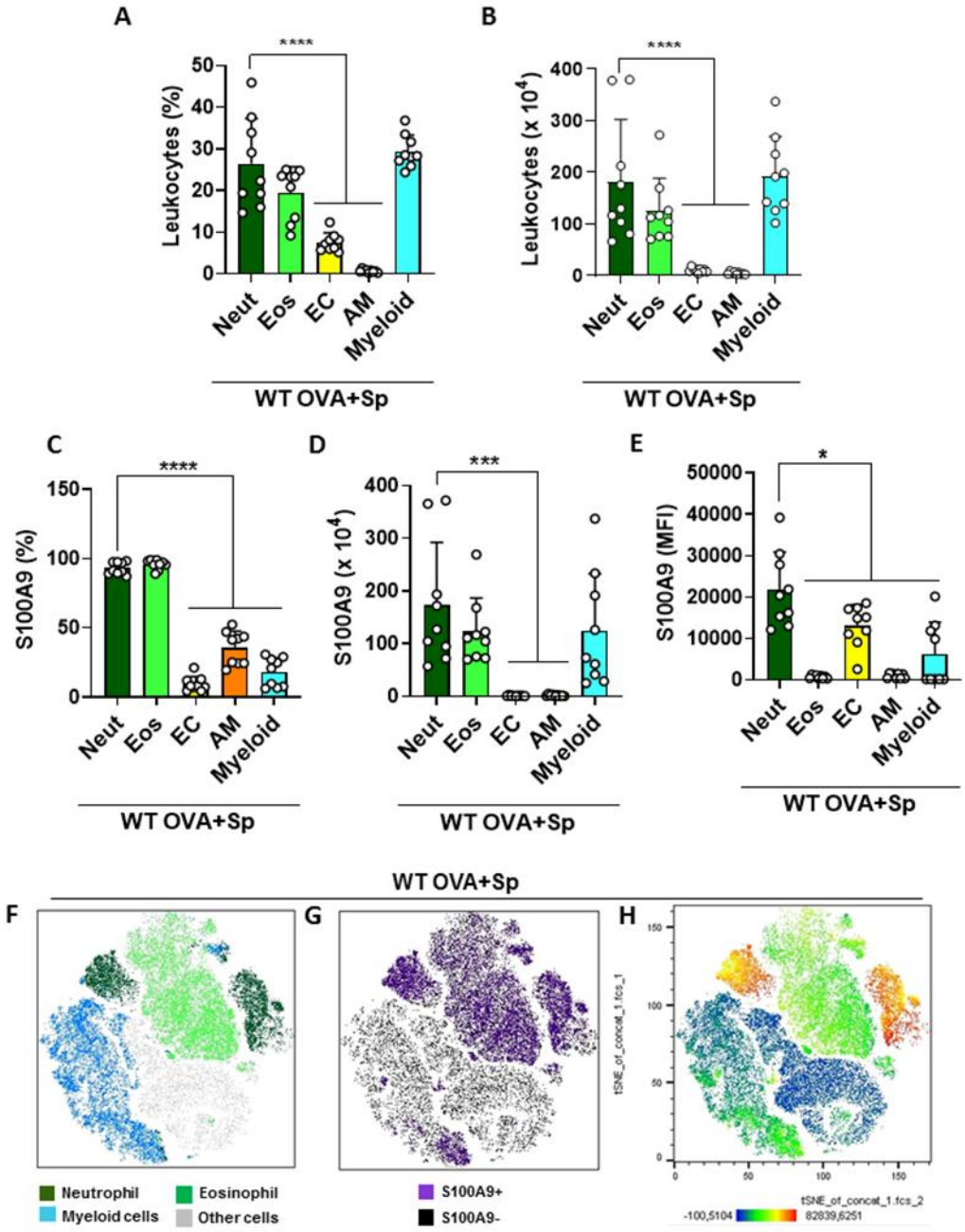


Figure 10. Evaluation of the production of S100A9 by epithelial cells and leukocytes during the comorbidity. (A, B, C, D and E) Percentage of lung leukocytes, S100A9^{+} cells and S100A9 expression in those cells determined by flow cytometry. **(F, G and H)** *t*SNE plots depicting the percentage of lung leukocytes, S100A9^{+} cells and S100A9 expression in those cells determined by flow cytometry. Data in A, B, C, D and E are from two independent experiments. Data are shown as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$. ****, $P < 0.0001$.

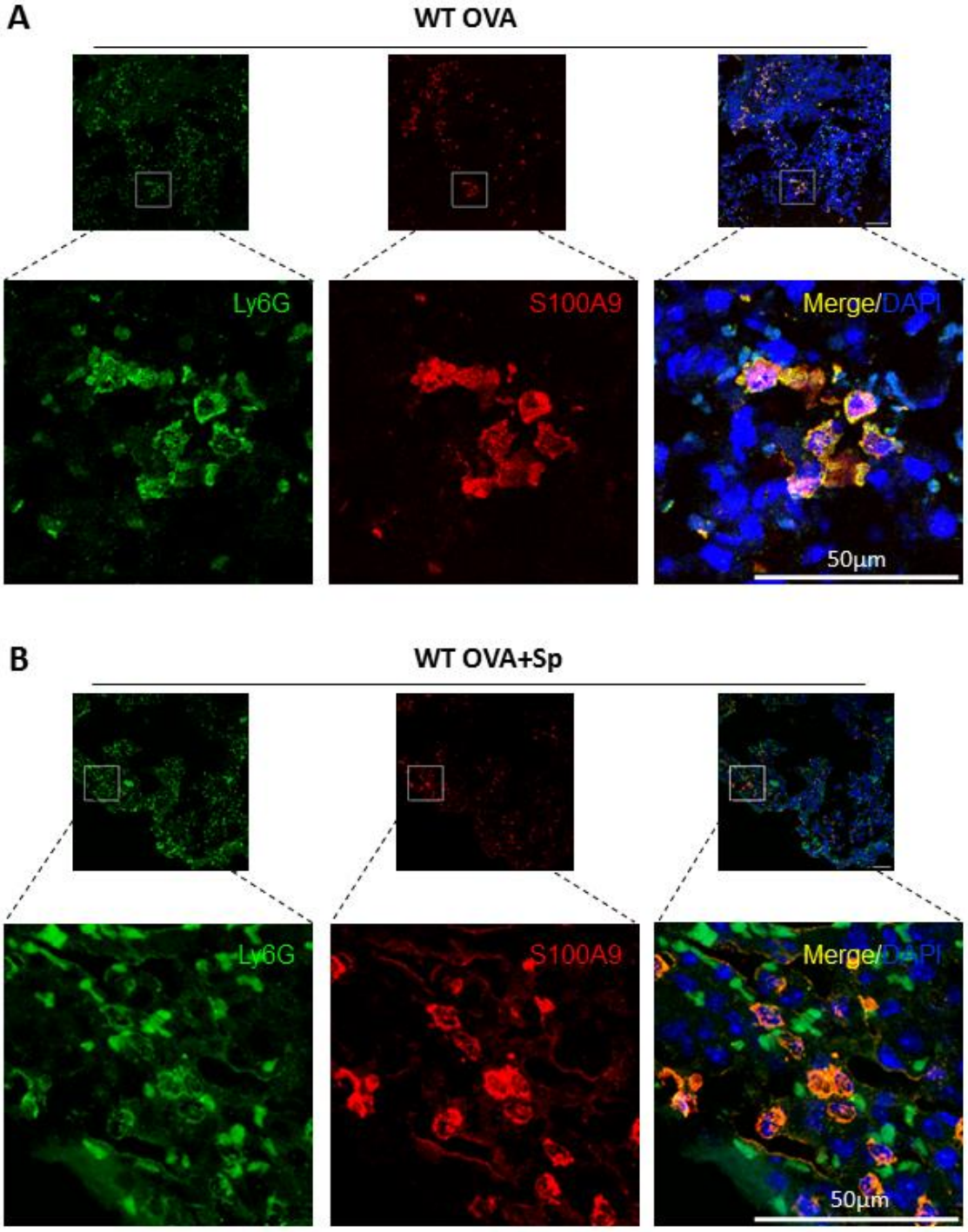


Figure 11. Expression of S100A9 by neutrophils. (A and B) Representative immunofluorescence analysis of S100A9^{+} neutrophils (DAPI, blue = nuclei), Ly6G (green), and S100A9 (red) in OVA and OVA+Sp groups. Scale bar: 20 µm. Data are from two independent experiments.

As *t*SNE plots reveal two populations of neutrophils, we sought to understand the difference between this two populations of neutrophils. For this we evaluated the expression of Ly6G in lung cells from OVA and OVA+Sp mice by flow cytometry **(Supplementary Fig. 7)**. We observed a high and low Ly6G expression, characterizing two populations of neutrophils in both groups, infected or not. However, the infection of animals previously subjected to OVA increases $Ly6G^{High}$ neutrophil population accompanied by a decrease of $Ly6G^{Low}$ neutrophil population compared to animal only exposed to OVA **(Supplementary Fig. 8 A and B)**. Next, we evaluated the expression of S100A9, CD11b, CD64, Ly6C, CD45 and SiglecF in each population of neutrophils. Among these cell markers, only CD11b were found to be expressed significantly in high neutrophils of OVA+Sp group **(Supplementary Fig. 8 C)**. These results suggest that infection with *S. pneumoniae* increases the ability of myeloid cells including $Ly6G^{High}$ neutrophils to migrate into the lung for host defense.

5.5. S100A9 deficiency reduces neutrophilic inflammation without impairing NET production and Th17 cells during the comorbidity

Next, we looked for the function of S100A9 using mice deficient for the expression of S100A9 (S100A9 KO), which were sensitized and challenged with OVA and infected or not with *S. pneumoniae*, as described in **Fig. 4A**. S100A9 deficiency resulted in a significant increase of eosinophil infiltration and in a significant reduction in neutrophilic inflammation in the BALF during the comorbidity compared to WT OVA+Sp group **(Fig. 12 A and B)**. Similar results were observed in total cell numbers **(Fig. 12 C and D)**. The concentration of the chemokine CXCL1 was significantly reduced in the absence of S100A9 during the comorbidity **(Fig. 12 E)** whereas MPO levels were not impaired **(Fig. 12 F)**. In WT mice, we observed a significant increase in the percentage of $IL\text{-}17^+$ and $IL\text{-}22^+$ lymphocytes in the OVA+Sp group compared to OVA group, as previously observed (**Fig. 5 G and H**). The deficiency of S100A9 did not affect IL-17- or IL-22-expressing $CD4^+$ cells obtained from OVA+Sp group. However, the deficiency of S100A9 increased the population of both $CD4^+IL\text{-}17^+$ and $CD4^+IL\text{-}22^+$ in the lungs obtained from OVA group **(Fig. 12 G and H)**.

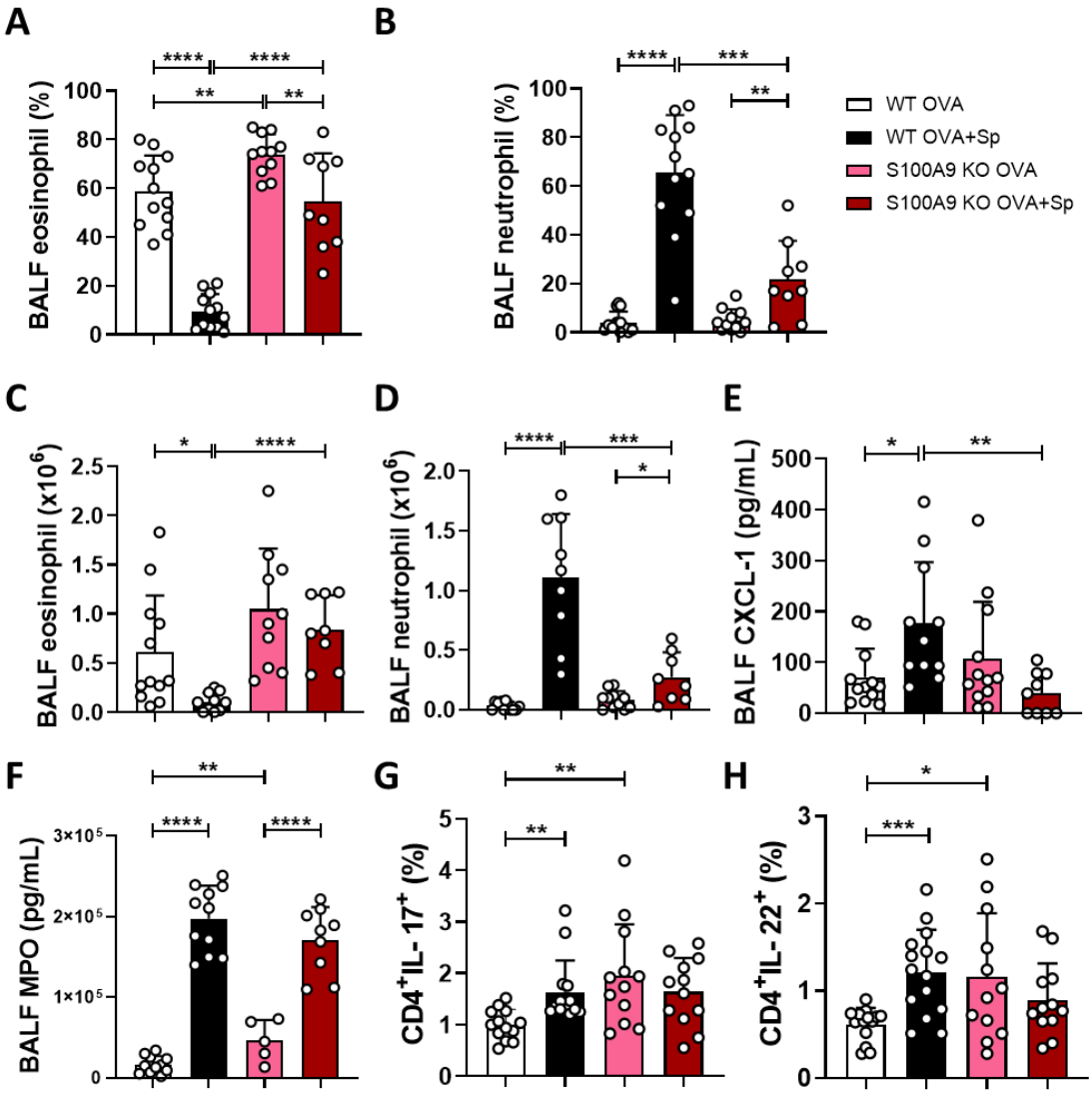


Figure 12. S100A9 deficiency reduces neutrophils and CXCL-1 in the lungs during the comorbidity. (A, B, C and D) Differential and total count of BALF leukocytes. **(E and F)** CXCL1 and MPO concentrations in the BALF supernatants. **(G and H)** Percentage of $CD4^+IL17^+$ and $CD4^+IL\text{-}22^+$ in the lungs. Data in A, B, C, D, E, F, G and H are from three independent experiments. Data are shown as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ****, $P < 0.0001$.

5.6. S100A9 induces neutrophil survival and exacerbates pulmonary inflammation during the comorbidity

Regarding histological analysis in the lungs, we found that inflammation was significantly impaired during the comorbidity in the absence of S100A9 compared to WT OVA+*S*p group **(Fig. 13 A and B)**.

Although we observed reduction of pulmonary inflammation, we also found an increase in the frequency of eosinophils in the lungs of S100A9$^{-/-}$ mice with comorbididy compared to their WT counterpart. These increase in the frequency of eosinophils was associated with reduced eosinophil death. The same was found for OVA groups **(Fig. 13 C, D and E)**.

However, the comparison between WT OVA+Sp and S100A9 KO OVA+Sp groups showed a significantly reduced percentage of lung neutrophils in the last one **(Fig. 13 G)**. As S100A9 induces neutrophil survival (Ryckman et al., 2003; Raquil et al., 2008), we evaluated neutrophil apoptosis in the lungs and found that S100A9 deficiency increased neutrophil death in the lungs during the comorbidity. **(Fig. 13 F and H)**.

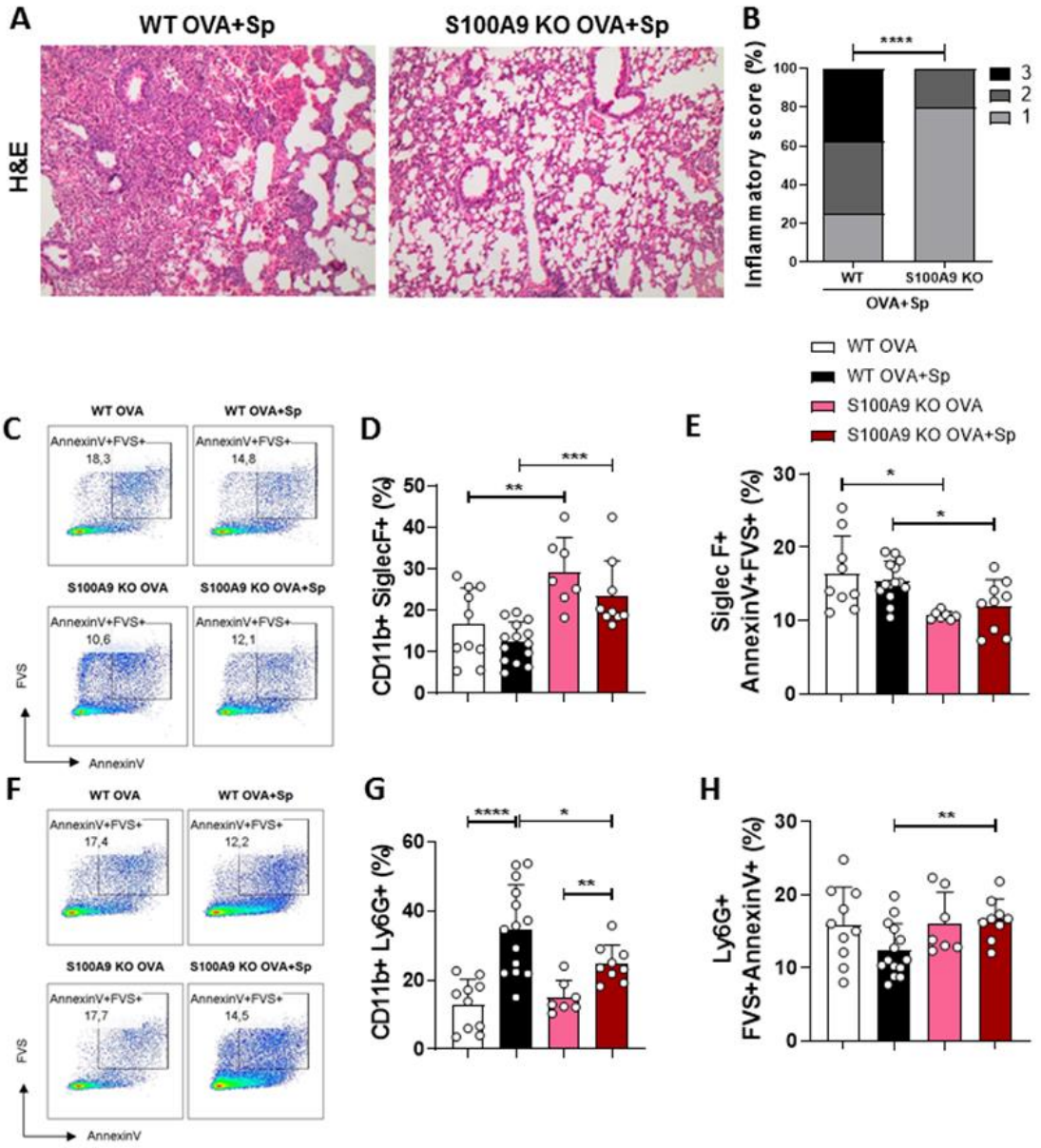


Figure 13. S100A9 induces neutrophil survival and pulmonary inflammation during the comorbidity. (A) Histopathological analysis of the lungs **(B)** Inflammation score. **(C)** Representative flow cytometry analysis of eosinophil apoptosis in the lungs. **(D and E)** Percentage of eosinophil and eosinophil apoptosis in the lungs determined by flow cytometry. **(F)** Representative flow cytometry analysis of neutrophil apoptosis in the lungs determined by flow cytometry. **(G and H)** Percentage of neutrophil and neutrophil apoptosis in the lungs determined by flow cytometry. Data in B, D, E, G and H are representative of two independent experiments. Data are shown as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ****, $P < 0.0001$.

5.7. Treatment with tasquinimod, but not RAGE inhibition, reduces neutrophilic inflammation in the BALF

Considering that S100A9 deficiency was associated to a significant reduction of neutrophilic inflammation during the comorbidity, we investigated the effect of tasquinimod, an inhibitor of S100A9. In neutrophils and monocytes, S100A9 interacts with TLR4 or Receptor for Advanced Glycation End products (RAGE) inducing the production of pro-inflammatory cytokines (Kessel et al., 2013). Therefore, we also evaluated the effect of azeliragon, an inhibitor of RAGE receptor.

C57BL/6 mice were treated by intraperitoneal route with tasquinimod 3 hours before OVA challenge and *S. pneumoniae* infection **(Fig. 14 A)**. Treatment with tasquinimod, but not with azeliragon, resulted in a significant reduction in the percentage and total number of neutrophils in the BALF compared to non-treated animals **(Fig. 14 B and C)**.

5.8. Treatment with tasquinimod and azeliragon reduce neutrophilic inflammation in the lungs

Histological analysis showed that pulmonary inflammation was significantly reduced in the lungs after tasquinimod and azeliragon treatments **(Fig. 15 A and B)**.

Treatment with tasquinimod or azeliragon resulted in a significant reduction in the percentage of neutrophils in the lungs **(Fig. 15 C and D)**. Reduced percentage of neutrophils was associated with an increased death of neutrophil in tasquinimod treated mice and in azeliragon treated mice compared to non-treated OVA+*S*p group **(Fig. 15 E and F)**.

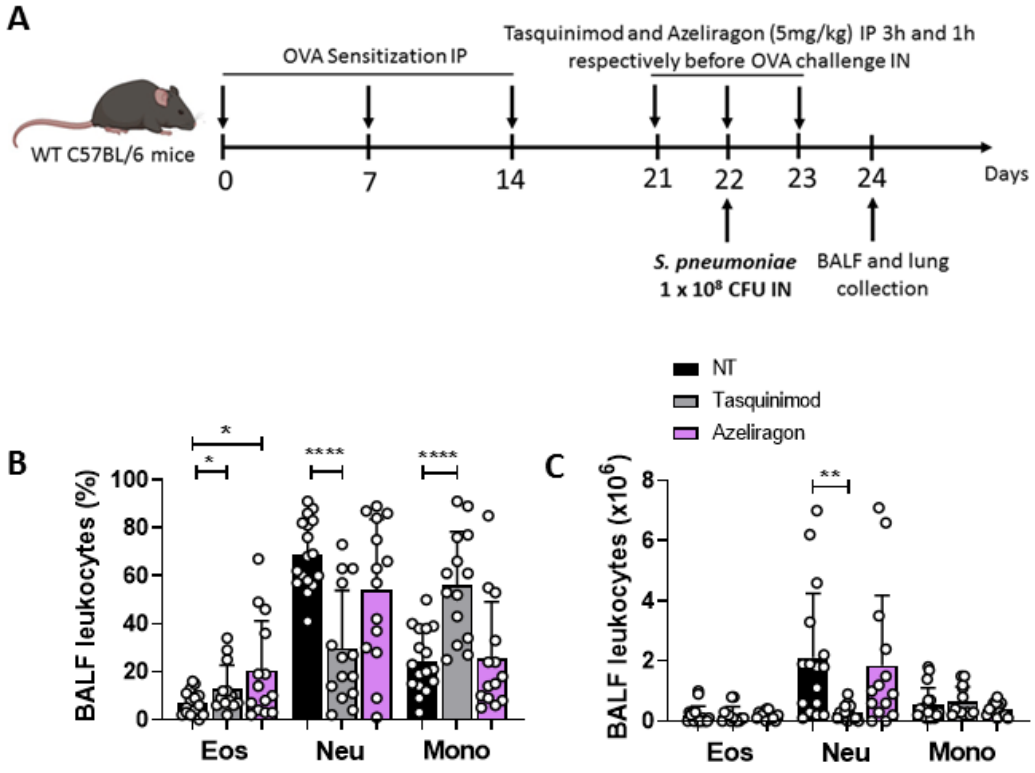


Figure 14. Evaluation of leukocytes in the BALF of animals treated with tasquinimod or azeliragon (A) C57BL/6 mice were sensitized and challenged with OVA and treated with tasquinimod or azeliragon 3h or 1h, respectively, before OVA challenges and infection with *S. pneumoniae*. **(B and C)** Differential and total count of BALF leukocytes. Data in B and C are representative from three independent experiments. Data are shown as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

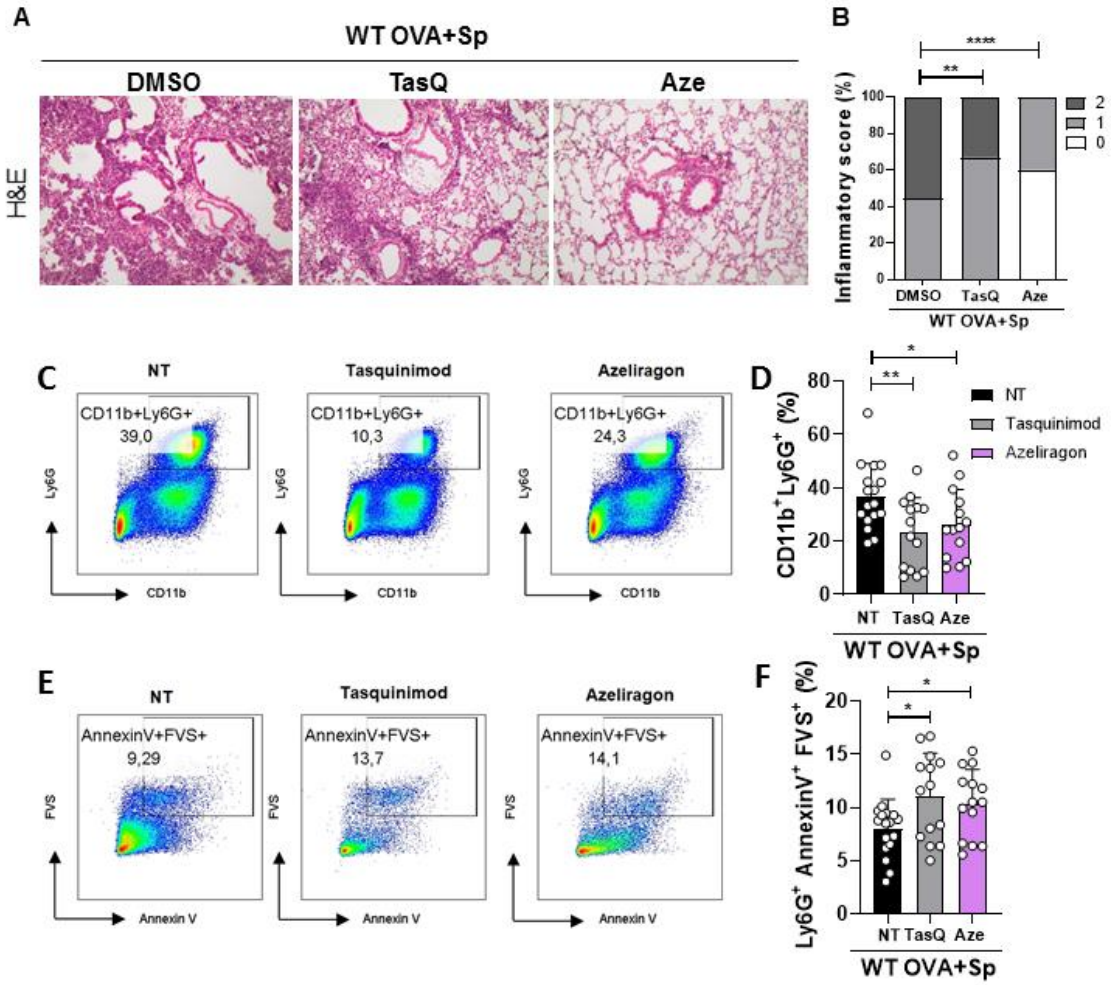


Figure 15 Histological analysis, neutrophils and neutrophil death in the lungs of animals treated with tasquinimod or azeliragon. (A) Histopathological analysis of the lungs. **(B)** Inflammation score. **(C and D)** Representative flow cytometry analysis and perentage of neutrophils in the lungs. **(E and F)**. Representative flow cytometry analysis and frequency of neutrophil apoptosis in the lungs. Data in B, D and F are representative from three independent experiments. Data are shown as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ****, $P < 0.0001$.

5.9. S100A9 and RAGE receptor inhibitions reduce NET production

Next we assessed the effect of tasquinimod or azeliragon on NET production and found that the MPO levels and the expression of extracellular MPO and citrullinated histone (H3Cit) in the lungs were reduced in response to tasquinimod or azeliragon treatments **(Fig. 16 A, B, C and D)**.

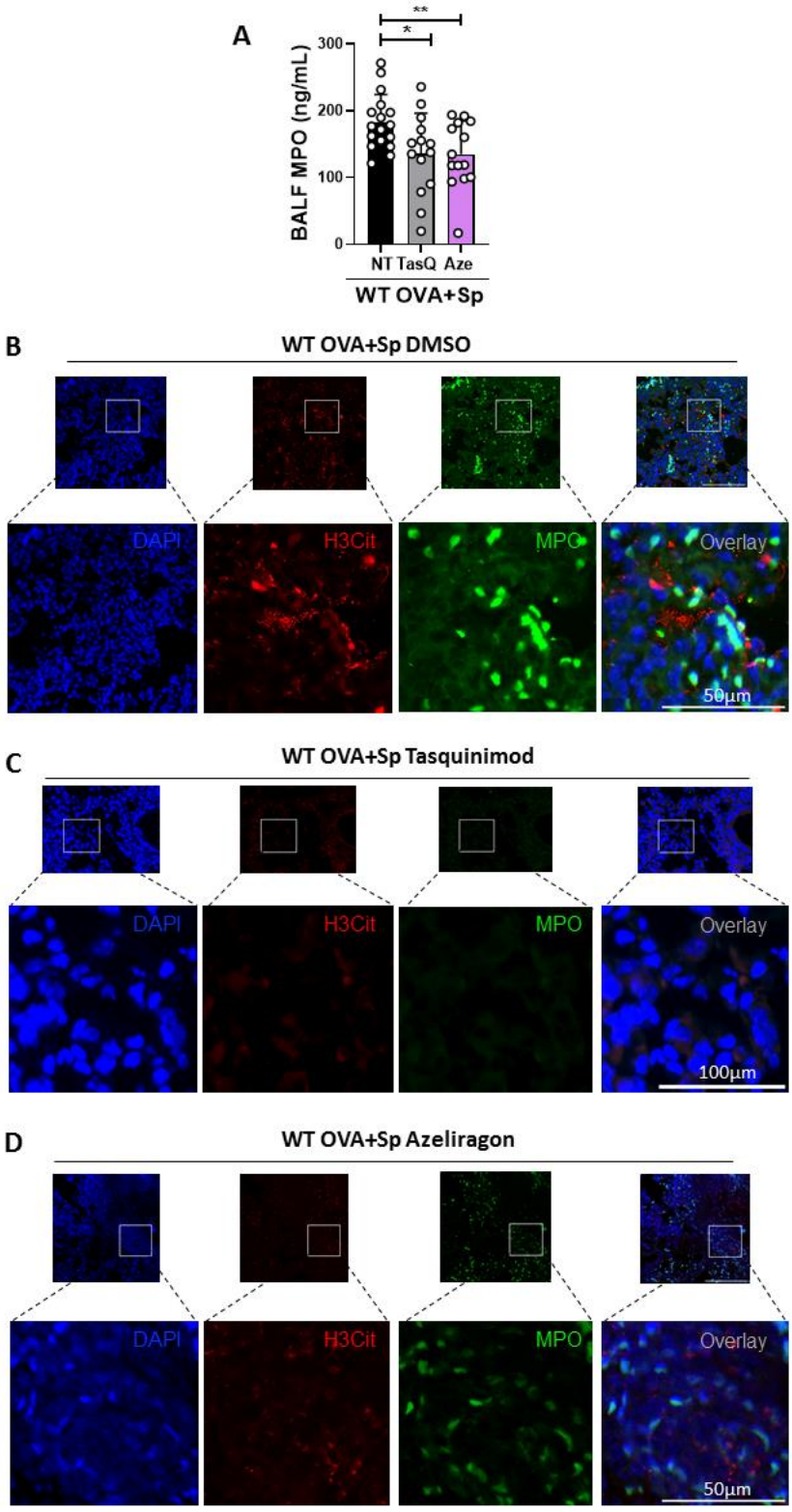


Figure 16. Evaluation of NET production in the lungs of animals treated with tasquinimod or azeliragon. (A) MPO concentrations in BALF supernatants. **(B, C and D)** Representative immunofluorescence analysis of NET production by lung neutrophils (DAPI, blue = nuclei), MPO (green), and H3Cit (red). Scale bar: 50 µm. Data in A is representative from three independent experiments. Data is shown as mean ± SEM. *, $P < 0.05$ **, $P < 0.01$.

6. Discussion

Most mouse model used to induce severe asthma are based on the administration of an usual OVA/CFA or OVA/LPS in mice (Quoc, 2021, Lee 2013, Lee 2017). In those asthma model, S100A9 was found as an alarmin that contribute to asthma exacerbation and was suggested as a therapeutic target for neutrophilic asthma (Lee et al., 2017; Quoc, 2021). However, these studies were performed with artificial condition, using adjuvants or part of bacteria, as LPS. Pneumonia caused by *S. pneumoniae* might improve or exacerbate asthma (Hartmann et al., 2015; Yang et al., 2015; Ray and Kolls, 2017; Fraga-Silva et al., 2023). The exacerbation in the comorbidity asthma and pneumococcal pneumonia could be due to the induction of neutrophilic inflammation. Here we established a model of comorbidity, a combination of two pulmonary diseases, infectious and allergic inflammation. Nethertheless, both asthma induction using OVA/CFA or OVA/LPS and our comorbidty model (OVA+*S. pneumoniae*) lead to neutrophilic inflammation. Besides, studies investigating the roles of S100A9 in allergic asthma have yielded conflicting results suggesting that the pro or anti-inflammatory role of this proteine may depend on the inhaled allergen that directs the inflammation in the lungs (Yin et al., 2010; Manni and Alcorn, 2019; Palmer et al., 2019). In experimental pneumococcal pneumonia, S100A9 was found to protect after infection (Filippo et al., 2014; Wilson et al., 2015). Regarding these functions of S100A9 in asthma and pneumococcal pneumonia, this alarmin may be related to the mechanisms of aggravation in the condition of comorbidity.

It has been reported that pulmonary infections might aggravate asthma, particularly rhinovirus infections, by a mechanism dependent on mixed recruitment of neutrophils and eosinophils (Message et al., 2008). In general, bacterial infections could protect against the development or exacerbation of asthma as postulated by the hygiene hypothesis (Strachan, 2000). However, infections caused by fungi and viruses and some bacteria are detrimental to asthma (Ray and Kolls, 2017). The host defense against pathogens depends on a plethora of factors, which involve the virulence of infectious agent, dose or inoculum, microenvironment and magnitude of inflammatory response that might induce tissue pathology.

It has already been shown that *S. pneumoniae* negatively regulates the allergic response in an OVA-induced asthma model and the attenuation of Th2 inflammation was associated with the induction of regulatory T cells (Hartmann et al., 2015). However, pneumococcus infection may also drive severe asthma through Th17 immune

responses and neutrophil infiltration (Yang et al., 2015; Ray and Kolls, 2017; Fraga-Silva et al., 2023).

Our group established a model of asthma and acute pneumonia comorbidity induced by *S. pneumoniae*, the bacteria that is the major cause of pneumonia. We showed that the comorbidity is characterized by neutrophilic and Th17 cell inflammation, and is resistant to treatment with corticosteroids (unpublished data). Using the comorbidity model we showed that pneumococcus stimulates the secretion of S100A9 alarmin, which induces the recruitment and survival of neutrophils that produce NET. The collective action of S100A9, neutrophils and NET induced by acute pneumonia aggravates the pulmonary inflammation that follows the allergen exposure. Moreover, the neutrophilic and Th17 cell inflammation that characterizes the asthma and acute pneumonia comorbidity is consistent with the phenotype of severe asthma.

Previous studies have reported a critical role of S100A9 in the pathogenesis of neutrophilic asthma induced by OVA/CFA or OVA/LPS in mice (Quoc, 2021, Lee 2017). Moreover, S100A9 was increased in sputum samples of asthma patients (Lee et al., 2013, 2017). Our results show that concomitant allergen exposure and pneumococcal infection lead to the induction of neutrophilic inflammation and S100A9 secretion. These results corroborate studies revealing an increased neutrophil counts and higher S100A9 levels in BALF and in the lungs in a neutrophilic asthma model induced by OVA/CFA or OVA/LPS compared to mice with eosinophilic and mixed granulocytic asthma (Lee et al., 2017; Quoc, 2021). Because S100A9 maintains neutrophil survival (Ryckman et al., 2003; Raquil et al., 2008) and neutrophils are resistant to corticosteroid-induced death (Liles et al., 1995), we evaluated neutrophil and eosinophil death and found reduced death of neutrophils in the lungs associated with increased conentrations of S100A9 in the OVA+Sp group. The deficiency of S100A9 or treatment with S100A9 antagonist resulted in the improvement of disease symptoms. Therefore, one of the reasons by which *S. pneumoniae* aggravates allergic asthma by inducing neutrophilic asthma is because the acute infection induces neutrophil recruitment associated with increased levels of CXCL-1 and S100A9, and S100A9 protects against neutrophil apoptosis.

S100A9 was predominantly secreted by neutrophils and epithelial cells during the comorbidity compared to mice exposed only to OVA. Indeed, the early production

of S100A9 by airway epithelial cells in response to infection by *S. pneumoniae* was already described (Raquil et al., 2008). S100A9 secreted by epithelial cells might engage TLR4 and RAGE in neutrophils inducing its own production that increase neutrophil recruitment (Kessel et al., 2013). These results corroborate the AEC-S100A9–neutrophil axis found in an OVA/LPS-induced asthma model resulting in increased airway hyperresponsiveness (Quoc, 2021) and reinforce the role of this molecule in the neutrophilic asthma.

As previously mentioned, S100A9 is known to induce recruitment, activation and survival of neutrophils, and we found a decrease in the frequency and in the number of lung neutrophils in animals deficient for S100A9 that were exposed to allergen and infected with *S. pneumoniae*. These results are similar to previous study in which the authors found a significant reduction in the percentage of neutrophils in an asthma model induced by OVA and CFA after treatment with anti-S100A9 monoclonal antibody compared to non-treated animals (Lee et al., 2017). Although the neutrophil recruitment was reduced, we observed that the eosinophil recruitment was significantly increased in the lungs of S100A9 deficient mice. Therefore, the deficiency of S100A9 changes the asthma phenotype to allergic or eosinophilic asthma even during acute pneumonia. Similarly, an increased recruitment of eosinophils was found in S100A9 deficient mice challenged with the allergen *Alternaria alternata*, suggesting that the absence of S100A9 increase the capacity of eosinophils to migrate to the lungs (Palmer et al., 2019). Despite the significant reduction of neutrophil observed in S100A9 deficient mice during the comorbidity, the concentration of MPO remains impaired compared to WT mice, suggesting that the absence of S100A9 might led to the migration of myeloid cells that produce MPO in response to *S. pneumoniae* infection.

TLR4 and RAGE are the receptors that engage S100A9 (Kessel et al., 2013). Therefore, we asked if one of them or both would be associated with the function played by S100A9. For that we used tasquinimod, that is a S100A9 antagonist, and we used RAGE antagonist (azeliragon). Studies investigating the effect of tasquinimod and azeliragon in cancer and alzheimer disease have yielded interesting results due to their anti-inflammatory effects (Raymond et al., 2014; Burstein et al., 2018). Both drugs were also tested in some diseases that affect the lungs. In a guinea pig granuloma model induced by the Bacillus Calmette–Guérin (BCG), increased numbers of lung granulomas were reduced when animals were treated with tasquinimod (Yoshioka et

al., 2016). However, RAGE inhibition using FPS-ZM1, a RAGE-specific blocker, did not result in decreased pulmonary inflammation in *Mycobacterium tuberculosis*-infected mice during the chronic phase of infection (Scott et al., 2020). However the action of both drugs in neutrophilic asthma has not already been tested. Using both drugs, tasquinimod and azeliragon that block the binding of S100A9 to its receptors, we found that the treatment with tasquinimod and azeliragon, reduced the frequency and number of neutrophils and NET production. This finding is corroborated by treatment with paquinimod, another inhibitor of S100A9, which resulted in a significant reduction of neutrophilic inflammation in the BALF and lung inflammation in an asthma model indued by OVA and CFA (Lee et al., 2021). Similar results were also found in COVID-19 pathogenesis in which treatment with paquinimod induced the suppression of aberrant neutrophils in the lungs accompanied by the improvement of disease symptoms, whereas inhibition of the RAGE pathways using azeliragon did not significantly prevent the recruitment of aberrant neutrophils (Guo et al., 2021).

Neutrophil recruitment in the airways during the comorbidity asthma and acute pneumonia was also accompanied by increased NET production compared to animals only exposed to OVA. There is evidence that DNA extracellular traps are released in human asthmatic airways (Dworski et al., 2011). The increase of NET production is quite possibly due to acute infection that is able to induce the production of NET (Beiter et al., 2006). The increase in the magnitude of NET in the lungs may contribute to asthma exacerbation. It should also be noted that $Ly6G^{Low}$ neutrophils significantly reduced during the comorbidity. These $Ly6G^{Low}$ neutrophils may present immature characteristics, reported as aberrant (Silvin et al., 2020; Wilk et al., 2020). Our results demonstrated that *S. pneumoniae* acute infection induces the migration of $Ly6G^{High}$ neutrophils to the lung, neutrophils capable of performing their functions, in contrast to $Ly6G^{Low}$ aberrant neutrophils observed in non-infected OVA mice.

The treatment with BB Cl-amidine, an NET inhibitor, confirmed the participation of NET in the neutrophilic asthma induced by *S. pneumoniae* acute infection. BB Cl-amidine is known to inhibit PAD4, a molecule that is involved in chromatin decondensation and NET production, thereby impeding NET formation. These results were consistent with a recent study showing that the treatment with Cl-amidine was followed by a significant reduction of NET production and lung inflammation in a murine asthma model induced by a pro-allergic low dose of

lipopolysaccaride (LPS) before exposure to House Dust Mite (HDM) (Radermecker et al., 2019).

Thus, our study show for the first time that tasquinimod, azeliragon and BB Cl-amidine might be potential drugs to dampen *S. pneumoniae*-induced difficult-to-treat asthma due to their capacity to reduce neutrophilic inflammation and NET production. Targeting NET was also associated to reduced lung inflammation. Thereby S100A9 and NET might be a therapeutic targets in severe asthma triggered by *S. pneumoniae* infection.

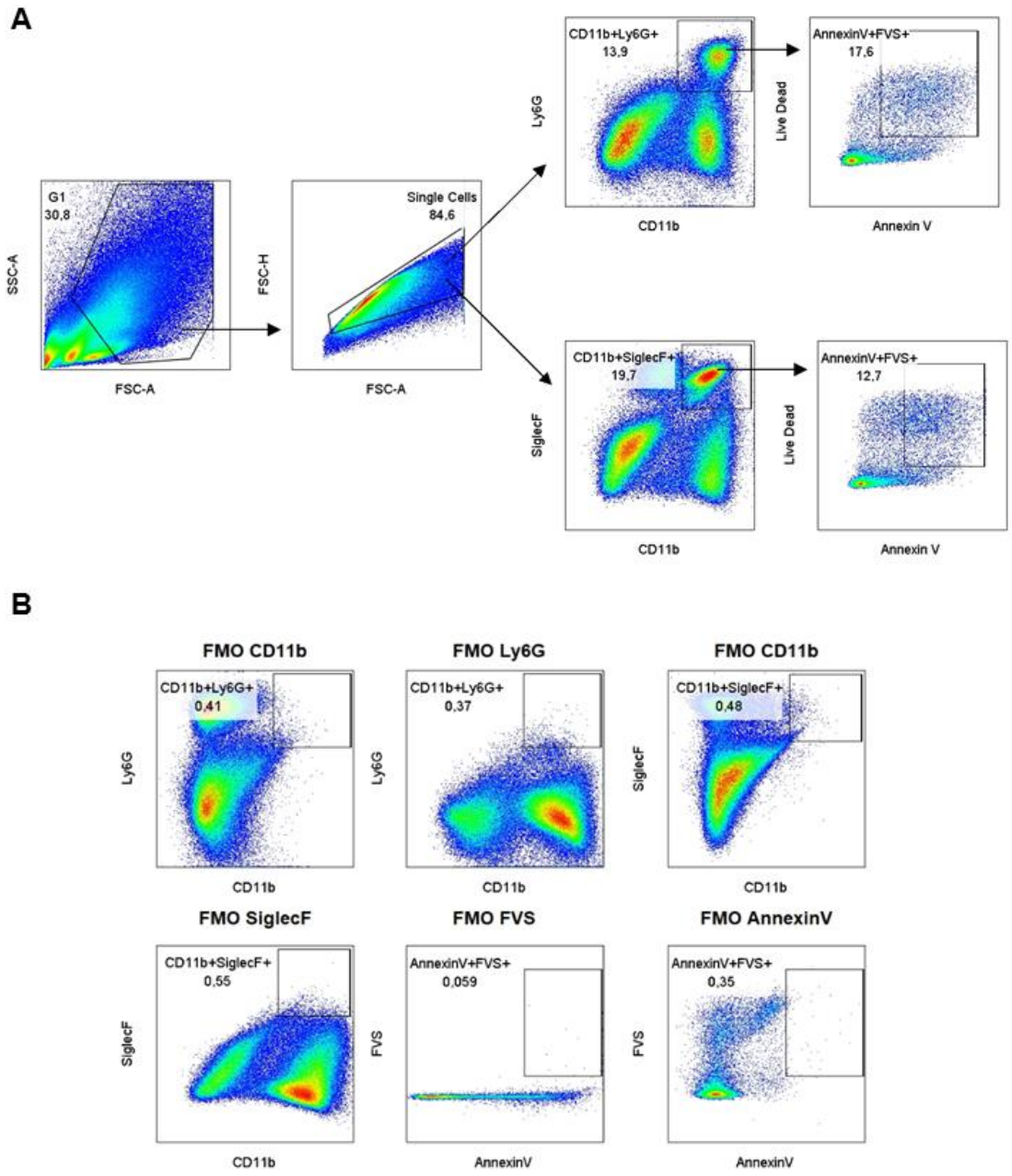
It has been reported that paquinimod induce the reduction of $CD4^+$ T cells, $CD115^+$ Ly6Chi monocytes, and $CD11b^+$ $F4/80^+CD206^+$ macrophages through modification of NKT-II cells (Fransén et al, 2018). Therefore the action of paquinimod in these immune response could be investigated.

The limitation of this study is the lack of additional difficult-to-treat asthma model induced by other bacteria strain. The role of S100A9 in other asthma related comorbidity also require investigations.

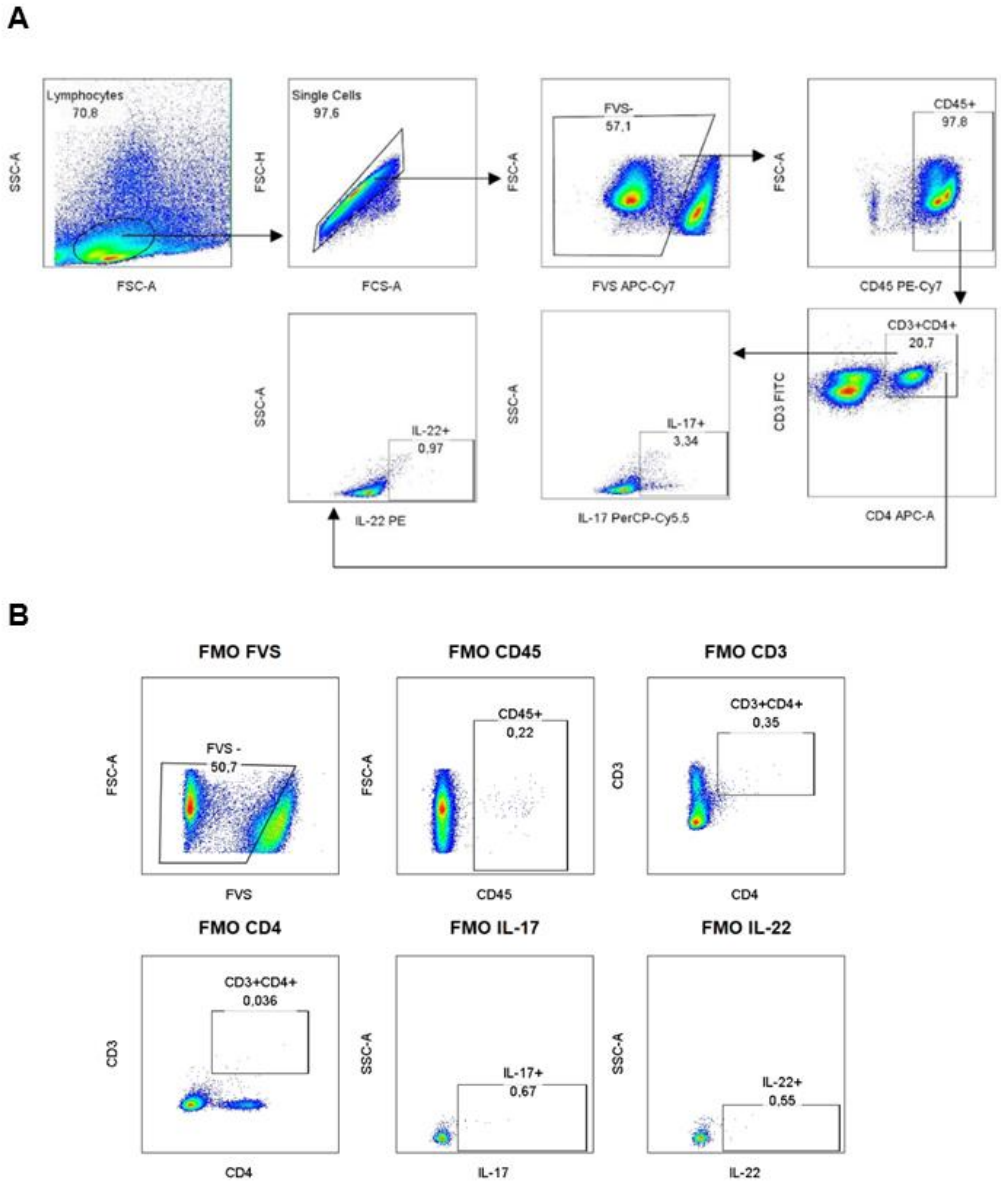
7. Conclusions

Acute *S. pneumoniae* infection is detrimental for asthma. Acute pneumonia exacerbates pulmonary inflammation by a mechanism dependent on S100A9 production that induces neutrophil recruitment and survival, and NET production in the lungs. S100A9 blocking reduces both neutrophilic inflammation and the survival of neutrophils in the asthma and acute pneumonia comorbidity. These results highlight the potential use of S100A9 antagonists in the treatment of asthma that courses with neutrophilic inflammation.

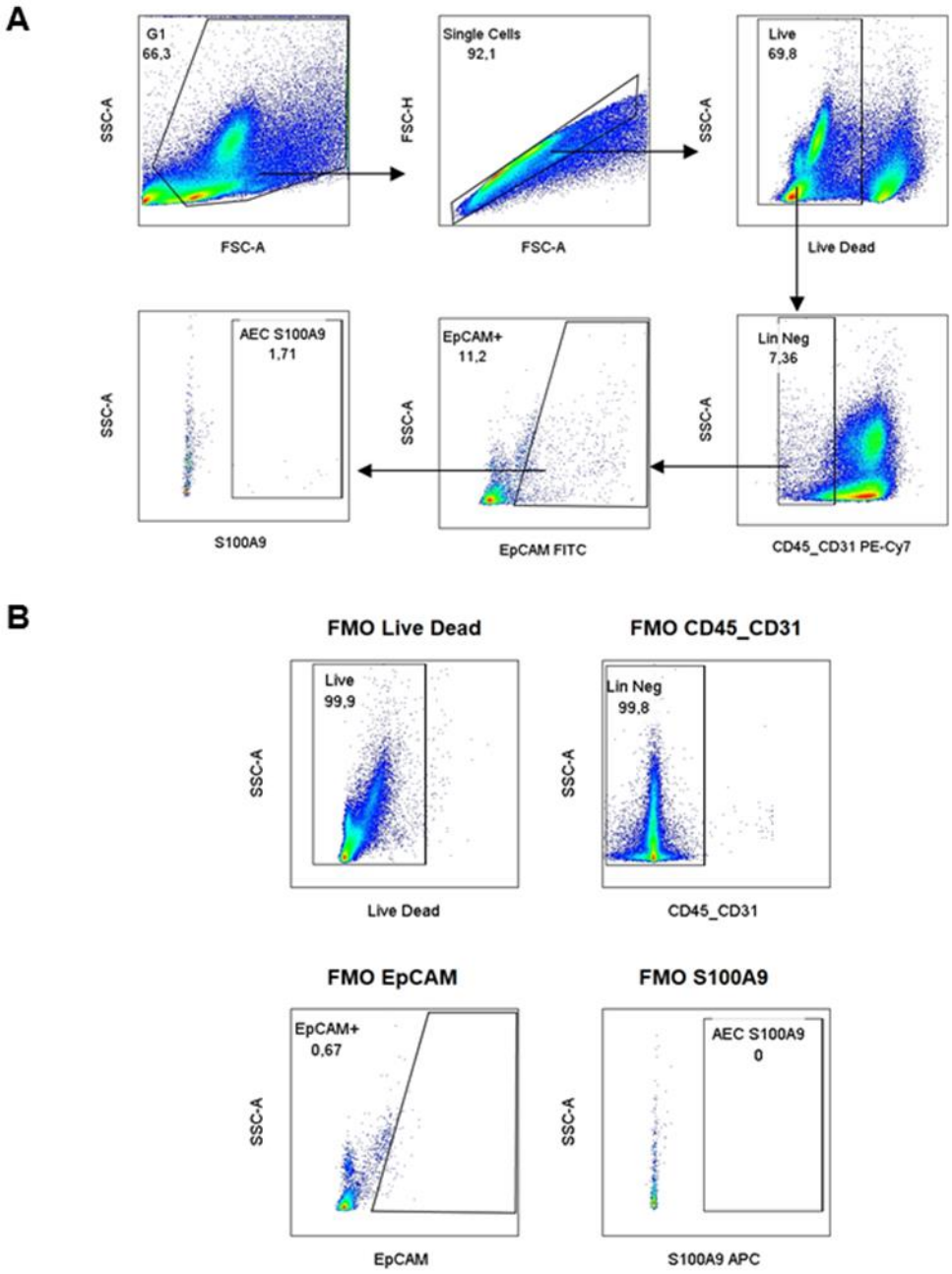
8. Supplementary figures



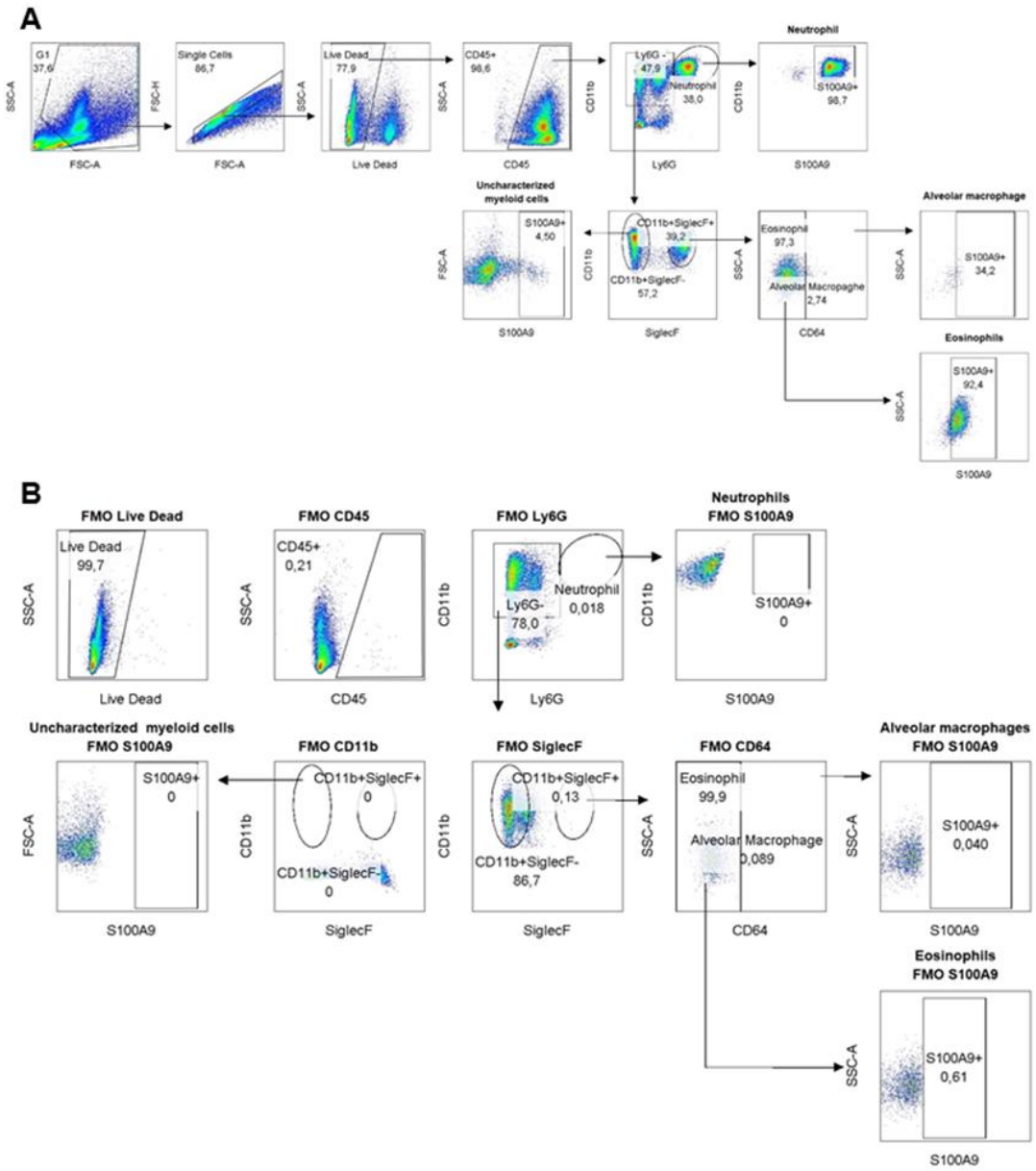
Supplementary figure 1. Neutrophils and eosinophils death characterization by flow cytometry. (A) Representative gate strategy for neutrophils and eosinophils characterization. First, polymorphonuclear leukocytes were discriminated by size (FSC-A) and internal complexity (i.e. granularity) (SSC-A). Then, single cells (FSC-H and FSC-A) were gated to evaluate the percentage of neutrophils ($CD11b^{+}Ly6G^{+}$) and eosinophils ($CD11b^{+}Siglec\text{-}F^{+}$). Finally, the percentage of death ($AnnexinV^{+}FVS^{+}$) was evaluated on neutrophils and eosinophils. **(B)** Fluorescence minus one (FMO, i.e. all fluorochromes used in the experiment except one) for neutrophils and eosinophils characterization showing one FMO control for each fluorochrome.



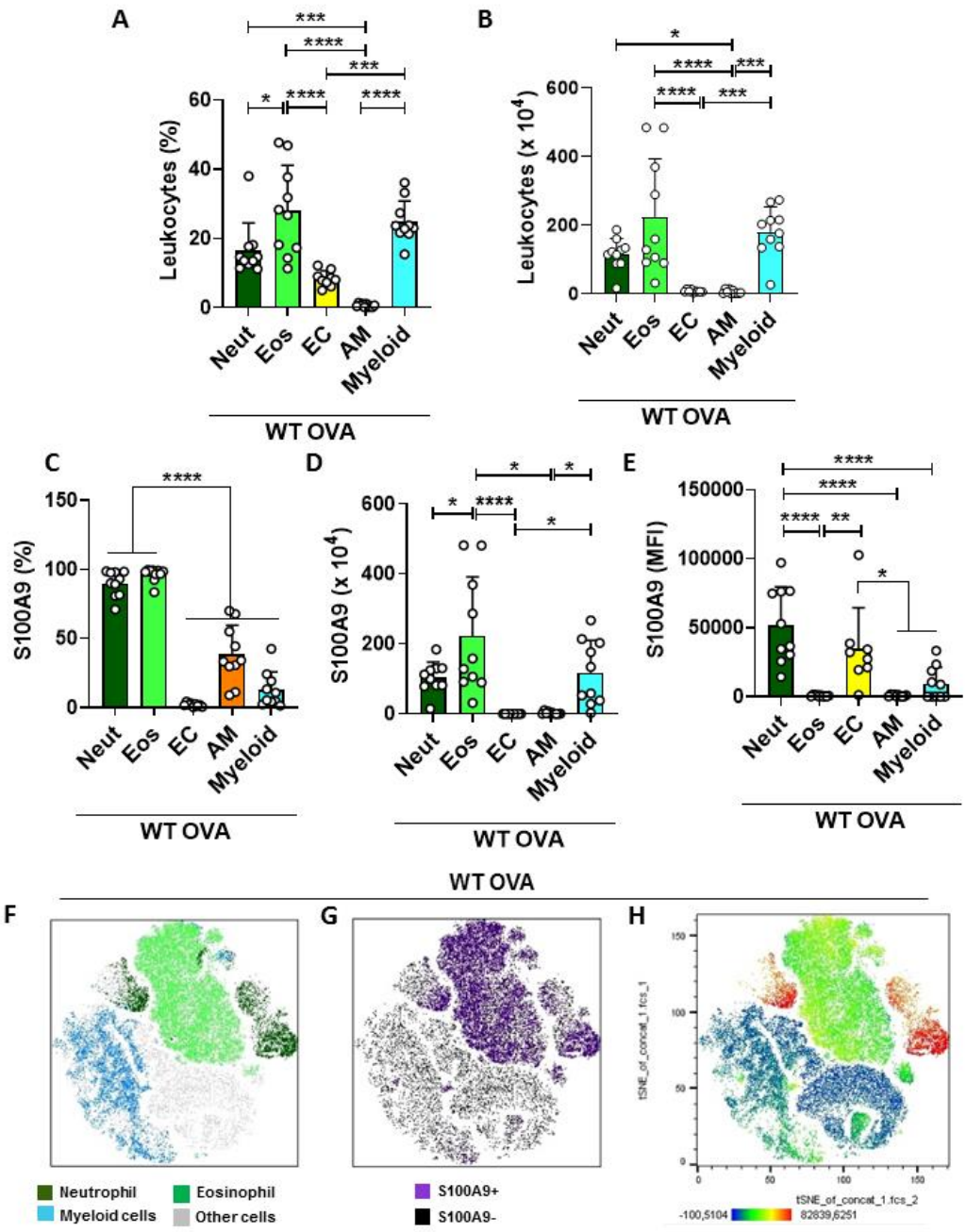
Supplementary figure 2. Characterization of IL-17 and IL-22 produced by T lymphocytes using flow cytometry. (A) Representative gate strategy for T lymphocytes and cytokine production. First, lymphocytes were discriminated by size (FSC-A) and internal complexity (i.e. granularity) (SSC-A). Then, single (FSC-H and FSC-A) and live (FVS^-) cells were gated to evaluate the percentage of total leukocytes ($CD45^+$). The percentage of T lymphocytes ($CD3^+CD4^+$), gated on total leukocytes, was used to evaluated intracellular production of IL-17 or IL-22. **(B)** Fluorescence minus one (FMO, i.e. all fluorochromes used in the experiment except one) for IL-17 and IL-22 producing T lymphocytes characterization showing one FMO control for each fluorochrome.



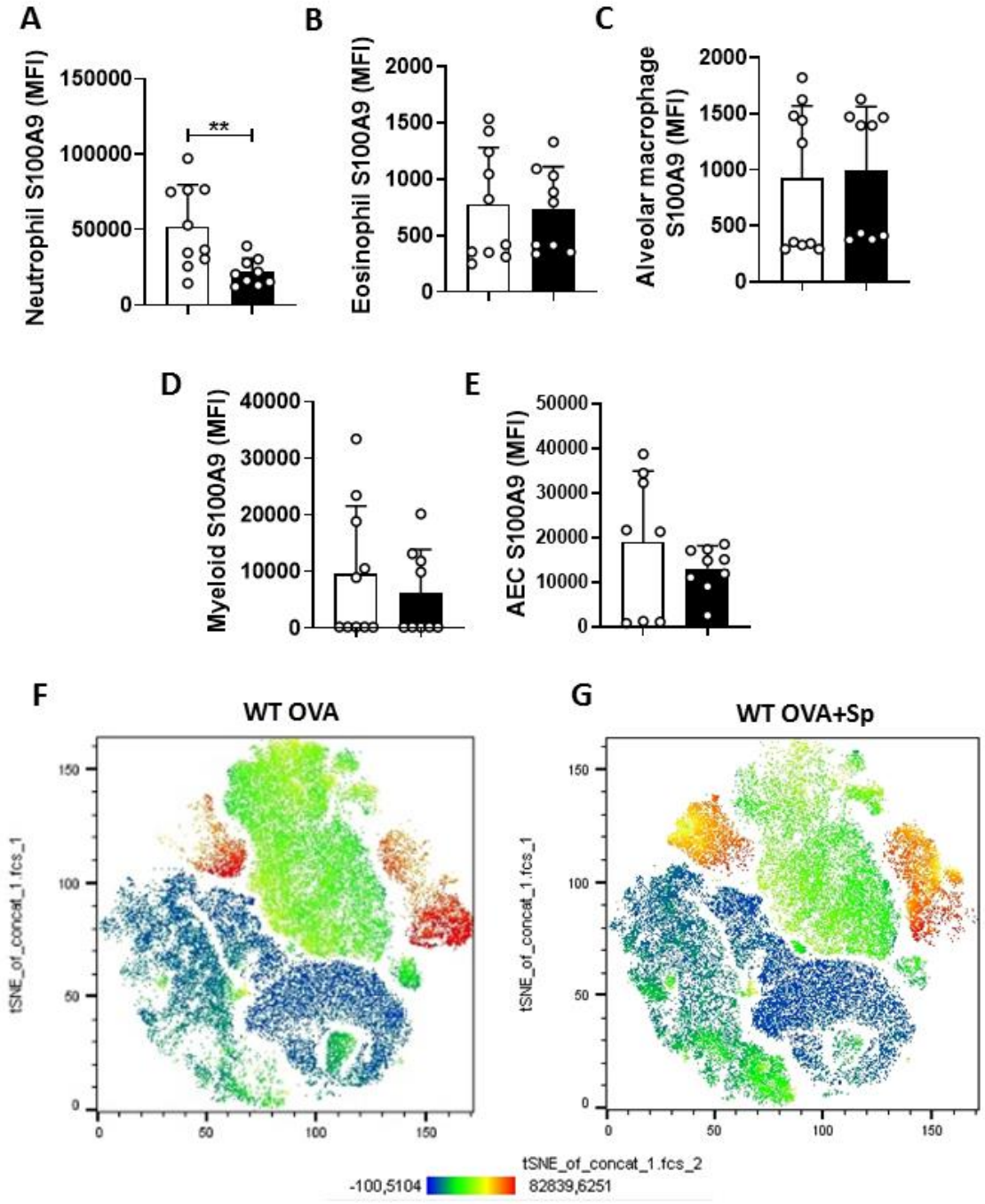
Supplementary figure 3. Characterization of S100A9 produced by airway epithelial cells using flow cytometry. (A) Representative gate strategy for airway epithelial cells (AEC) and S100A9 production. First, whole cells were discriminated from cell debris by size (FSC-A) and internal complexity (i.e. granularity) (SSC-A). Then, single (FSC-H and FSC-A) and live (Live/Dead$^-$) cells were gated to excluded leukocytes and endothelial cells (CD45$^-$CD31$^-$), called leneage negative (Lin Neg). The percentage of AEC (EpCAM$^+$), gated on Lin Neg, was used to evaluated intracellular production of S100A9. **(B)** Fluorescence minus one (FMO, i.e. all fluorochromes used in the experiment except one) for AEC characterization showing one FMO control for each fluorochrome.



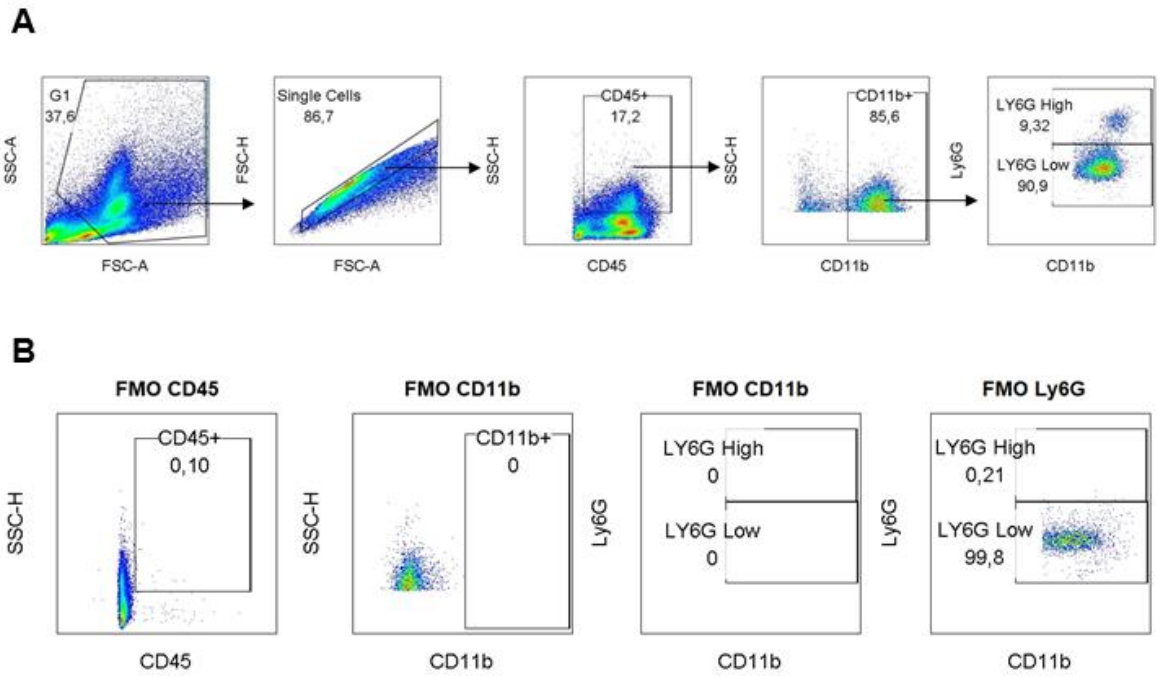
Supplementary figure 4. Characterization of S100A9 produced by myeloid cells using flow cytometry. (A) Representative gate strategy for lung cells and S100A9 production. First, myeloid cells were discriminated from cell debris and lymphocytes by size (FSC-A) and internal complexity (i.e. granularity) (SSC-A). Then, single (FSC-H and FSC-A) and live (Live/Dead$^-$) cells were gated to evaluate the percentage of total leukocytes (CD45$^+$). The percentage of neutrophils (CD11b$^+$Ly6G$^+$), gated on total leukocytes, was used to evaluated intracellular production of S100A9. Uncharacterized myeloid cells were gated on non-neutrophils (CD11b$^+$Ly6G$^-$) and non-eosinophils (CD11b$^+$Siglec-F$^-$) to evaluated intracellular production of S100A9. The percentage of eosinophils (CD11b$^+$Siglec-F$^+$CD64$^-$) and alveolar macrophages (CD11b$^+$Siglec-F$^+$CD64$^+$), gated on total non-neutrophils (CD11b$^+$Ly6G$^-$), was used to evaluated intracellular production of S100A9. **(B)** Fluorescence minus one (FMO, i.e. all fluorochromes used in the experiment except one) for myeloid cells characterization showing one FMO control for each fluorochrome. This gate strategy was used to construct t-distributed stochastic neighbor embedding (t-SNE) analysis.



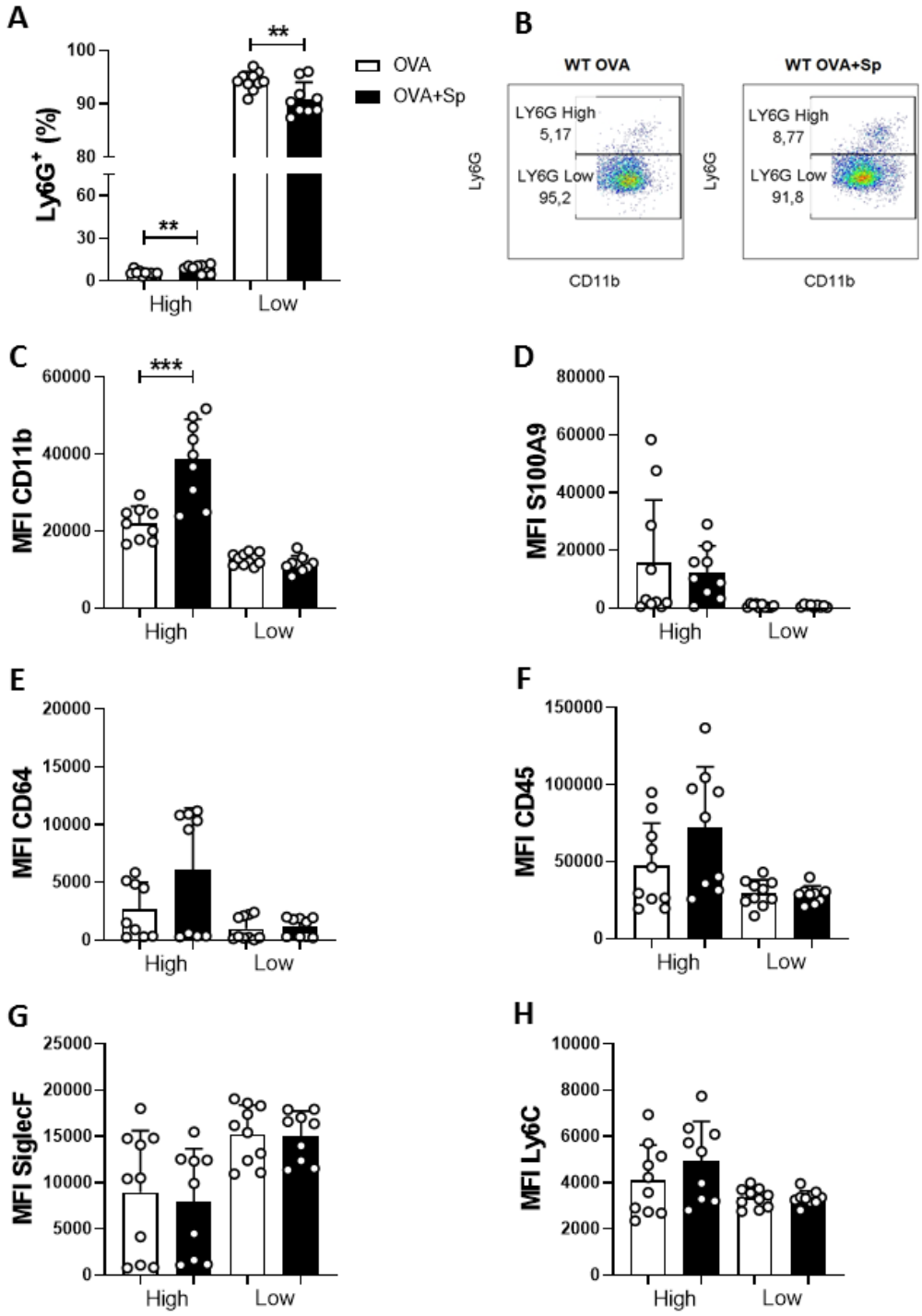
Supplementary figure 5. Evaluation of S100A9 production by epithelial cells and leukocytes in WT OVA mice. (A, B, C, D and E) Percentage of lung leukocytes, S100A9^{+} cells and S100A9 expression in those cells determined by flow cytometry. **(F, G and H)** *t*SNE plots depicting the percentage of lung leukocytes, S100A9^{+} cells and S100A9 expression in those cells determined by flow cytometry. Data are from two independent experiments. Data are shown as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$. ****, $P < 0.0001$.



Supplementary figure 6. S100A9 expression in lung cells in WT OVA and WT OVA+Sp groups. (A, B, C, D and E) S100A9 expression in lung leukocytes. **(F and G)** *t*SNE plots depicting S100A9 expression in OVA and OVA+Sp groups. Data in A, B, C, D and E are from two independent experiments. Data are shown as mean ± SEM. *, $P < 0.05$, ****, $P < 0.0001$.



Supplementary figure 7. Neutrophil populations characterization using flow cytometry. (A) Representative gate strategy for lung cells and neutrophil populations characterization. First, myeloid cells were discriminated from cell debris and lymphocytes by size (FSC-A) and internal complexity (i.e. granularity) (SSC-A). Then, single (FSC-H and FSC-A) and leukocytes ($CD45^+$) cells were gated). The percentage of $CD11b^+$ cells, gated on total leukocytes, was used to evaluated $Ly6G^+CD11b^+$ cells. **(B)** Fluorescence minus one (FMO, i.e. all fluorochromes used in the experiment except one) for neutrophil populations characterization showing one FMO control for each fluorochrome.



Supplementary figure 8. Evaluation of aberrant neutrophils in the lung during the comorbidity. (A) Percentage of conventional ($CD45^{+}CD11b^{+}Ly6G^{High}$) and aberrant ($CD45^{+}Ly6G^{+}Ly6G^{Low}$) neutrophils in the lungs determined by flow cytometry. **(B)** Representative flow cytometry analysis of aberrant neutrophil in the lungs determined by flow cytometry. **(C, D, E, F, G and H)** Expression of CD11b, S100A9, CD64, CD45, SiglecF and Ly6C determined by flow cytometry. Data in A, C, D, E, F, G and H are representative of two independent experiments. Data are shown as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ****, $P < 0.0001$.

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10. Annex

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Article

IL-22 Is Deleterious along with IL-17 in Allergic Asthma but Is Not Detrimental in the Comorbidity Asthma and Acute Pneumonia

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Abstract: There is evidence that IL-22 and IL-17 participate in the pathogenesis of allergic asthma. To investigate the role of IL-22, we used IL-22 deficient mice (IL-22 KO) sensitized and challenged with ovalbumin (OVA) and compared with wild type (WT) animals exposed to OVA. IL-22 KO animals exposed to OVA showed a decreased number and frequency of eosinophils, IL-5 and IL-13 in the airways, reduced mucus production and pulmonary inflammation. In addition, IL-22 KO animals exhibited a decreased percentage and number of lung $CD11c^+CD11b^+$ cells and increased apoptosis of eosinophils. Th17 cell transfer generated from IL-22 KO to animals previously sensitized and challenged with OVA caused a reduction in eosinophil frequency and number in the airways compared to animals transferred with Th17 cells generated from WT mice. Therefore, IL-22 is deleterious with concomitant secretion of IL-17. Our findings show a pro-inflammatory role for IL-22, confirmed in a model of allergen-free and allergen-specific immunotherapy. Moreover, during the comorbidity asthma and pneumonia that induces neutrophil inflammation, IL-22 was not detrimental. Our results show that targeting IL-22 would negatively affect the survival of eosinophils, reduce the expansion or migration of $CD11c^+CD11b^+$ cells, and negatively regulate allergic asthma.

Keywords: asthma; IL-22; IL-17; eosinophil; dendritic cell; apoptosis

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1. Introduction

Asthma affects approximately three hundred million people worldwide [1]. The disease is characterized by bronchial hyperresponsiveness and airflow limitation, caused by airway inflammation [2]. There are different asthma phenotypes. Allergic asthma is characterized by type 2 inflammation, which follows with Th2 cell activation and production of IL-4, IL-5 and IL-13 that cause eosinophilia, pulmonary eosinophilic inflammation, and mucus overproduction, leading to airway dysfunction [3,4]. These characteristics causes coughing, wheezing, and pressure in chest [1]. Severe asthma or type 2 low is characterized by pulmonary neutrophilic inflammation or granulocytic inflammation, Th17 activation and, generally, resistance to corticosteroid treatment [4–6].

Studies have shown that Th17 cells may accentuate the severity of the disease, especially in patients who have an intense neutrophil infiltrate in the lungs [4,7]. IL-17A secreted by Th17 cells was detected in samples of bronchial epithelial tissue from patients with more severe symptoms of asthma [8,9]. IL-22, another cytokine of Th17 cells, was also elevated in the serum of asthmatic patients compared to healthy individuals [7,10]. IL-22 acts on epithelial cells, enhances lung tissue integrity and mediates defense against airway infections [11–15]. IL-22 is critical in maintaining the intestinal barrier because it induces the expression of anti-apoptotic proteins responsible for the survival of epithelial cells in the intestine [16]. Moreover, IL-22 production might be regulated by intestinal bacteria [17] and IL-22 modulates intestinal microbiota [18,19].

IL-22 might play a pro- or anti-inflammatory role depending on the microenvironment [10,20,21]. In the airway inflammation after allergen exposure, IL-22 functions are still controversial. The neutralization of IL-22 positively modulated eosinophil recruitment to the lungs in an ovalbumin (OVA)-induced asthma model [22]. Pretreatment of OVA-pulsed dendritic cells (DC) with recombinant IL-22 abolished the eosinophil recruitment induced by those non-treated and OVA-pulsed DC [22]. Administration of anti-IL-22 monoclonal antibody augmented eosinophil recruitment and IL-13 secretion while recombinant IL-22 reduced eosinophilic inflammation and indirectly decreased the secretion of IL-25 by airway epithelial cells [23]. These studies showed that IL-22 acts as a negative regulator of type 2 inflammation in the airways. The pro- or anti-inflammatory action of IL-22 was described to be time-dependent given that the neutralization of this cytokine during the sensitization with allergen or sensitization of IL-22 deficient mice ameliorated and reduced allergic inflammation, and decreased eosinophilic inflammation while recombinant IL-22 given during the allergen challenge was protective [10].

In an attempt to investigate the role of IL-22 in asthma, which is still controversial, we use IL-22 knockout mice to evaluate its role on airway inflammation, eosinophil survival and number of dendritic cells.

2. Results

2.1. IL-22 Has a Pro-Inflammatory Function in Airway Allergic Inflammation

To confirm the pro- or anti-inflammatory function of IL-22 in the airway allergic inflammation, WT and IL-22 KO mice sensitized and challenged with OVA had their lungs evaluated, as depicted in Figure 1A. The total number of cells in the BALF was quantified and showed a significant decrease in numbers in the BALF of IL-22 KO mice exposed to OVA (IL-22 KO OVA) compared to their counterpart WT (WT OVA) (Figure 1B). The frequency and the number of eosinophils and lymphocytes were also reduced in the BALF of the IL-22 KO OVA group compared to WT OVA animals (Figure 1C,D). There was no difference in the number of neutrophils, as they were too low in all groups, and macrophages among the groups (Figure 1C,D). Considering the decrease in BALF cells in IL-22 KO OVA animals, we evaluated the production of Th2 cytokines. Corroborating the data of lymphocytes, concentrations of IL-5 and IL-13 were also reduced in the BALF of IL-22 deficient mice exposed to the allergen (Figure 1E,F). In addition, the IL-22 KO OVA group exhibited reduced levels of circulating OVA-specific IgE compared to those detected in the WT OVA group (Figure 1G). Histological analysis of WT OVA group revealed increased inflammatory cells, including eosinophils (arrows) (Figure 2A-top panel). The inflammatory infiltrate was predominantly peribronchial (Figure 2A-bottom panel). WT animals exposed to OVA exhibited hyperplasia of goblet cells and mucus production (Figure 2B). IL-22 KO OVA group exhibited mild pulmonary inflammation (Figure 2A) and no mucus production (Figure 2B) compared to the respective WT group. However, the number of inflammatory cells in IL-22 deficient animals sensitized and challenged with OVA were increased compared to IL-22 deficient animals non-exposed to the allergen (Figure 2A).

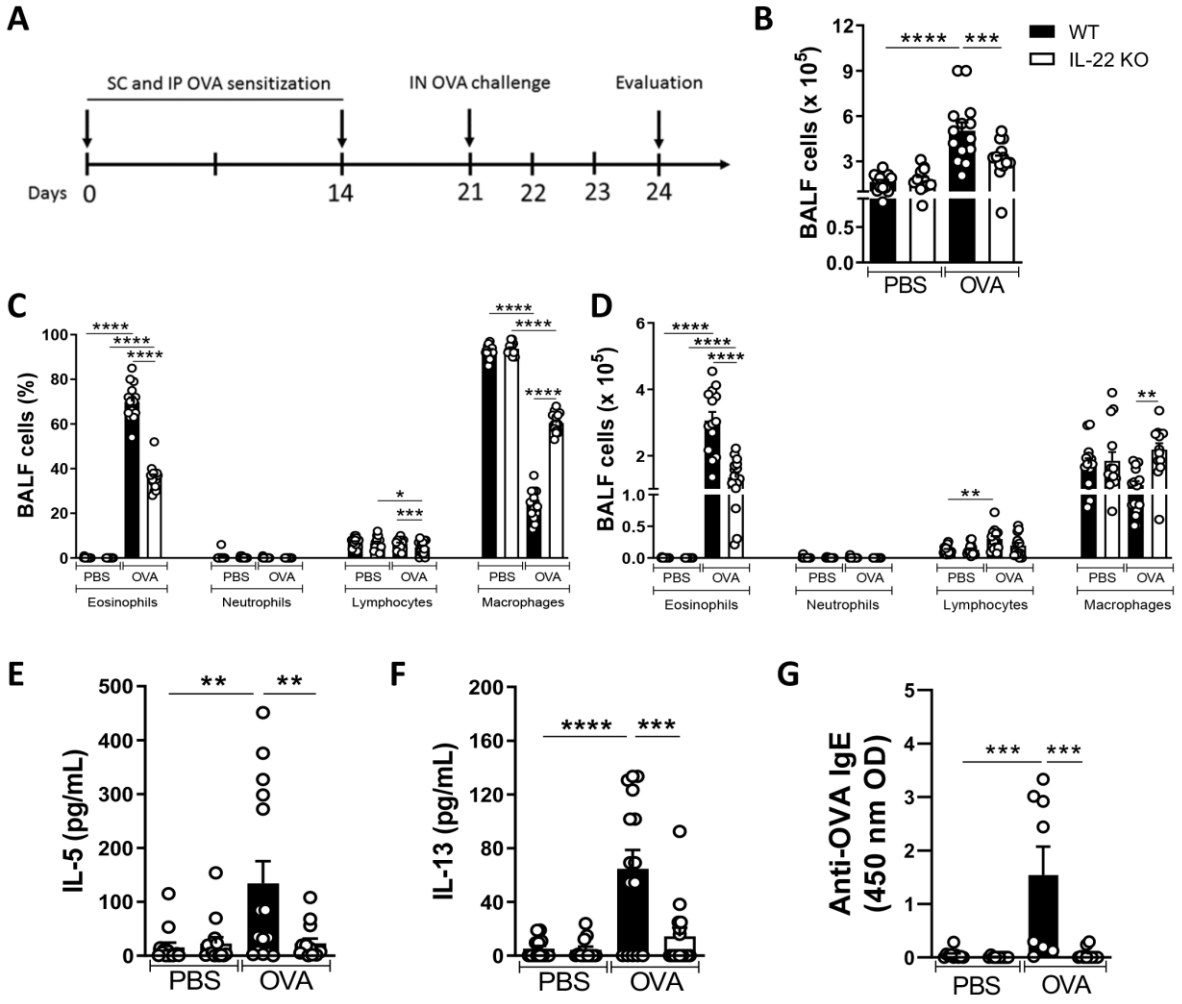

Figure 1. The allergic response is improved in the absence of IL-22. (**A**) WT and IL-22 KO mice were sensitized by administration of OVA (100 μg) and Aluminum Hydroxide (1.6 mg) by subcutaneous route (SC). On day 14, the animals received the second OVA sensitization (50 μg) via intraperitoneal route (IP). On day 21, the animals were challenged with OVA (100 μg) via the intranasal route (IN). (**B**) Total number of cells in BALF. (**C**,**D**) Differential and total count of BALF leukocytes. (**E**,**F**) Concentrations of IL-5 and IL-13 in the BALF supernatants. (**G**) Concentrations of anti-OVA IgE in the serum. The results are expressed as mean ± standard deviation of the individual values obtained for each experimental group. Results are representative of three independent experiments for BALF and cytokines and two independent experiments for antibodies. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. IP = intraperitoneal route, SC = Subcutaneous, IN = Intranasal.

These results attribute a pro-inflammatory function to IL-22 in the allergic airway inflammation given that airway allergic type 2 inflammation was impaired in IL-22 deficient mice.

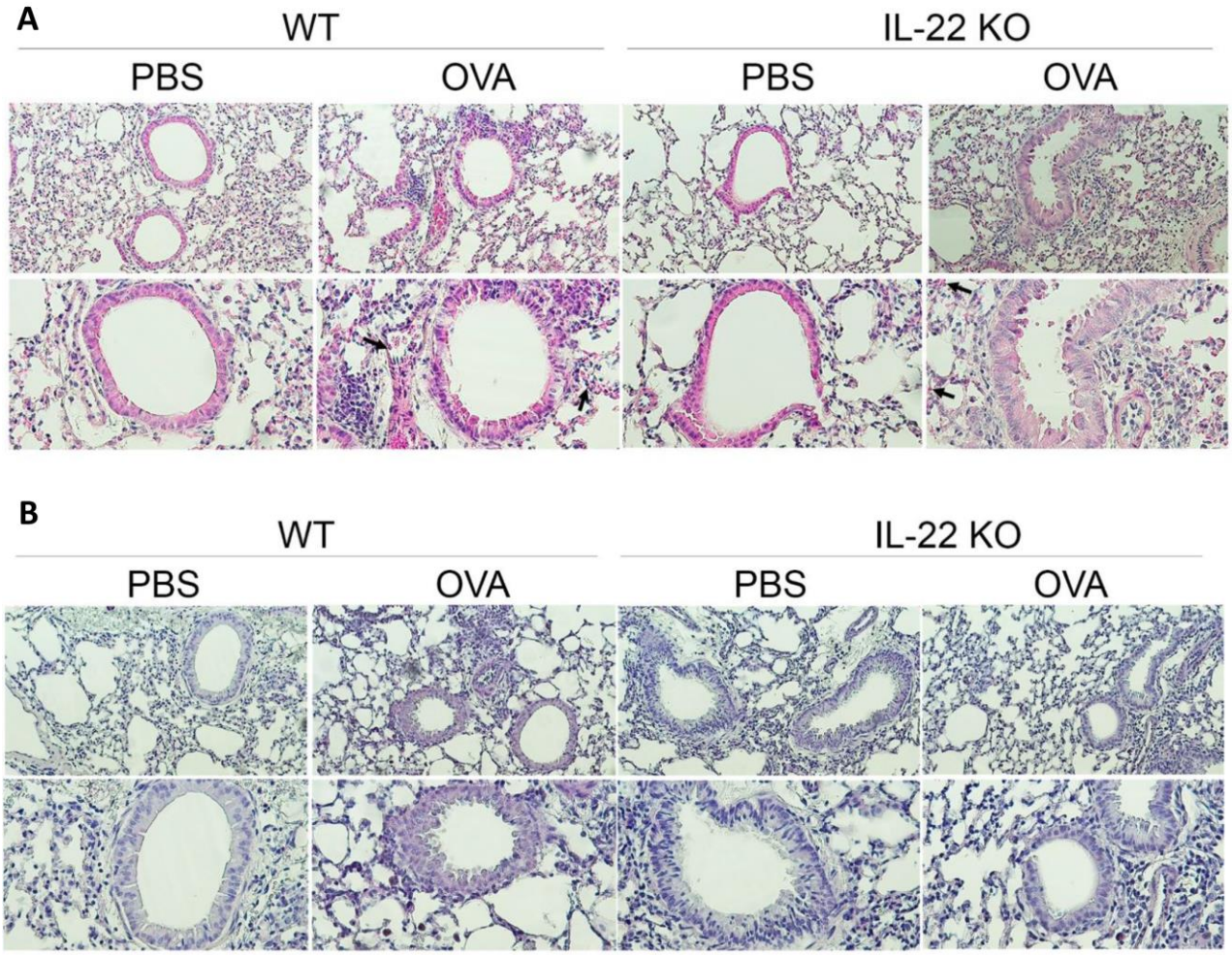

Figure 2. Reduction in pulmonary inflammation and mucus production in the absence of IL-22. (**A**) Histopathological analysis of the lungs. Arrows show eosinophils. (**B**) Mucus production. Results are representative of three independent experiments. Top = magnification 200×; bottom = magnification 400×.

2.2. Increase in IL-17 in the Lungs of Animals Exposed to the Allergen Is Not Affected by the Deficiency of IL-22

IL-17 and IL-22 are increased in the lungs and in the serum of asthmatics subjects compared to healthy subjects [7,10]. We evaluated the production of IL-17 in immune competent and in IL-22 deficient-mice exposed to the allergen. WT mice exposed to the allergen (WT OVA group) showed an increase in IL-22 and IL-17 concentrations in the BALF compared to the control group (WT PBS). IL-22 KO mice exposed to the allergen (IL-22 KO OVA) also secreted significant concentrations of IL-17 compared to their control counterpart (IL-22 KO PBS) (Figure 3A,B).

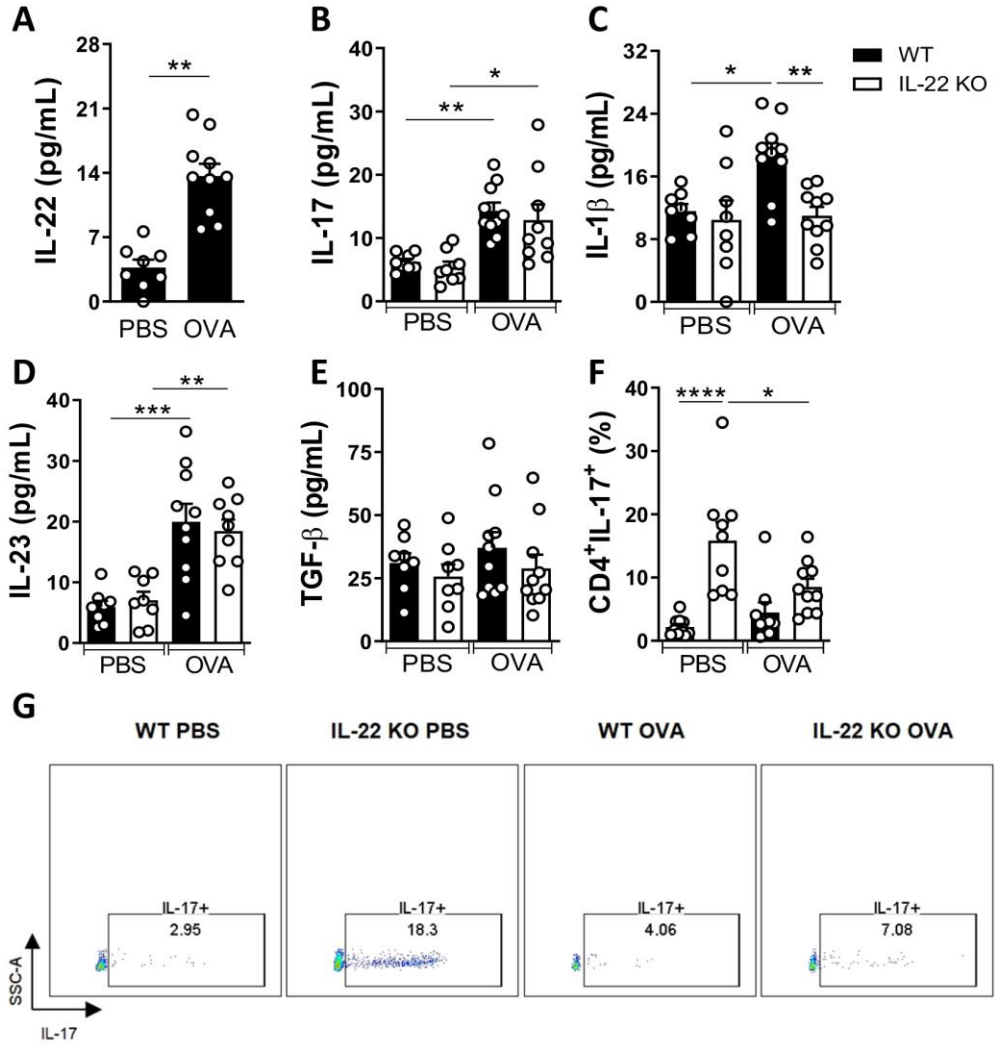

Figure 3. Th17 response is induced in allergic lung inflammation. (**A**,**B**) Concentrations of IL-22 and IL-17 in the BALF supernatants. (**C**–**E**) Concentrations of IL-1β, IL-23 and TGF-β in the BALF supernatants. (**F**,**G**) Frequency and representative analysis of $CD4^+IL\text{-}17^+$ cells, gated previously on $CD4^+$ cells, in the lungs of mice exposed or not (PBS) to the allergen (OVA). Results were expressed as mean ± standard deviation of the individual values obtained for each experimental group. Results are representative of two independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

IL-1β, described as an important cytokine in the Th17 cell differentiation [24], was enhanced in WT OVA group, but not in the IL-22 KO OVA group (Figure 3C). In addition, IL-23, which maintains the Th17 differentiation [25], was enhanced in both WT and IL-22 KO mice exposed to the allergen (Figure 3D). TGF-β concentrations were not altered among experimental groups (Figure 3E).

Next, we evaluated the frequency of IL-17-producing $CD4^+$ cells in the lungs and we found no difference between WT OVA and IL-22 KO OVA groups. However, the deficiency of IL-22 increased the frequency of IL-17-producing $CD4^+$ cells in the control group (PBS) compared to the WT PBS group (Figure 3F,G). Although the deficiency of IL-22 increases the frequency of IL-17-producing $CD4^+$ cells in steady-state (IL-22 KO PBS group), in the presence of allergens, IL-22 KO animals (IL-22 KO OVA group) exhibited lower frequency of IL-17-producing $CD4^+$ cells (Figure 3F,G).

2.3. Th17 Cells Negatively Regulate Eosinophil Recruitment in the Absence of IL-22

Neutralization of IL-17 in IL-22 KO mice exposed to the allergen restores eosinophil recruitment in the lungs [10]. In an attempt to confirm that the concomitant secretion

of IL-17 and IL-22 by Th17 cells play a pro-inflammatory role, we differentiated Th17 cells in vitro from naive $CD4^+$ lymphocytes obtained from spleens of WT or IL-22 KO mice and performed Th17 cell transfer at the moment of challenge with OVA (Figure 4A). First, we confirmed that Th17 cells differentiated from WT animals produced both IL-17 and IL-22 (Figure 4B–D). As expected, Th17 cells from IL-22 KO animals produced only IL-17 (Figure 4B–D). Th17 cell transfer from WT mice did not significantly affect total cell number, frequency and number of eosinophils in the BALF compared to mice sensitized and challenged with OVA with no cell transfer (Figure 4E–G). However, Th17 cell transfer from IL-22 KO mice to mice sensitized and challenged with OVA significantly reduced total cell number, frequency and number of eosinophils in the BALF (Figure 4E–G). These findings show that IL-17 and IL-22 act concomitantly to induce a type 2 inflammation in allergic asthma.

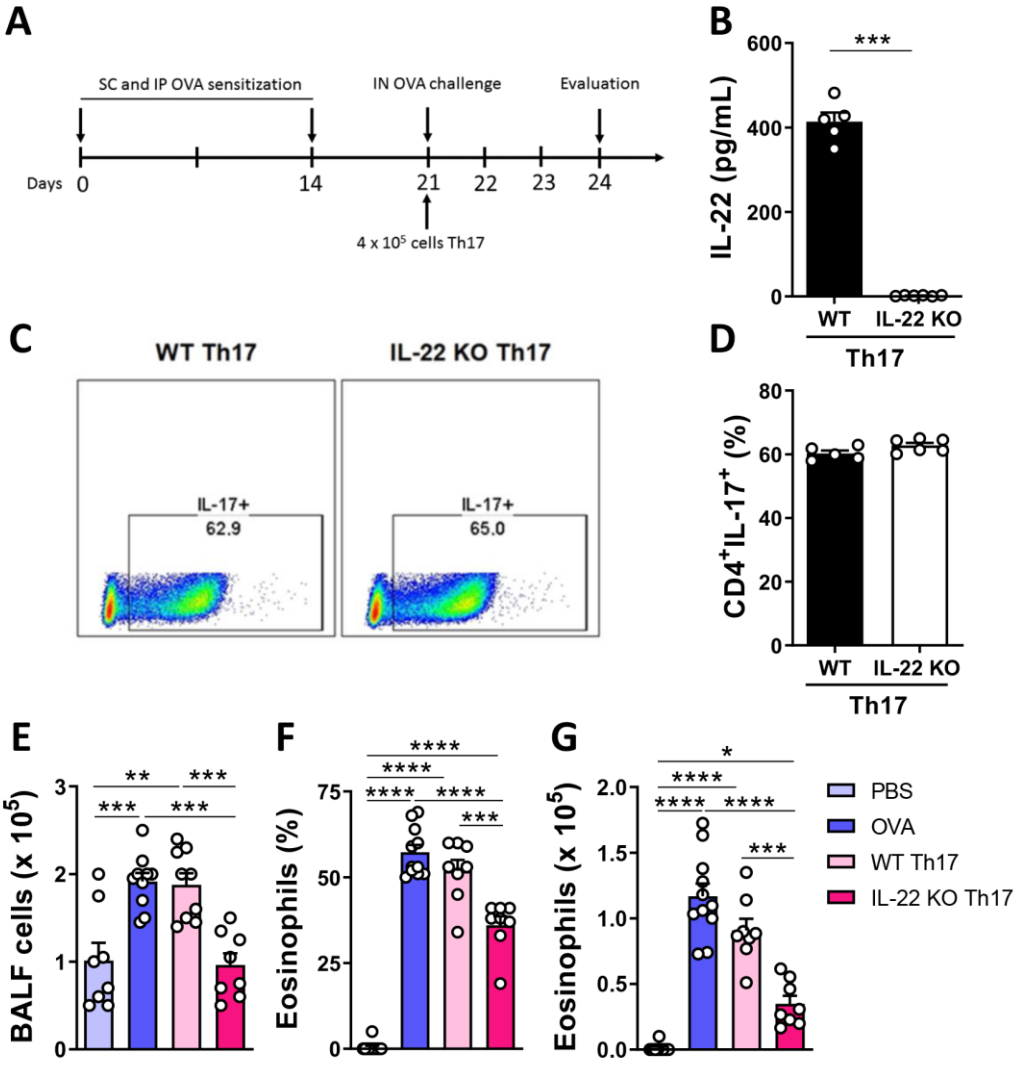

Figure 4. In the absence of IL-22, Th17 cells are protective in allergic asthma. (**A**) WT animals were sensitized and challenged, as previously described, and during the challenge, they received 4×10^5 Th17 cells differentiated from naive $CD4^+CD62L^+$ cells from the spleen of WT or IL-22 KO animals. (**B**) Concentrations of IL-22 secreted by differentiated Th17 cells in vitro. (**C**,**D**) Representative flow cytometry analysis and frequency of differentiated Th17 cells in vitro. (**E**) Total number of cells in the BALF. (**F**,**G**) Frequency and total number of eosinophils in the BALF. Results are expressed as mean ± standard deviation of the individual values obtained for each experimental group. Results are representative of two independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. IP = intraperitoneal route, SC = Subcutaneous, IN = Intranasal.

2.4. IL-22 Increases the Frequency of Lung CD11c⁺CD11b⁺ Dendritic Cells and Promotes Viable Eosinophils

IL-22-treated and OVA-pulsed DC impaired the recruitment of eosinophils to the airways [22]. Given that IL-22 affects the role of dendritic cells, we asked whether IL-22 plays a role in the DC infiltration of the airways following allergen exposure. We found a decrease in the frequency and in the number of $CD11c^+CD11b^+$ DC, which is the DC subset that orchestrates Th2 immune response in asthma [26,27], in the lungs of IL-22 deficient mice exposed to OVA compared to the WT OVA group (Figure 5A–C). No differences were observed in the frequency of $CD11c^+CD103^+$ DC.

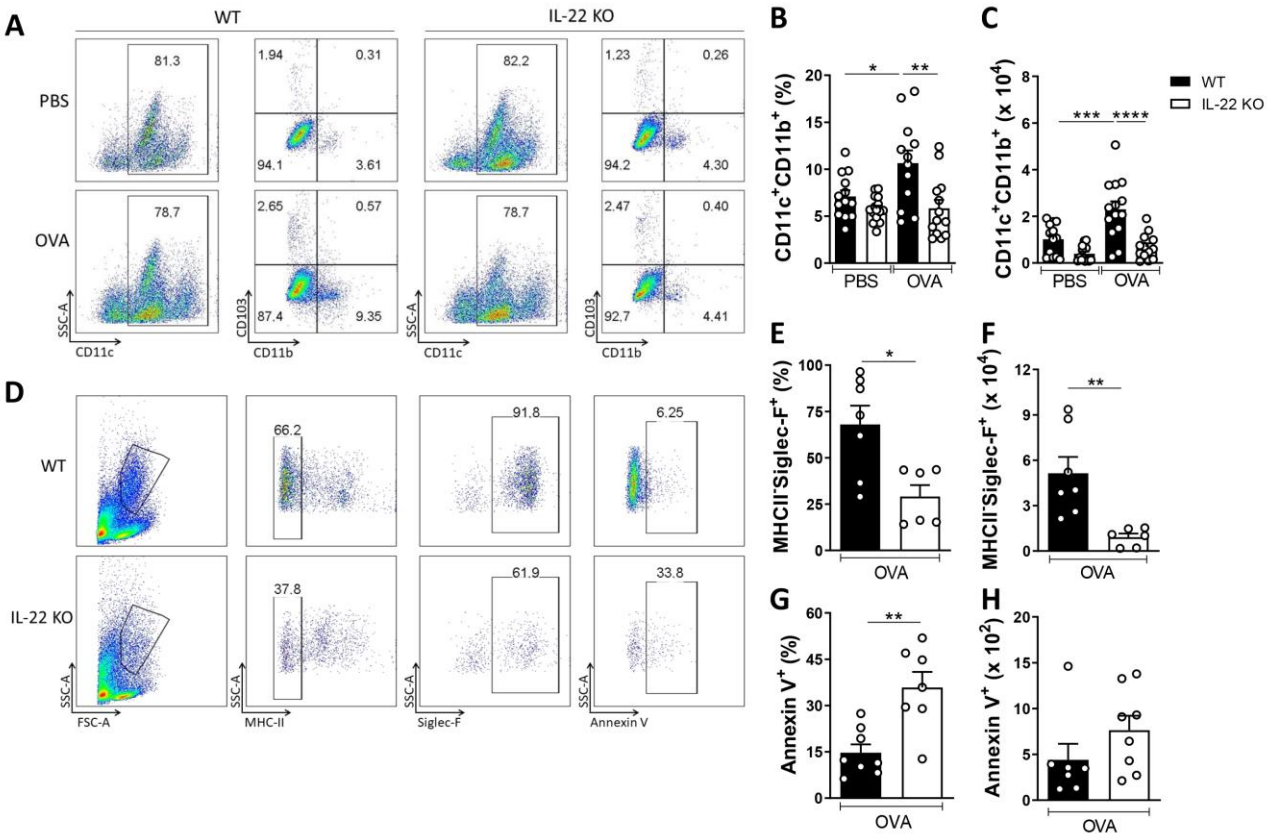

Figure 5. IL-22 modulates the frequency and number of dendritic cells, and eosinophil apoptosis in lungs of mice exposed to the allergen. WT and IL-22 KO mice were sensitized and challenged with OVA, as described in the legend of Figure 1A. (**A**) Representative flow cytometry analysis of $CD11c^+CD11b^+CD103^-$ cells in the lungs. (**B**,**C**) Frequency and number of $CD11c^+CD11b^+CD103^-$ cells in the lungs. (**D**) Representative flow cytometry analysis of MHC-II^-Siglec-F^+ cells and MHC-II^-Siglec-F^+AnnexinV$^+$ cells. (**E**,**F**) Frequency and number of MHC-II^-Siglec-F^+ cells. (**G**,**H**) Frequency and number of MHC-II^-Siglec-F^+AnnexinV$^+$ cells. The results are expressed as mean ± standard deviation of the individual values obtained for each experimental group. Results are representative of three independent experiments for DC and two independent experiments for eosinophil apoptosis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

IL-22 also participates in the induction of anti-apoptotic factors [28,29]. Considering the roles of IL-22 and that IL-22 may also promote the in vitro survival of epithelial cell [11,16], we asked whether IL-22 would promote eosinophil survival. Pulmonary eosinophils were evaluated as MHC class II- and Siglec-F^+ cells [30]. Our results show that IL-22 deficient mice exhibited a percentage and number of eosinophils lower than the WT OVA group (Figure 5D–F). We found that in a lower frequency and number the population of eosinophils in the lungs of IL-22 KO animals exhibited greater annexin V

staining, confirming that the absence of IL-22 causes an increase in apoptosis in eosinophils (Figure 5D,G,H).

These results suggest that the pro-inflammatory function of IL-22 is associated with the recruitment and/or expansion of $CD11b^+$ DC that favor Th2 immune response and that IL-22 prevents eosinophil apoptosis.

2.5. Allergen-Specific and Allergen-Free Treatment Reduces Eosinophil, Dendritic Cell and IL-22 Secretion in Airway Lung Inflammation

Our group reported previously the mechanisms by which CpG plus mycobacterial protein, an allergen-free immunotherapy, conferred protection against pulmonary eosinophilic inflammation [31–33]. To confirm the role of IL-22 as a pro-inflammatory cytokine in the airway lung inflammation, we evaluated if the levels of this cytokine would be reduced after a mixed allergen-specific and allergen-free immunotherapy. WT animals were sensitized and challenged with OVA and treated with increasing concentrations of OVA by sublingual route, or CpG oligodeoxynucleotides, agonist for TLR9, by subcutaneous route, or treated simultaneously with OVA by sublingual route and CpG by subcutaneous route (Figure 6A). Treated animals showed a significant decrease in IL-22 in the lung homogenates (Figure 6B). Concomitantly, treated groups exhibited a significant reduction in the allergic inflammatory response, characterized by decrease in frequency and number of eosinophils in the BALF (Figure 6C,D) associated with the reduction in frequency and number of $CD11c^+CD11b^+$ DC (Figure 6E–G). These findings confirm the role of IL-22 as a pro-inflammatory mediator in lung allergic inflammation.

2.6. IL-22 Plays No Role in the Comorbidity Asthma and Acute Pneumonia

Next, we evaluated whether the IL-22 deficiency would affect allergic asthma that courses with neutrophil inflammation. For this, we employed a model of comorbidity of allergic asthma with acute pneumonia caused by *Streptococcus pneumoniae*, as depicted in Figure 7A, because pneumococcus changes the pattern of airway eosinophilic inflammation to a mixed neutrophilic/eosinophilic inflammation. We observed a significant increase in the recruitment of neutrophils accompanied by a reduction in the recruitment of eosinophils in the BALF of WT mice exposed to OVA and infected with *S. pneumoniae* (OVA+Sp group) compared to the animals exposed only to the allergen (WT OVA group) (Figure 7B,C). Additionally, the concentrations of CXCL-1, a neutrophil chemoattractant, the frequency of $CD4^+IL\text{-}17^+$ cells and the pulmonary inflammation were increased in the lungs during the comorbidity compared to the WT OVA group (Figure 7D–G). The induction of comorbidity in IL-22 KO group increased the percentage of $CD4^+IL\text{-}17^+$ cells in the lung compared to WT counterpart (WT OVA+Sp group) (Figure 7E,F). Neutrophilic inflammation, CXCL-1 levels and pulmonary inflammation were not significantly different when comparing WT OVA+Sp and IL-22 KO OVA+Sp groups (Figure 7B–G), suggesting that IL-22 is not involved in the comorbidity asthma and acute pneumonia that courses with neutrophilic inflammation. These findings suggest that IL-22 plays a pivotal role in allergic asthma but is dispensable for asthma that coursed with an increase in neutrophils.

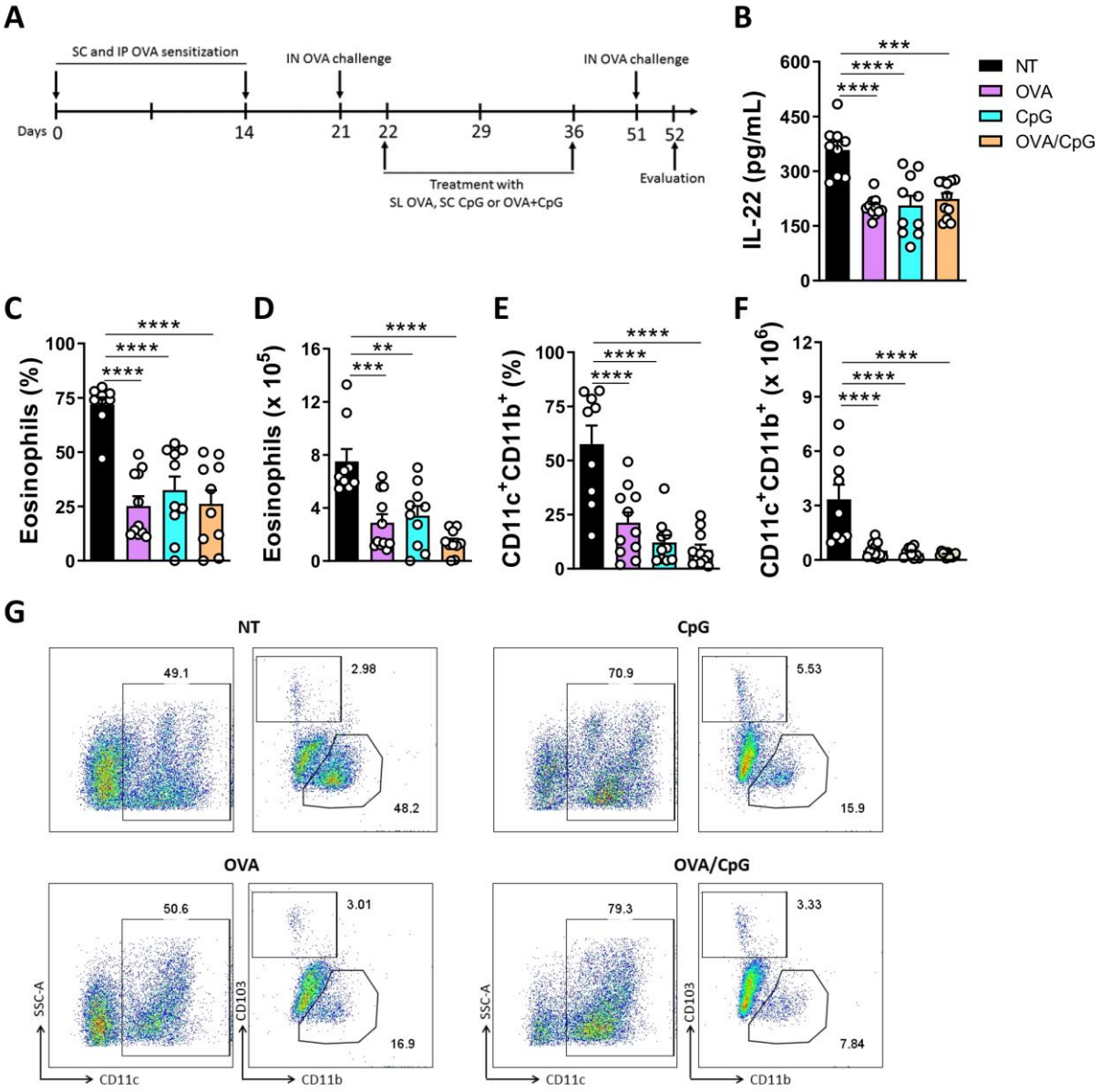

Figure 6. Allergen-specific, allergen-free or simultaneous treatment reduces IL-22 production and dendritic cells in the lungs. (**A**) Mice were sensitized and challenged with OVA, followed by treatment with OVA by sublingual route, or CpG by subcutaneous route or with OVA and CpG simultaneously. Fifteen days after the end of immunotherapy, mice were challenged again with OVA, and evaluated 72 h post challenge. (**B**) Levels of IL-22 in the BALF. (**C**,**D**) Frequency and number of eosinophils in the BALF. (**E**,**F**) Frequency and number of $CD11c^{+}CD11b^{+}$ cells in the lungs of treated or non-treated animals. (**G**) Representative flow cytometry analysis of $CD11c^{+}CD11b^{+}$ cells in the lungs of treated or non-treated animals. The results are expressed as mean ± standard deviation of the individual values obtained for each experimental group. Results are representative of two independent experiments. The bars show the significant differences between groups ** $p < 0.02$; *** $p < 0.01$; **** $p < 0.0001$. IP = intraperitoneal route, SC = Subcutaneous, IN = Intranasal, SL = Sublingual.

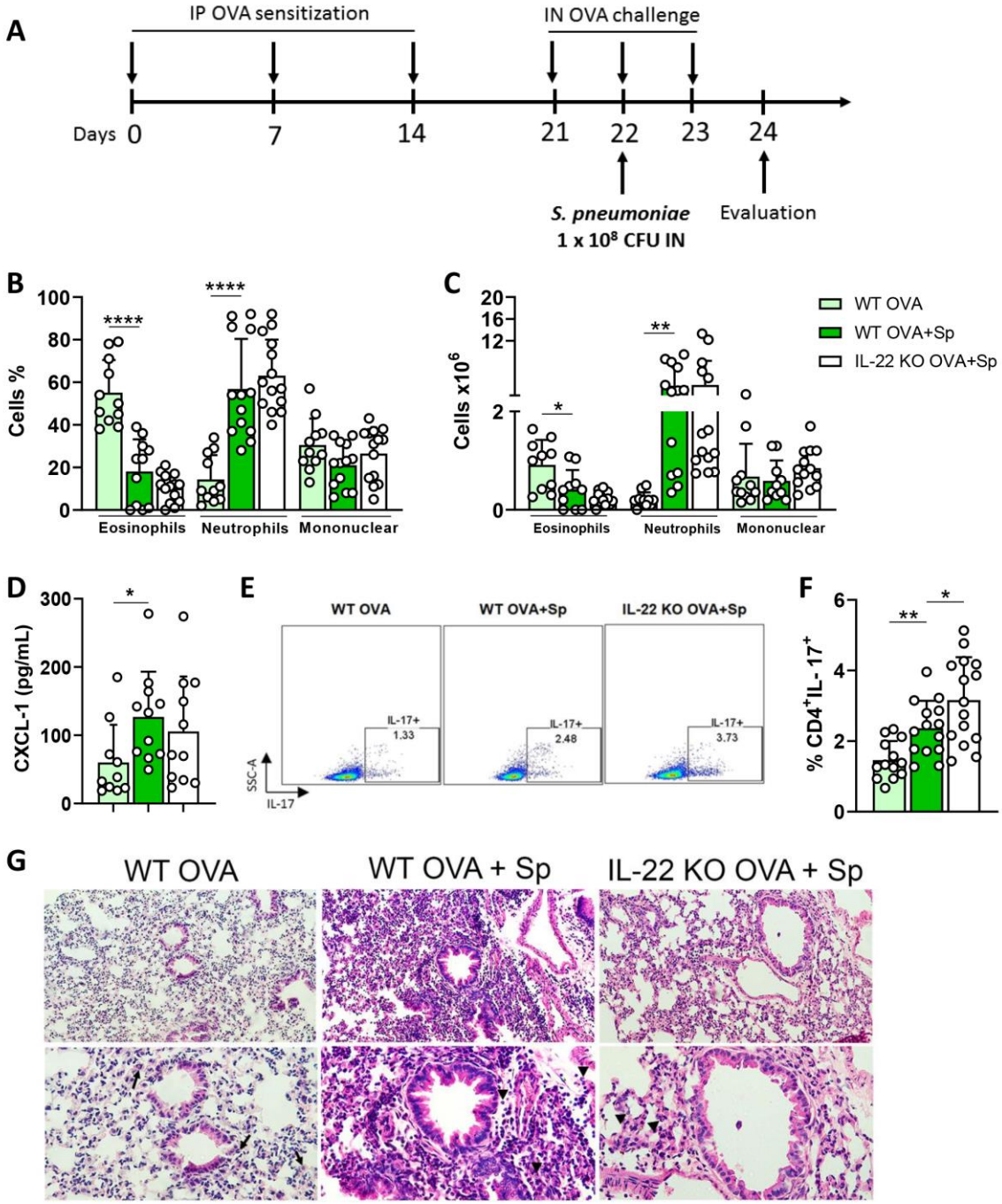

Figure 7. IL-22 is not required for the exacerbation of neutrophilic inflammation in the comorbidity asthma and acute pneumonia. (**A**) WT and IL-22 KO animals were sensitized and challenged with OVA and infected or not with *S. pneumoniae*. (**B**,**C**) Differential and total count of leukocytes in the BALF. (**D**) Concentrations of CXCL-1 in the BALF supernatants. (**E**,**F**) Frequency of $CD4^+IL\text{-}17^+$ cells in the lungs. (**G**) Histopathological analysis of the lungs. Arrows and arrowheads show eosinophils and neutrophils, respectively. The results are expressed as mean ± standard deviation. Results are representative of three independent experiments. Top, magnification 200×; bottom, magnification 400×. The bars show the significant differences between groups * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

3. Discussion

Studies show that the IL-22 function in airway allergy appears to be time-dependent, whereas IL-22 was protective during the allergen challenge and deleterious during the sensitization [10]. Moreover, the deleterious role of IL-22 was dependent on the simultane-

ous presence of IL-17 [10]. However, two studies attributed an anti-inflammatory function to IL-22 [22,23], while one study showed a pro-inflammatory role to this cytokine [10]. The findings reported in these studies show that the IL-22 function in the airway allergy remains to be elucidated given that the collecting findings are controversial [10,22].

Our results show a significant increase in the production of IL-22 and IL-17 in the BALF of mice exposed to the allergen, confirming the studies of other groups [7,10]. The deficiency of IL-22 did not affect the frequency of IL-17-producing $CD4^+$ cells in the lungs of mice exposed to the allergen. However, mice deficient in IL-22 exhibited a higher frequency of IL-17-producing $CD4^+$ cells, as already described in a model of lung inflammation induced by silica [34]. Therefore, IL-22 appears to negatively regulate Th17 cells in steady state. In the silica model, IL-17 is mostly produced by T cells in the lungs, and it is essential for an increase in inflammatory infiltrate in the airways [34]. Our study shows an original finding when we described that the pro-inflammatory function of IL-22 was dependent on simultaneous secretion of IL-17 by Th17 cells in allergic asthma.

The increase in IL-17 and IL-22 occurred along with the significant increase in IL-1β and IL-23 in the BALF. IL-1β and IL-23 participate in the differentiation and maintenance of Th17 cells [35]. As IL-17 and IL-22 are elevated in mice exposed to the allergen, as well as in patients presenting symptoms of severe or moderate asthma, our study and our results suggest that IL-22 plays a pro-inflammatory function in airway allergic inflammation modulating it positively, as described by Besnard et al. [10], because IL-22 deficiency caused a reduction in eosinophils and lymphocytes in the BALF, IL-5 and IL-13 reduced inflammatory cell infiltrate in the airways and decreased mucus production by goblet cells in the lungs compared to WT group exposed to the allergen.

The studies with IL-22 in asthma are scarce and suggest that this cytokine negatively reduces the pro-allergenic role of dendritic cells (DC) [22]. Because we showed a pro-inflammatory function for IL-22 in allergic asthma, we evaluated the frequency and number of $CD11c^+CD11b^+$ DC ex vivo, which were negatively regulated in IL-22 KO mice exposed to the allergen compared to WT OVA group. $CD11c^+CD11b^+$ DC were described as main players in generating Th2 immune response following allergen contact [26,27]. Therefore, we show an ex vivo finding that IL-22 might downregulate the recruitment or expansion of $CD11c^+CD11b^+$ DC, which contribute to the differentiation and proliferation of IL-5- and IL-13-producing effector and memory Th2 cells and eosinophil recruitment. It remains to be evaluated if the treatment with recombinant IL-22 would be capable of increasing the infiltration of $CD11c^+CD11b^+$ DC in the lungs of animals exposed to the allergen.

We also found that the deficiency of IL-22 increased the frequency of eosinophils in apoptosis ($MHC\text{-}II^-Siglec\text{-}F^+AnnexinV^+$ cells), suggesting that IL-22 is also a survival factor for eosinophil and not only for epithelial cells [16]. This result suggests that IL-22 may indirectly influence the maintenance of lung allergic inflammation, acting on the survival of the most important readout of experimental allergic inflammation, the eosinophil. It remains to be confirmed in vitro if IL-22 increases the survival of eosinophils.

Previously, our group used allergen-free immunotherapy and showed that CpG plus proteins secreted by *Mycobacterium tuberculosis* [31] or CpG plus mycobacterial protein [33] were capable to negatively regulate allergic asthma, reduce eosinophilic and Th2 inflammation by a mechanism dependent on the deviation of immune response to Th1 cells and by a mechanism dependent on TLR9 and increase the influx of inflammatory monocytes. Moreover, Fonseca et al. [32] showed that mice with pulmonary allergic inflammation treated with CpG exhibited a decrease in IL-17 concentrations in the BALF compared to the group exposed to the allergen but non-treated. This study attributes to IL-17 a pro-inflammatory role in allergy of the airways. However, the role of IL-22 had not yet been assessed [32]. To confirm that IL-22 has a pro-inflammatory function, we treated mice exposed to the allergen with different treatments: allergen-free therapy (CpG treatment), allergen-specific therapy (increasing doses of OVA), and a combination of both CpG and increasing doses of OVA, and evaluated if the immunotherapy would be able to reduce IL-22 concentrations as well as eosinophilic inflammation on the BALF, and influx of $CD11c^+CD11b^+$ cells in the

lungs. We used sublingual, specific-allergen immunotherapy because it has been shown to be effective in reducing pulmonary allergic inflammation by promoting peripheral tolerance, regulatory T lymphocytes, and IL-10 production [36,37]. The role of IL-22 in the sublingual allergen-specific immunotherapy had not been elucidated. As the two forms of therapy had already been described in the literature, we sought to assess whether combined allergen-specific therapy and allergen-free therapy could potentiate the reduction in allergic inflammation and IL-22 production. Our results show that the reduction in frequency and number of eosinophils and $CD11c^+CD11b^+$ cells were accompanied by a reduction in IL-22 concentrations, confirming that IL-22 acts as a pro-inflammatory mediator in the airway allergy inflammation.

Although we show that IL-22 positively regulates the allergic asthma inducing eosinophil survival and $CD11c^+CD11b^+$ cell infiltration, IL-22 plays no pro-inflammatory role in neutrophilic inflammation induced during the comorbidity asthma and acute pneumonia. During the comorbidity asthma and acute pneumonia, we found an increase in $CD4^+IL\text{-}17^+$ cells in the lungs of WT animals exposed to the allergen and infected with *S. pneumoniae*. However, the deficiency of IL-22 neither affects pulmonary inflammation nor neutrophil or eosinophil frequencies. These results corroborate data from patients with severe asthma that have an increased neutrophilic inflammation and Th17 cells, suggesting that IL-17, and not IL-22, might contribute to asthma severity that courses with neutrophil infiltration [4,7].

Although our study has limitations, as it would have been of great importance to investigate the death by apoptosis of human eosinophils differentiated in vitro or obtained from BALF of asthmatics patients, beyond the frequency of $CD11c^+CD11b^+$ cells in the peripheral blood and in the BALF of asthmatics patients, our study adds new clues about the pro-inflammatory role of IL-22 in allergic asthma.

4. Materials and Methods

4.1. Mice

Female C57BL/6 wild type (WT) and IL-22-KO mice, 8–12 weeks, were obtained from the local breeding facility at the Ribeirao Preto Medical School, University of Sao Paulo, Brazil, and housed under specific pathogen-free conditions. Mice were treated according to the Animal Welfare guidelines of the Ribeirao Preto Medical School, University of Sao Paulo. The local Animal Research Ethics Committee approved all of the procedures (Process number 140/2014 and 245/2019).

4.2. Induction of Allergic Asthma

WT and IL-22-KO mice were sensitized with 100 µg of OVA Grade V (Sigma-Aldrich, St. Louis, MO, USA, A5503) and aluminum hydroxide (1.6 mg) subcutaneously on day 0 and sensitized again with OVA (50 µg) intraperitoneally on day 14. The animals were challenged with OVA (100 µg) via the intranasal route on day 21, and 72 h after the challenge (day 24), the animals were euthanized, and lungs and serum were collected. The euthanasia of the animals was performed by retro-orbital inoculation of 100 µL of anesthetic solution containing 20% ketamine hydrochloride (Agener, Embu-Guaçu, Sao Paulo, Brazil) and 10% xylazine (Laboratorios Calier SA, Barcelona, Spain).

4.3. Induction of Th2 Low Asthma

WT and IL-22-KO mice were sensitized three times, at 7-day intervals, with 10 µg of OVA Grade VI (Sigma-Aldrich, St. Louis, MO, USA, A2512) and aluminum hydroxide (2 mg) (Thermo Scientific, Waltham, MA, USA, 77161) intraperitoneally. On days 21, 22 and 23 mice were challenged with 30 µg OVA Grade V (Sigma, A5503), intranasally, and samples were collected 24 h after the last challenge. TIGR4 strain of *S. pneumoniae* (ATCC BAA-334) frozen at −80 °C in tryptic soy broth (TSB) (BD, cat. 211825) containing 10% glycerol was thawed, plated on blood agar and incubated overnight at 37 °C, 5% CO_2. After growth, colonies were inoculated in TSB medium, when the growth was checked at OD 600 = 0.06, following incubation for 4 to 5 h. When OD 600 = 0.3, bacteria was counted,

and the concentration was adjusted to 50×10^8/mL of colony-forming unit (CFU) in sterile Phosphate Buffered Saline (PBS). Mice were infected with 1×10^8 CFU of *S. pneumoniae* by intranasal route (20 μL/animal) before the second challenge with OVA. The euthanasia of the animals was performed 48 h after the infection.

4.4. BALF

Mice were anesthetized with ketamine (100 mg/kg, Sespo Industry and Commerce, Paulínia, SP, Brazil) and xylazine (10 mg/kg, Vetecia Laboratory of Veterinary Products, Jacareí, SP, Brazil) 72 h after OVA challenge or 24 h after the last OVA challenge. The trachea was exposed and cannulated with a subsequent injection of 5 mL of RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) for bronchoalveolar lavage fluid (BALF) collection. Samples were centrifuged at 450× *g* for 10 min and the supernatants were stored at −20 °C for cytokine measurement. Cells were resuspended in 500 μL of RPMI 1640 and centrifuged at cytocentrifuge (Thermo Fisher Scientific, Waltham, MA, USA) at 18× *g* for 3 min. Next, cells were stained with rapid panoptic (Labor Clin, Sao José do Rio Preto, SP, Brazil) for differential cell count.

4.5. Th17 Cell Differentiation

Naive T cells ($CD4^+CD62L^+$) were isolated from total spleen cells of WT or IL-22-KO animals by MACS $CD4^+CD62L^+$ cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified 1.5×10^6 cells ($CD4^+CD62L^+$) were distributed in 96-well plates (Greiner Bio-one, Kremsmünster, Austria) in IMDM medium (Sigma, St. Louis, MO, USA) (Sigma, St. Louis, MO, USA), Sodium Pyruvate (400 μM) (Sigma, St. Louis, MO, USA), L-glutamine (0.88 μM) (Sigma-Aldrich, St. Louis, MO, USA), 2-Mercaptoethanol (55 μM) (Sigma, St. Louis, MO, USA) and gentamycin (10 μg/mL) (Gibco-Invitrogen, Grand Island, NY, USA). For the differentiation of Th17 cells, naive cells were cultured with antibodies required for polyclonal stimulation, mAb anti-CD3 (2 μg/mL) (BD Biosciences, Franklin Lakes, NJ, USA) and mAb anti-CD28 (2 μg/mL) (BD Biosciences, Franklin Lakes, NJ, USA), recombinant cytokines for Th17 polarization: IL-6 (20 ng/mL) (R&D Systems, Minneapolis, MN, USA), TGF-β (5 ηg/mL) (R&D Systems, Minneapolis, Minnesota), in addition to mAb anti-IL-4 (20 μg/mL) (R&D Systems, Minneapolis, MN, USA), mAb anti-IL-2 (20 μg/mL) (R & D Systems, Minneapolis, MN, USA) and mAb anti-IFN-γ (20 μg/mL) (R&D Systems, Minneapolis, MN, USA). After 72 h of culture, the supernatants were collected for detection of IL-17 and the cells were analyzed by flow cytometry for quantification of CD4+ IL-17+ cells. Th17 cell transfer (4×10^5 cells) was performed via the intra-tracheal route.

4.6. Lung Cells

After euthanasia, lungs were perfused with 5 mL of PBS through the right ventricle. Perfused lower right lobes lungs were cut into small pieces and digested for 50 min at 37 °C, 5% CO_2 with collagenase type IV (2.2 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) and DNAse (0.055 mg/mL) (Roche, Basel, Switzerland). The digestion was stopped using EDTA (10 mM). Then, cells suspension was passed through a cell strainer, centrifuged, lysed with ACK, and resuspended in complete RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA).

4.7. Flow Cytometry

Isolated lung cells were stained with FVS780 viability dye (BD Pharmingen, San Diego, CA, USA) and specific antibodies for the characterization of Th17 cells ($CD4^+$IL-17^+), dendritic cells ($CD11c^+CD11b^+CD103^-$) and eosinophils ($MHCII^+$Siglec-F^+) were used according to manufacturer's instructions (BD Pharmingen, San Diego, CA, USA and Thermo Fisher Scientific, Waltham, MA, USA). To evaluate eosinophils death, lung cells were stained with Annexin V (BD Pharmingen, San Diego, CA, USA) after antibodies staining. Samples were fixed with paraformaldehyde 1% (Labsynth, Diadema, SP, Brazil). Samples were acquired in FACS Canto (BD Biosciences, San Jose, CA, USA) and analyses

were performed in the FlowJo X 10.0.7r2 software for Windows (Becton Dickinson and Company, Franklin Lakes, NJ, USA).

4.8. IgE Levels

OVA-specific IgE antibodies were assayed in the mouse serum by sandwich ELISA using biotin-conjugated anti-mouse IgE (Clone R35-118, BD Pharmingen, San Diego, CA, USA). First, a 96-well plate was coated with OVA Grade V (Sigma, A5503) and incubated overnight at 4 °C. After washing, nonspecific binding was blocked with PBS-10% fetal bovine serum and 0.05% Tween 20 during 1 h at 37 °C. The plate was washed again, and serum samples were added and incubated for 2 h at 37 °C. Biotin-conjugated anti-mouse IgE was added after washing and the plate was incubated for 1 h at 37 °C. The reaction was revealed using tetramethylbenzidine substrate (BD Pharmingen, San Diego, CA, USA) and stopped with 16% sulfuric acid solution. The results were expressed as absolute absorbance values measured at 450 nm.

4.9. Pulmonary Inflammation and Mucus Production Histological Analysis

The upper right lobe of the lung was preserved in buffered formalin for the preparation of tissue slides. Pulmonary tissue samples were cut (5 µm) and stained with hematoxylin-eosin (HE). The analysis of mucus production was performed by periodic acid staining of Schiff (PAS).

4.10. Statistical Analysis

All data were initially analyzed for normal/parametric distribution (Kolmogorov–Smirnov test). If there was a parametric distribution, the analysis of variance was applied to evaluate the differences among groups. To compare 2 groups, Student's T-test was applied. If no parametric distribution was found, the Kruskal–Wallis test was applied to evaluate differences among groups. To compare 2 groups, the Mann–Whitney U test was applied. Statistical analyzes were performed using GraphPad PrismVersion 8.1 (GraphPad 8.0.2 software for Windows, Inc., San Diego, CA, USA). Values of $p < 0.05$ were considered significant.

5. Conclusions

In summary, our data suggest that IL-22 acts as a pro-inflammatory cytokine in allergic asthma, contributing to the survival of eosinophils and increase in $CD11b^+$ DC in the lungs. However, IL-22 does not play a function in Th2 low asthma. These findings suggest that IL-22 could be targeted as adjuvant therapy for allergic asthma.

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Institutional Review Board Statement: The animal study protocol was approved by local Animal Research Ethics Committee (protocol code and date: 140/2014 and 245/2019).

Data Availability Statement: The data can be requested from the corresponding author.

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Asthma-associated bacterial infections: Are they protective or deleterious?

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Eosinophilic, noneosinophilic, or mixed granulocytic inflammations are the hallmarks of asthma heterogeneity. Depending on the priming of lung immune and structural cells, subjects with asthma might generate immune responses that are T_H2-prone or T_H17-prone immune response. Bacterial infections caused by *Haemophilus*, *Moraxella*, or *Streptococcus* spp. induce the secretion of IL-17, which in turn recruit neutrophils into the airways. Clinical studies and experimental models of asthma indicated that neutrophil infiltration induces a specific phenotype of asthma, characterized by an impaired response to corticosteroid treatment. The understanding of pathways that regulate the T_H17-neutrophils axis is critical to delineate and develop host-directed therapies that might control asthma and its exacerbation episodes that course with infectious comorbidities. In this review, we outline clinical and experimental studies on the role of airway epithelial cells, S100A9, and high mobility group box 1, which act in concert with the IL-17-neutrophil axis activated by bacterial infections, and are related with asthma that is difficult to treat. Furthermore, we report critically our view in the light of these findings in an attempt to stimulate further investigations and development of immunotherapies for the control of severe asthma. (J Allergy Clin Immunol Global 2023;2:14-22.)

Key words: *Severe asthma, bacterial infections, neutrophil, IL-17, lung inflammation*

Abbreviations used

AEC: Airway epithelial cell
AHR: Airway hyperresponsiveness
CCL: Chemokine (C-C motif) ligand
DC: Dendritic cell
HDM: House dust mite
HMGB1: High mobility group box 1
NE: Neutrophil elastase
NET: Neutrophil extracellular trap
NF-kB: Nuclear factor kappa B
OVA: Ovalbumin
PRR: Pattern recognition receptors
TLR: Toll-like receptor
Treg: Regulatory T

Asthma is a chronic disease characterized by airway inflammation, reversible or irreversible airway obstruction, airway hyperresponsiveness (AHR), and lung remodeling. Coughing, wheezing, shortness of breath, and chest tightness are the symptoms of the disease.[1,2] Asthma is viewed as a complex and heterogeneous syndrome, which phenotypically may be classified as mild, moderate, and difficult to treat to severe, that is most likely caused by a combination of genetic predisposition, changes in lifestyle, and environmental factors. Despite this heterogeneity and complexity, 2 endotypes of asthma are defined: allergic (or atopic) and nonallergic asthma.[3]

Asthma affects children, teenagers, and adults. According to the Global Asthma Network, 339 million people are affected worldwide.[4,5] The prevalence and mortality associated with asthma changes from one region to another. Indeed, although the prevalence of asthma is elevated in high-income countries, the highest number of deaths is recorded in low- and middle-income countries, which corresponds to more than 80% of asthma-related deaths.[2,6] The global prevalence of asthma also depends on sex and age. The incidence and prevalence of asthma are higher in children, but its morbidity and mortality are higher in adults. Sex prevalence varies along life, with higher prevalence among boys than among girls. However, for all adulthood, females are more affected than males.[5]

In this review, we will address current knowledge about the mechanisms that modulate neutrophilic inflammation in a specific asthma phenotype, which is difficult to treat, in the context of bacterial infectious comorbidities in an attempt to highlight the challenges and to overcome them for the development of host-directed therapies.

PATHOGENESIS OF ASTHMA

Allergic asthma is defined by eosinophilic infiltrate, mast cell activation, and IgE production.[7] Usually, allergic asthma has an

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early onset and is associated with other allergic conditions such as allergic rhinitis and atopic dermatitis.[3] Nonallergic asthma has a late onset and might be characterized by a T_H2-high immune response with eosinophilic infiltrate, or T_H2-low immune response. T_H2-low endotype is characterized by neutrophilic inflammation that could be due to obesity, environmental exposure to pollutants such as diesel particles and cigarette smoke, or infections.[3,8,9]

In T_H2-high asthma, eosinophilic inflammation is one of the main readouts of the disease. Allergens are recognized by PRRs, including Toll-like receptor (TLR)-4, on airway epithelial cells (AECs) and stimulate the secretion of IL-25, IL-33, thymic stromal lymphopoetin, GM-CSF, IL-1, chemokine (C-C motif) ligand (CCL)2, CCL20, and β-defensins. AECs also release ATP, lysophosphatidic acid, uric acid, and reactive oxygen species. Cytokines and chemokines recruit and activate dendritic cells (DCs) and type 2 innate lymphoid cells, which secrete IL-5, IL-9, and IL-13 and stimulate the differentiation of T_H2 cells. T_H2 cells secrete IL-4, IL-5, and IL-13 and induce allergen-specific IgE production.[7,10] IgE binds to high-affinity IgE receptor on mast cells. Subsequent contacts with the allergen induce mast cell degranulation and immediate allergic reaction mediated by histamine, cysteinyl leukotrienes, and cytokines as tumor necrosis factor.[11] T_H2 cells, eosinophils, and basophils induce late allergic reaction. Although memory T_H2 cells recognize antigens that usually are innocuous to the host and mediate eosinophil differentiation and recruitment, and collaborate for mucus production, eosinophils and basophils play a role in the induction of tissue damage mediated by major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin.[12]

The identification of additional T_H-cell subsets brought new insight about the complexity of asthma pathogenesis. Higher number of circulating T_H9 cells and increased levels of IL-9 were detected in allergic asthmatic patients compared with nonallergic subjects and in the asthma murine model.[13-15]

T_H17 cells also participate in the immunopathology of asthma. Although IL-17 is associated with neutrophilic asthma, high levels of IL-17 in the airways and increased levels of IL-22 in the serum of patients with asthma compared with healthy individuals suggested that these cytokines also participate in the immune response of allergic asthma.[16-19] Fig 1 summarizes the description of immunopathology in the allergic asthma.

The heterogeneity of asthma endotypes includes eosinophilic (allergic and nonallergic), noneosinophilic (neutrophilic, T_H1/T_H17, and paucigranulocytic), and mixed granulocytic inflammation.[20] Subjects with asthma might generate a T_H2-prone and/or T_H17-prone immune response depending on the inflammatory milieu. The persistent neutrophilic inflammation, which might be complete or partially mediated by T_H17 cells, or mixed eosinophilic/neutrophilic inflammation has been reported as severe asthma.[21,22] Subjects with neutrophilic airway inflammation were refractory to inhaled corticosteroid treatment.[23]

High levels of IL-17 in serum[19] and in the lung[16,24] were reported in allergic asthmatic patients, but few clinical studies involving T_H17-related cytokines (IL-17, IL-23, and IL-22) were performed. In a randomized, double-blind, placebo-controlled study, the blocking of IL-17 signaling using brodalumab (anti–IL-17RA mAb) in subjects with inadequately controlled moderate to severe asthma had no evidence of an effect in the overall study population; however, an increase in symptom-free days was observed in the high-reversibility subgroup treated with brodalumab, encouraging further study of IL-17 blockade in this asthma subgroup.[25] Concerning IL-23 signaling, a recent data of a randomized, phase 2a, multicenter, and double-blind clinical trial with risankizumab (anti–IL-23p19 mAb) showed that the treatment was not beneficial in severe asthma.[26] Although anti–IL-22 therapies have not been evaluated in asthma yet,[27] the use of fezakinumab (anti–IL-22 mAb) was efficient in severe atopic dermatitis, which has similar inflammatory features of asthma.[28] A metadata study showed that a subset of subjects with asthma may respond to anti–IL-22 antibody therapy.[29] Nevertheless, a deeper investigation is needed to ascertain the role of IL-17 and IL-22 in the modulation of T_H2 immune responses. Considering that IL-22 activates DCs,[30,31] it will be relevant to investigate whether IL-22 plays a direct or an indirect role in other innate cells, such as eosinophils. Interestingly, the presence of IL-22 was detrimental during the sensitization phase, but it was protective during the allergen challenge,[18] suggesting a time-dependent effect of IL-22.

In moderate to severe asthma, AECs secrete neutrophil chemoattractant CXCL8 (IL-8), CCL2, and CCL20, which recruit DCs.[32] Activated DCs migrate to the mediastinal lymph node where they prime naive T cells to differentiate into T_H17 cells through the expression of IL-1β, TGF-β, IL-6, and IL-23 by DCs. Primed T_H17 cells migrate into the lungs and produce IL-17 and IL-22.[22] T_H17 cells increase mucus production (IL-17) and exacerbate AR (IL-17/IL-22) in severe asthma.[33] In addition, IL-17 directly acts on bronchial smooth muscle contraction through the upregulation of Ras homolog family member A protein, associated with actomyosin contractility.[34] During severe asthma, type 3 innate lymphoid cells are early providers of IL-17 and IL-22. Type 3 innate lymphoid cells may play a critical role in severe asthma phenotypes by producing IL-17 and IFN-γ, which stimulate neutrophil recruitment,[10] besides IL-22. IL-17 sustains neutrophilic inflammation by stimulating CXCL1 and CXCL8 production by AECs. CXCL1 and CXCL8 enhance airway smooth muscle contraction and stimulate collagen synthesis.[21,35] Furthermore, T_H17 cells and IL-17 induce the expression of polymeric immunoglobulin receptor in the airway epithelium and a subsequent increase in airway IgM and IgA levels in mice.[36] Therefore, IL-17 plays a key role in the mucosal immune response by regulating IgA production and transport to the lungs and gut.[36-38] This pathway if unbalanced might be deleterious to the host and cause pathology.

IL-22 enhances airway smooth muscle contraction, promotes collagen deposition and production of S100A9, an alarmin that induces neutrophil influx, by AECs, and is associated with neutrophil survival by inhibiting apoptosis pathway. S100A9 may stimulate AECs to produce neutrophil survival cytokines such as MCP-1, IL-6, and CXCL8.[39-42] Increased levels of S100A9 were reported in the sputum of subjects with severe uncontrolled asthma compared with subjects with controlled asthma,[43] suggesting that S100A9 could be a biomarker of neutrophilic inflammation in severe asthma.[43,44] In an animal model of neutrophil-dominant asthma generated using ovalbumin (OVA) and complete Freund's adjuvant, S100A9 was reported to generate and amplify neutrophilic inflammation followed by a high production of IL-1β, IL-17, and IFN-γ in the lungs lysates.[44] S100A9 interacts with TLR4 or receptor for advanced glycation end products in granulocytes and induces its own production in these cells. S100A9 may also interact with TLR4 or receptor

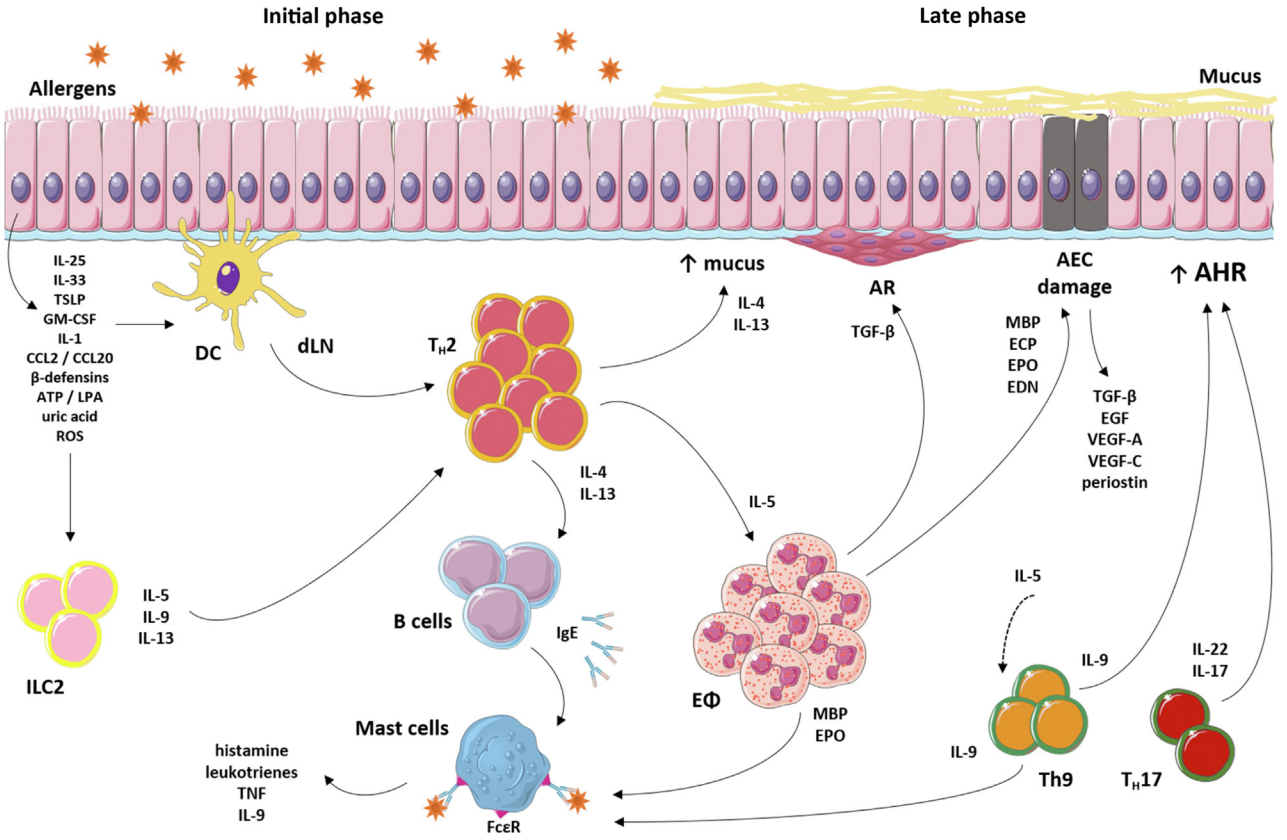

FIG 1. Immunopathology of allergic asthma. Inhaled allergens activate AECs that recruit and activate DCs and ILC2s by production of IL-25, IL-33, TSLP, ATP, uric acid, LPA, and ROS. DCs and ILC2s induce T_H2 differentiation and activation. T_H9 and T_H17 might also undergo differentiation. Eosinophil (EΦ) recruitment and activation followed by activation of T_H2, T_H9, and T_H17 promote AHR and mucus production, AEC damage, and AHR. *ILC2*, Type 2 innate lymphoid cell; *LPA*, lysophosphatidic acid; *ROS*, reactive oxygen species; *TSLP*, thymic stromal lymphopoetin.

for advanced glycation end products in monocytes. In both cells, S100A9 induces the production of IL-1β, IL-6, IL-18, and tumor necrosis factor. IL-1β has an autocrine and paracrine effect on both granulocytes and monocytes.[45]

In severe asthma, S100A8 and S100A9 induce also the production of high mobility group box 1 (HMGB1), a proinflammatory alarmin produced by inflammatory and airway cells.[46] Sputum levels of HMGB1 were significantly higher in children with severe asthma compared with children with mild and moderate asthma.[47] In addition, in a model of neutrophilic asthma induced by OVA combined with LPS, anti–HMGB1-neutralizing antibody administered intranasally before OVA sensitization reduced neutrophilic inflammation, IL-17 production, and AHR.[48] As S100A9, HMGB1 also interacts with receptor for advanced glycation end products and TLR4 and induces the release of neutrophil extracellular traps (NETs).[49,50] NETs play a detrimental effect because their compounds histones, neutrophil elastase (NE), myeloperoxidase, cathepsin G, and DNA cause tissue damage.[51]

The role of NETs in severe asthma is not limited to tissue damage in AECs. NETs might indirectly stimulate the recruitment of neutrophils into the airways by a mechanism dependent on NET-activated AECs, which secrete CXCL1, CXCL2, and CXCL8 and drive neutrophilic inflammation through the TLR4/nuclear factor kappa B (NF-kB) pathway.[52] Likewise, another study showed that during severe asthma, NETs induce a second wave of neutrophil infiltration in the airway by stimulating macrophages to produce IL-1β, which intensifies the recruitment of neutrophils and the production of NETs. The accumulation of NETs may amplify tissue damage and aggravate asthma pathology.[53] Activated neutrophils produce reactive oxygen species, antimicrobial peptides, elastase, and matrix metalloproteinase-9.[54,55] Reactive oxygen species, NE, and matrix metalloproteinase-9 may be involved in tissue damage during severe asthma.[56] NE augments CXCL8 production by AECs, promoting a loop in neutrophil recruitment as well as the inactivation of tissue inhibitor of metalloproteinase-1. Increased levels of matrix metalloproteinase-9 and NE and decreased levels of tissue inhibitor of metalloproteinase-1 contribute to bronchoconstriction in asthma.[57] Fig 2 summarizes the description of immunopathology in severe asthma.

ASTHMA AND INFECTIOUS COMORBIDITIES

Viral, bacterial, and fungal infections might accelerate the progression of asthma and induce severe disease.[57] Therefore, the therapeutic management of asthma is more difficult during infectious comorbidities. The relationship between asthma and infections is complex and involves the pathogen and factors associated with the host genetic background. Overall, the failure in mechanisms of pathogen tolerance induces host cell death and might cause tissue injury. The recognition of microbial-

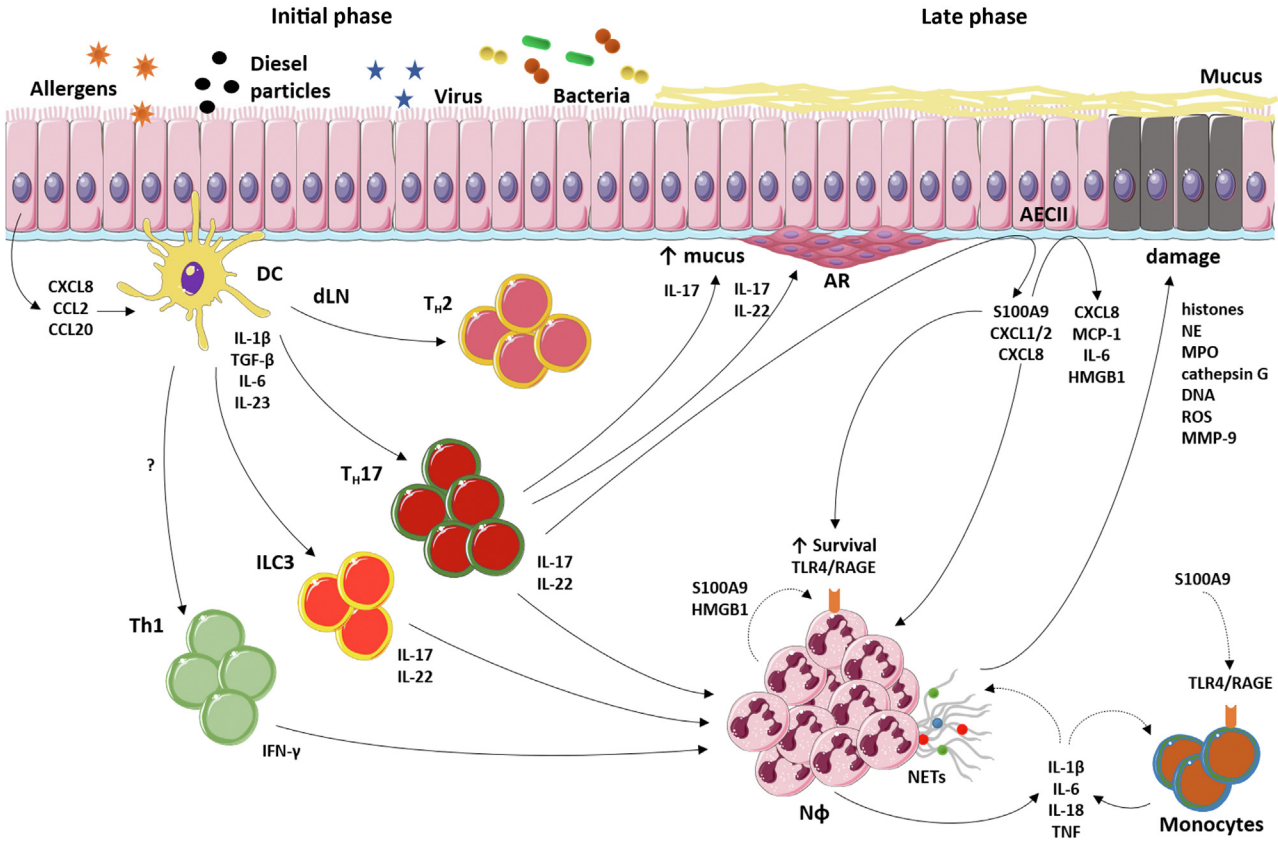

FIG 2. Immunopathology of severe asthma. Allergens, pollutants, and viral/bacterial infections promote the activation of AECs that recruit and activate DCs by production of IL-1β, IL-6, CXCL8, CCL2, and CCL20. DCs migrate to draining lymph node (dLN) and induce T_H1, T_H2, and T_H17 cell differentiation. ILC3s provide the early sources of IL-17 and IL-22. ILC3, T_H17, and T_H1 contribute to recruit neutrophils (NΦ). NΦ cause epithelial damage by release of NETs. AEC-derived signals (S100A9, CXCL1, CXCL2, and CXCL8) stimulate the NΦ survival and recruitment, respectively. The interface of neutrophils and AECs amplifies the local inflammation and aggravates asthma immunopathology. *ILC3*, Type 3 innate lymphoid cell.

associated molecular patterns by pattern recognition receptors on host cells activates innate leukocytes that secrete proinflammatory cytokines and regulate positively the expression of MHC and costimulatory and adhesion molecules. Signaling through cytokines and PRRs drives the differentiation of $CD4^+$ T-cell subsets. This is the critical outcome in the landscape of comorbidities that might affect the type and magnitude of inflammation in subjects with asthma. Although the immune response and inflammatory response against the infection is important for pathogen control, they may worsen the episodes of asthma and cause severe disease. The comprehension of the mechanisms by which microbial components are detrimental or protective in asthma might provide the basis for host-directed therapies for severe asthma. In this sense, 2 detrimental possibilities may be considered in asthma and infection comorbidities: (1) asthma impairs and weakens the immune response against pathogens and (2) infections change asthma phenotype and induce a difficult-to-treat or severe asthma endotype.

It should be noticed that in opposition to this notion, the hygiene hypothesis elegantly explored and supported the idea that infections might negatively regulate type 2 inflammation, the hallmark of allergic asthma. The hygiene hypothesis might be briefly summarized by stating that the exposure to pathogens, or infectious agents or their molecules, drives the immune response to the T_H1 profile or toward regulatory T (Treg) cells, and counteracts or reduces the T_H2 profile.[58] In the former decade, we evaluated the therapeutic potential of experimental vaccines against tuberculosis in an asthma model. Subunit vaccines and a DNA vaccine with mycobacterial antigens, used as allergen-free immunotherapies in murine asthma model with OVA or *Dermatophagoides pteronyssinus* protein (Der-p1), reduced airway inflammation and improved pulmonary function.[59-62]

Although infections might regulate diversely the immunopathology of asthma, in the following section, we shall discuss how the infections drive the severity of asthma and we will focus on how airway and pulmonary bacterial infections might be detrimental in asthma. This field deserves investigations to advance in the search of targets for immunotherapies. Furthermore, in our critical view, the order of stimuli (allergen-infection or infection-allergen) and the time-dependency of infection are critical factors that drive the exacerbation or reduction of asthma immunopathology.

BACTERIAL INFECTIONS AND ASTHMA EXACERBATIONS

Pathogenic bacteria, atypical bacteria, and lung microbiota might affect the immunopathology of asthma.[20,22,63-66] The

association between atypical bacteria, as *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*, and asthma exacerbation is strongly associated with the enhancement of allergic asthma phenotype and type 2 inflammation induced by the infection.[64]

Clinical evidence and experimental studies support a significant association between asthma severity and changes in bacterial community profiles. A recent clinical trial with 214 children showed alterations in the airway microbiota at time of early loss of asthma control. The airway microbiota dominated by *Corynebacterium* and *Dolosigranulum* was associated with favorable clinical outcomes compared with microbiota dominated by pathogenic bacteria, including *Staphylococcus*, *Streptococcus*, and *Moraxella*.[67] These data reinforce the results obtained in a study with adults classified as adults with mild asthma, which revealed a negative correlation between the relative abundance of *Corynebacterium* and *Moraxella* in nasal brush. In addition, this study showed that the relative abundance of *Moraxella* and *Streptococcus* correlated positively with systemic eosinophilia and lower airway eosinophils and bronchial levels of tumor necrosis factor and IL-7, respectively.[68] In 17 of 28 treatment-resistant patients with severe asthma, the dominant species within the airway bacterial community were *Moraxella catarrhalis* and members of *Haemophilus* and *Streptococcus* genera. Chronic infections with these species were associated with longer asthma duration and positively correlated with higher IL-8 concentrations and neutrophil counts in the sputum.[69] If there is such relationship, further studies are necessary to indicate which one comes first, asthma severity or bacterial chronic infections.

Chronic bacterial infections are possible factors that contribute to the development of neutrophilic asthma[20,22] and the neutrophil-mediated inflammation could explain the inefficacy of corticosteroids in a T_H2-low asthma phenotype.[20] Considering this, antibiotic therapy has been suggested. Treatment with macrolide (azithromycin) as an immunomodulator and anti-inflammatory agent for 6 months reduced the exacerbation in those with noneosinophilic asthma, suggesting that chronic bacterial infections in T_H2-low patients is a significant contributing factor to exacerbation risk.[70]

Airway neutrophils from subjects with asthma spontaneously release lower levels of IL-8, IL-1β, and tumor necrosis factor compared with healthy controls, suggesting an impaired role of these cells, which may impact the susceptibility to airway infections.[71] Both bacteria from *Haemophilus* and *Streptococcus* genera require neutrophils to control chronic infections,[72,73] and the function of neutrophils is compromised in asthma milieu.

Haemophilus influenzae

H influenzae is a Gram-negative, nonencapsulated bacterium that chronically infects the airways and is the most common isolated bacteria from patients with asthma, associated with neutrophilic asthma and corticosteroid-reduced response in asthma.[74] Accordingly, abundance of *H influenzae* in the airways is associated with predominance of neutrophils in the sputum of patients with asthma.[75]

The cell type mostly infected by *H influenzae* is AECs. Nontypeable *H influenzae* is recognized in the airways by TLR2, resulting in Myd88 recruitment with NF-κB activation. However, the subsequent effector mechanisms responsible for the clearing of the infection and the control of inflammatory response remain elusive.[76] Mice previously infected with *H influenzae* and submitted to OVA-induced asthma exhibited a higher bacterial load compared with single infected mice. Furthermore, chronic *H influenzae* infection and OVA exposure induced neutrophilic inflammation and T_H17 responses that promoted bacterial persistence, leading to the development of a phenotype similar to steroid-resistant neutrophilic asthma.[77] Anti–IL-17 treatment of OVA-sensitized and *H influenza*–infected mice completely inhibited airway neutrophilic inflammation induced by the infection, suggesting that neutrophilic inflammation is dependent on IL-17.[73] In addition, infection by nontypeable *H influenzae* in mice exposed to allergen promotes increase of AHR, MUC5A, and MUC5B expression and is associated with hyperphosphorylation of p38 mitogen-activated protein kinase. The combined treatment with dexamethasone and SB203580, a specific inhibitor of p38, substantially suppressed the *H influenzae*–induced asthma exacerbation.[78] On the basis of these findings, the authors suggested that cotreatment with dexamethasone and SB203580 could be a novel strategy against steroid-resistant asthma.

Acute *H influenzae* infection induces T_H17 cells, neutrophil influx, increased mucus production, and attenuation of the lung function in mice.[79] Long-term exposure to low dose of *H influenzae* after OVA challenge resulted in increased tissue remodeling and reduced number of Treg cells in the lungs and IL-10 levels in the bronchoalveolar lavage fluid, suggesting an impaired anti-inflammatory response during the comorbidity.[79]

In a recent randomized clinical trial, azithromycin treatment for 48 weeks of adults with uncontrolled asthma reduced *H influenzae* load in sputum and efficiently reduced the asthma exacerbations rate.[80] However, this long-term treatment favored the antibiotic resistance to *Staphylococcus aureus*, but not to *H influenza* and *S pneumoniae*. These findings show that the comorbidity asthma and bacterial infections must be treated in 2 ways: short-term antibiotic and regulation of inflammatory response. Therefore, the search for new anti-inflammatory targets to control bacterial infection–associated asthma exacerbations is critical.

Moraxella catarrhalis

M catarrhalis is a Gram-negative diplococcus, nonencapsulated bacterium that colonizes the mucosal membranes of nasopharynx. *M catarrhalis* has emerged as a transmittable pathogen, causing infections such as pneumonia, laryngitis, endocarditis, meningitis, and otitis media.[81] Particularity, *M catarrhalis* is associated with chronic obstructive pulmonary disease and asthma exacerbations. Bacteriome characterization of hospitalized children with asthma in consequence of an episode of asthma exacerbation showed the presence of *M catarrhalis* in the nasopharyngeal swabs.[82] Moreover, children with asthma with nasal airway microbiota colonized predominantly by *Moraxella* genera have an increased risk of asthma exacerbations.[83]

Epithelial cells from mucosal surface are the main targets of *M catarrhalis*. In the alveoli, *M catarrhalis* interacts with TLR2, TLR4, and TLR9 from AECs, resulting in NF-κB activation. Consequently, AECs increase the expression of adhesion molecules (intercellular adhesion molecule 1 and vascular cell adhesion molecule 1), and produce CCL2 and MCP-1, which recruit monocytes and DCs.[81] Lipooligosaccharidae from the bacterial membrane activates TLR4 in macrophages by a mechanism dependent on CD14, leading to NF-κB activation and production of IL-6, IL-8, and tumor necrosis factor.[84] IL-6, tumor necrosis

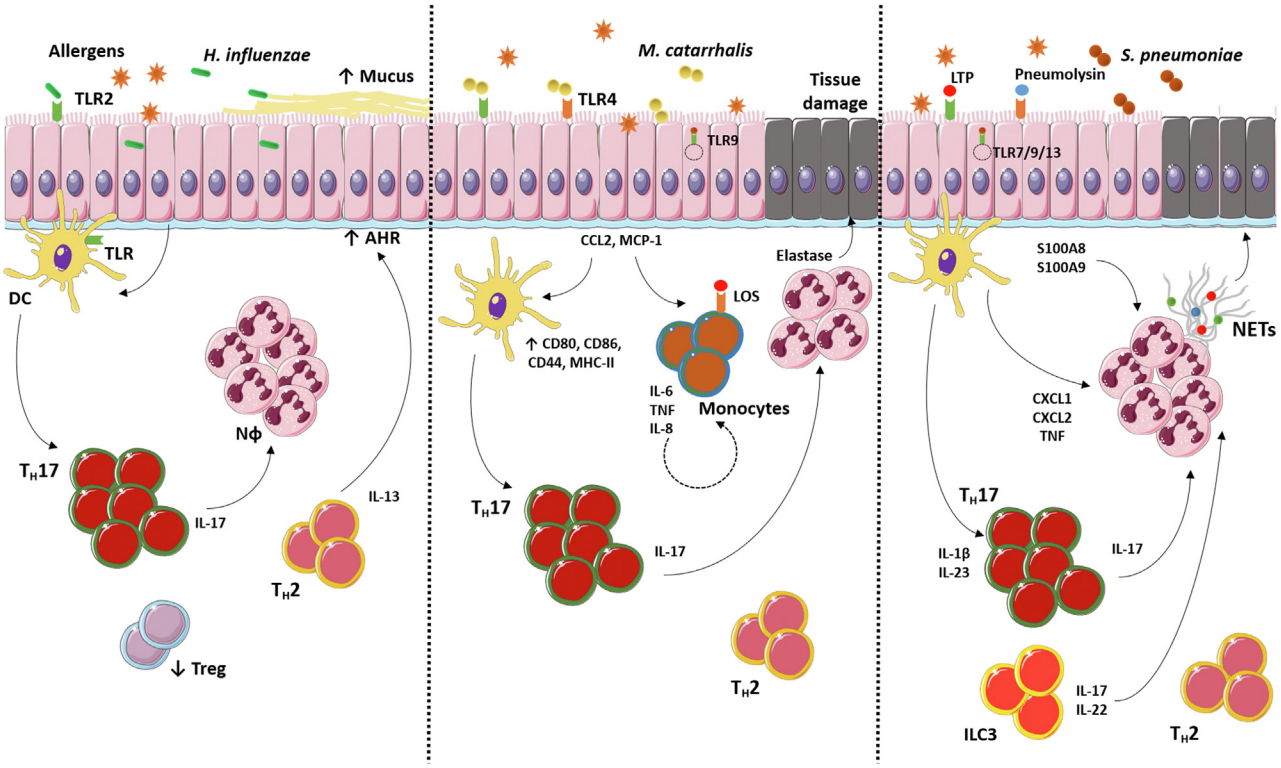

FIG 3. Bacterial infections and asthma exacerbation. Bacterial infections activate TLRs on DCs and might drive the allergic immune response from T_H2 to T_H2/T_H17 cells. Activated eosinophils and neutrophils produce inflammatory mediators that activate smooth muscle cells and damage epithelial cells, which release S100A8 and S100A9 alarmins. T_H17 cells might indirectly aggravate the immunopathology of asthma, inducing the recruitment of neutrophils that produce NETs.

factor, and IL-8 produced by macrophages result in recruitment and activation of monocytes, and exacerbation of inflammatory response.[81,85] The recognition of pathogens by DCs results in an increase in costimulatory molecules (CD80 and CD86), CD44, an adhesion molecule, and MHC-II expression. In addition, activated DC promotes both $CD8^+$ and $CD4^+$ T-lymphocyte activation.[81] $CD4^+$ lymphocytes secrete cytokines that induce recruitment and activation of granulocytes. As a consequence, activated neutrophils release elastase, which induces tissue damage.

Asthma exacerbations caused by *M catarrhalis* infection are also associated with recruitment and activation of T_H17 cells. In a mouse model of HDM-induced asthma, *M catarrhalis* infection in different time points of allergic airway inflammation induced severe airway inflammation and mucus production and added to increased concentrations of IL-17 and neutrophils in the bronchoalveolar lavage fluid.[86] IL-17 knockout mice exposed to allergen and infected displayed a significant reduction in neutrophils in the bronchoalveolar lavage fluid, in the lung inflammation, and in mucus production, indicating that IL-17–producing $CD4^+$ cells were a key mediator of *M catarrhalis*–induced asthma exacerbations.[86]

Streptococcus pneumoniae

S pneumoniae is a Gram-positive diplococcus bacterium that frequently colonizes the human nasopharynx. Pneumococcal infections lead to serious invasive diseases such as meningitis, septicemia, and pneumonia, and are an important cause of morbidity and mortality among children and in older adults.[87,88]

The susceptibility to *Streptococcus* infection was already described in patients with asthma. A study with 224 patients with asthma and 668 males without asthma showed that asthma was the only significant risk factor for *S pneumoniae* carriage.[89] A nested case-control study conducted with 635 subjects with invasive pneumococcal disease and 6350 controls showed that subjects with asthma had an increased risk of invasive pneumococcal disease.[90]

In the alveoli, *S pneumoniae* interacts with type II AECs, alveolar macrophages, and DCs through the recognition of pathogen-associated molecular patterns (PAMPs) by PRRs; pneumococcal lipoteichoic acid and cell wall peptidoglycanas are recognized by TLR2, pneumolysin is recognized by TLR4, and CpG motifs are recognized by TLR9.[91] An important synergy among TLRs has been described in response to *S pneumoniae*, especially TLR2/4/9 for induction of cytokines and chemokines[92] and TLR7/9/13 associated with susceptibility to infection.[93] TLR activation induces the production of tumor necrosis factor, CXCL1, and CXCL2, which recruit neutrophils. AEC also release alarmins, as S100A8 and S100A9, in the early phase of *S pneumoniae* infection that precedes neutrophil recruitment.[94] Although the activation of individual TLRs has a limited role in pneumococcal infection, MyD88, the adaptor protein of the signaling pathway of TLR, is crucial for initiating proinflammatory cytokine release and for the enhanced production of antimicrobial peptide to restrict *S pneumoniae* outgrowth.[95] DC recognize and phagocyte

pneumococcal mainly by pneumococcal adherence and virulence factor A and release IL-1β, IL-6, IL-8, IL-12, and tumor necrosis factor.[96] Airway DC, specially $CD103^+$ subset, might induce the release of IFN-γ and IL-17 by invariant natural killer cells,[97] whereas it has been shown that bone marrow–derived DC are important to induce T_H17-adaptive immune response,[98,99] both important to pneumococcal clearance. During pneumococcal infection, T_H17, T_H1, and T_H2 cells are critical to clear *S pneumoniae* infection. T_H1 and T_H17 cells recruit and activate macrophages and neutrophils, and T_H2 cells release IL-4 and induce the production of antibodies.[72] A potential synergistic effect of T_H17 cells and antibodies induces, respectively, increased recruitment of neutrophils and opsonization, which improve the control of chronic bacterial infection.[100] Besides antimicrobial defense mechanisms, neutrophils use NETs to restrict bacterial spreading.[101] Furthermore, IL-17 amplifies type II AEC activation by increasing the production of antimicrobial peptides and chemokines.[102] IL-17 and IL-22 are also produced by type 3 innate lymphoid cells by a mechanism dependent on DC, IL-1β, and IL-23.[41,103,104]

Although the susceptibility to *S pneumoniae* infection is increased in patients with asthma, the effect of infection during asthma episodes is still controversial and needs to be clarified. The induction of T_H1, T_H2, and T_H17 cells and the recruitment of neutrophils are potential risks for the development of severe asthma, and as we discussed previously, the exacerbation of pulmonary inflammation generates tissue damage.

Epidemiological and experimental findings showed a protective role for *S pneumoniae* infection in asthma. Children with asthma treated with sulfisoxazole and pneumococcal vaccine showed 56% reduction in the frequency of acute asthmatic attacks and 90% decrease in hospitalizations associated with otitis media.[105] Similarly, a retrospective cohort study, which evaluated the effectiveness of a 23-valent pneumococcal polysaccharide vaccine, showed that the vaccination decreased the risk of hospital admission for asthma compared with nonvaccinated subjects.[106] Experimentally, it was demonstrated that killed or live *S pneumoniae* administered before, during, or after OVA sensitization attenuated allergic inflammation.[107,108]

The protective role of infection after OVA sensitization increased T_H1 immune response in BALB/c mice, whereas *S pneumoniae* infection during OVA sensitization induced Treg cells and increased IL-10 production.[108] In addition, intranasal administration of pneumococcal conjugate vaccine, but not the polysaccharide vaccine, suppressed allergic immune response and increased Treg cells in the draining lymph nodes, lungs, and spleen.[109] Later, these authors identified pneumococcal components, 3 polysaccharides and pneumolysoid, as key immunoregulators of *S pneumoniae*–induced Treg cells. These components of *S pneumoniae* drove the differentiation of highly suppressive Treg cells, which inhibited T_H2 immune response, prevented the induction of T_H17 immune response, and disabled DC response, resulting in the effective suppression of OVA-induced inflammation,[110] supporting the hygiene hypothesis.[58] Experimental *S pneumoniae* infection in neonatal mice 21 days before OVA sensitization enhanced AHR and increased neutrophil recruitment and T_H17 cells into the airways, whereas IL-17 depletion alleviated airway inflammation mediated mostly by neutrophils, and decreased AHR.[111]

CONCLUSIONS

The interplay between asthma and bacterial infections is complex and involves both asthma and pathogen-specific immune responses. The comorbidities might affect the lung milieu and increase a preexisting T_H2 profile in subjects with allergic asthma (eosinophil inflammation) or drive the immune response to T_H17 profile and generate severe asthma (neutrophil inflammation or granulocytic inflammation). Specific pathogens might activate T_H17 cells that mediate neutrophil influx to the airways, which in turn exacerbates asthma (Fig 3).

It is likely that exacerbation of airway inflammation might require host-directed therapy, depending on the phenotype of asthma and on the comorbidity. The investigation of mediators and receptors using experimental models might provide new molecular targets. The confirmation of these targets is important to delineate immunotherapies or adjuvant therapies based on the concept of host-directed therapies for neutrophilic asthma endotype.

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Artepillin C Reduces Allergic Airway Inflammation by Induction of Monocytic Myeloid-Derived Suppressor Cells

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Abstract: Propolis is a natural product produced by bees that is primarily used in complementary and alternative medicine and has anti-inflammatory, antibacterial, antiviral, and antitumoral biological properties. Some studies have reported the beneficial effects of propolis in models of allergic asthma. In a previous study, our group showed that green propolis treatment reduced airway inflammation and mucus secretion in an ovalbumin (OVA)-induced asthma model and resulted in increased regulatory T cells (Treg) and polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC) frequencies in the lungs, two leukocyte populations that have immunosuppressive functions. In this study, we evaluated the anti-inflammatory effects of artepillin C (ArtC), the major compound of green propolis, in the context of allergic airway inflammation. Our results show that ArtC induces in vitro differentiation of Treg cells and monocytic MDSC (M-MDSC). Furthermore, in an OVA-induced asthma model, ArtC treatment reduced pulmonary inflammation, eosinophil influx to the airways, mucus and IL-5 secretion along with increased frequency of M-MDSC, but not Treg cells, in the lungs. Using an adoptive transfer model, we confirmed that the effect of ArtC in the reduction in airway inflammation was dependent on M-MDSC. Altogether, our data show that ArtC exhibits an anti-inflammatory effect and might be an adjuvant therapy for allergic asthma.

Keywords: propolis; artepillin C; allergic asthma; M-MDSC; therapies

1. Introduction

Propolis is a resin made by working *Apis mellifera* Linnaeus, 1758 bees from exudates and new buds of different plant species, which is traditionally used in alternative and complementary medicine. The Brazilian green propolis has the shrub *Baccharis dracunculifolia* DC as its primary botanical source and is rich in prenylated derivative compounds. It has gained notoriety for displaying anti-inflammatory, immunomodulatory, antitumoral, antibacterial, and antiviral proprieties [1–4]. Experimental evidence of anti-inflammatory actions of propolis was demonstrated in several murine models of inflammatory diseases. Those actions include the reduction in neutrophils and the levels of pro-inflammatory cytokines IL-6 and TNF-α in the bronchoalveolar lavage fluid (BALF) during LPS-induced pulmonary inflammation [5]; reduction in neutrophil infiltration and serum levels of IL-6,

IL-1β and TNF-α in experimental pancreatitis [6], and decreased production of reactive oxygen species (ROS) in ethanol-induced gastric ulcers [7]. In the type 2 inflammation induced by inhaled allergen, the treatment with propolis during the sensitization phase resulted in reduction in allergen-specific IgE, airway inflammation, and hyper-reactivity [8] and in a decreased number of inflammatory cells in the BALF [9]. Our research group characterized the anti-inflammatory action of green propolis in sensitized and challenged mice and showed a reduction in pulmonary inflammation and mucus secretion [10], suggesting a protective effect for propolis treatment in asthma.

Asthma is a chronic inflammatory lung disease that affects 339 million people worldwide [11]. Bronchial hyper-reactivity, mucus overproduction and airway remodeling induce symptoms such as shortness of breath, cough, and wheezing [12,13]. Although there are different disease phenotypes, the hallmark of allergic asthma is a type 2 immune response, characterized by IL-4, IL-5, and IL-13 secretion by T helper 2 (Th2) lymphocytes, IgE secretion, recruitment and activation of eosinophils, mast cells, dendritic cells and epithelial cells [12]. Currently, therapy with corticoids configures the most efficient treatment to control the disease symptoms but it does not cure asthma. Moreover, the recurrent use of corticoids favors the development of infections and impairs childhood development [12,14]. Therefore, there is an urgent need for new therapies for allergic asthma, both to control disease symptoms as well as to improve the life quality of asthmatic patients.

Although propolis treatment represents a promising strategy for immunotherapy, it is also challenging considering the isolation of the compounds that display the anti-inflammatory effects. Artepillin C (ArtC) (3,5-Diprenyl-4-hydroxicinnamic) is a prenylated phenolic compound derived from cinnamic acid and is one of the main components in green propolis [15]. Some in vitro studies demonstrated that ArtC inhibits ROS production, cytokine secretion and blocks NF-κB expression in IFN-γ-stimulated RAW264.7 macrophage cell lineage [16]. Additionally, ArtC exhibits an anti-inflammatory role in an experimental model of peritonitis, in which it induces reduction in leukocyte and PGE_2 levels in peritoneal exudate [17].

In this study, we aimed to evaluate the anti-inflammatory activity of ArtC in allergic airway inflammation. We hypothesized that ArtC induces regulatory $CD4^+$ T (Treg) cells and myeloid-derived suppressor cells (MDSC), which negatively regulates allergic pulmonary inflammation. Treg cells are involved in the regulation of immune response by suppressing the function of effector T cells (Th1, Th2, Th17, and $CD8^+$ T cells) [18]. MDSC comprises a heterogeneous cell population derived from bone marrow that expands in situations of physiological stress, such as chronic inflammation and tumors, and also display immunosuppressive actions [19–21]. There are two subsets of MDSC: the granulocytic (PMN-MDSC) and the monocytic (M-MDSC), which are classified according to their origin, either from granulocytic or monocytic myeloid cells lineages [22,23].

We showed that ArtC treatment reduced eosinophilic lung inflammation in an ovalbumin (OVA)-induced allergic asthma model and induced M-MDSC differentiation in vivo and in vitro. Finally, we performed ArtC treated-M-MDSC transfer to animals sensitized and challenged with the allergen and confirmed that the decrease in eosinophilic lung inflammation was dependent on ArtC.

2. Materials and Methods

2.1. Artepillin C (ArtC) Isolation

ArtC was isolated from the crude extract of green propolis provided by Apis Flora Company (Ribeirao Preto, SP, Brazil). First, the green propolis was frozen, pulverized in a blender, and extracted with hydroalcoholic solution (9:1) to furnish the crude hydroalcoholic extract. It was fractioned using a silica gel open chromatographic column with a gradient elution consisting of hexane–ethyl acetate 95:05 to 90:10 *v/v* furnishing approximately 100 fractions. Each fraction was analyzed by TLC (thin-layer chromatography), concentrated under vacuum, and combined according to its chromatographic similarities. The fractions were analyzed by HPLC-DAD (high-performance liquid chromatography

with a diode-array detector) using authentic ArtC standard. Then, the fractions rich in ArtC were purified by preparative HPLC-UV at 275 nm furnishing 68 mg of pure ArtC with relative purity of 95%. The elution program consisted of 7 min gradient with 80% B (methanol) and 20% A (99% water, 1% formic acid), 12 min with 85%B, 20 min with 90% B, 22 min with 95% B, and a flow rate of 8.0 mL/min, and detection at 275 nm, using a reverse-phase column (Shimadzu Shim-pack prep.-ODS 15 μm, 20 × 250 mm) [24]. ArtC chemical structure was confirmed by spectroscopic (1H NMR), and spectrometric (High-resolution ESI-MS) analyses. ArtC was solubilized in DMSO and maintained at −80 °C until the moment of use. For in vivo experiments, ArtC-DMSO was diluted in phosphate buffered sSaline (PBS) with a final concentration of 0.05% of DMSO. For in vitro experiments, ArtC-DMSO was diluted in RPMI 1640 (Sigma-Aldrich, St Louis, MO, USA) with a final concentration ≤1.8% DMSO.

2.2. Animals

Female C57BL/6 mice (6–8 weeks old) were obtained from the breeding facility of Ribeirao Preto Medical School, University of São Paulo (Ribeirao Preto, SP, Brazil). All animals were maintained in sterile environmental conditions in a ventilated rack (Alesco, Monte Mor, SP, Brazil) and received sterile food and water. Body weight was measured weekly. All experiments were performed according to the local Ethics Committee on Animal Experimentation (Protocol Number 216/2019).

2.3. OVA-Induced Asthma Model

Mice were sensitized three times with 10 μg of ovalbumin (OVA) Grade-VI emulsified in 2 mg of aluminum hydroxide (alum) (all from Sigma-Aldrich, St Louis, MO, USA) by intraperitoneal route with seven-day intervals. Mice were challenged three consecutive days with 30 μg of OVA Grade V (Sigma-Aldrich, St Louis, MO, USA) by intranasal route seven days after the third sensitization. For challenge, mice were anesthetized with ketamine (100 mg/kg, Sespo Industry and Commerce, Paulínia, SP, Brazil) and xylazine (10 mg/kg, Vetecia Laboratory of Veterinary Products, Jacareí, SP, Brazil).

2.4. Treatment

Mice were treated with seven doses of vehicle (PBS, 0.05% DMSO) or ArtC (80 μg, 0.05% DMSO) by intranasal route 24 h after the third allergen challenge in alternate days. After the end of treatment, the mice were challenged with the allergen three more times. For challenge, mice were anesthetized with ketamine (100 mg/kg, Sespo Industry and Commerce, Paulínia, SP, Brazil) and xylazine (10 mg/kg, Vetecia Laboratory of Veterinary Products, Jacareí, SP, Brazil).

2.5. Bronchoalveolar Lavage

To assess bronchoalveolar lavage fluid (BALF), animals were anesthetized with ketamine (100 mg/kg, Sespo Industry and Commerce, Paulínia, SP, Brazil) and xylazine (10 mg/kg, Vetecia Laboratory of Veterinary Products, Jacareí, SP, Brazil). Then, the trachea was exposed and cannulated with Angiocath with subsequent injection of 4 mL of PBS. Next, samples were centrifuged at 450× *g* for 5 min. The supernatant was stored at −20 °C for cytokine measurement. Cells were resuspended in 500 μL of RPMI 1640 (Sigma-Aldrich, St Louis, MO, USA), centrifuged at cytocentrifuge (Thermo Fisher Scientific, Waltham, MA, EUA) at 18× *g* for 3 min, and stained with rapid panoptic (Labor Clin, Sao José do Rio Preto, SP, Brazil) for differential cell count.

2.6. Lung Homogenate

After collection of BALF, animals were perfused with 5 mL of PBS. Left lung lobules were mechanically macerated in microtubes containing protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 3220× *g* for 15 min. The supernatant was collected and maintained at −20 °C for cytokine measurement.

2.7. Histopathology

The histopathological analysis was performed as previously described [25]. Briefly, right lung lobules were stained with hematoxylin and eosin (H&E) to evaluate the inflammatory infiltrate and periodic acid-Schiff (PAS) to evaluate mucus production. Histological images were performed using the objective lens of 20× (magnification 200×). Pulmonary inflammation score was determined following the criteria: 0–without inflammation; 1—mild inflammation, and 2—moderate to severe inflammation. Mucus score was determined following the criteria: 0—without mucus production, and 1—mild mucus production.

2.8. MDSC Differentiation

Bone marrow-derived MDSC were generated according to Solito and colleagues [26] from female C57BL/6 mice. Briefly, cells were stimulated with recombinant IL-6 (40 ng/mL, BD Pharmingen, San Diego, CA, USA) and GM-CSF (40 ng/mL, BD Pharmingen, San Diego, CA, USA) in the presence or absence of ArtC (0.01, 0.1, 5, 10, 20, 50, or 100 μM). Cells were cultured for 96 h, at 37 °C and 5% CO_2.

2.9. Treg Cell Differentiation

Naive T cells ($CD4^+CD62L^+$) from spleen and lymph nodes of female C57BL/6 mice, purified using magnetic beads (Mylteni Biotec, Bergisch Gladbach, NRW, Germany), were distributed in plates previously coated overnight with monoclonal antibody anti-CD3 (5 μg/mL, BD Pharmingen, San Diego, CA, USA) in the presence of anti-CD28 (1 μg/mL), rTGF-β (3 ng/mL, BD Pharmingen, San Diego, CA, USA) and rIL-2 (10 ng/mL, BD Pharmingen, San Diego, CA, USA), treated or not with ArtC (0.01, 0.1, 5, 10, 20, 50 or 100 μM). For Treg cell differentiation, cells were cultured for 96 h, at 37 °C and 5% CO_2.

2.10. Flow Cytometry

Lung cells were isolated by proper right lung lobules digestion using collagenase (2.2 mg/mL) (Sigma-Aldrich, St Louis, MO, USA) and DNAse (0.055 mg/mL) (Roche, Basel, Switzerland). Characterization of MDSC and Treg cells was performed by flow cytometry according to antibodies fabricant instructions (BD Pharmingen, San Diego, CA, USA). Samples were stained with FVS780 viability stain, CD45, CD11b, Ly6C, and Ly6G to characterize MDSC and CD45, CD4, and Foxp3 to characterize Treg cells (Table 1). Samples were fixed using PBS containing 1% paraformaldehyde (Labsynth, Diadema, SP, Brazil). The samples were acquired in FACS Melody (BD Biosciences, San Jose, CA). On average, two hundred and fifty thousand events per sample were collected within the gate of viable cells ($FVS780^-$). Analyses were performed in FlowJo software (Becton Dickinson and Company, Franklin Lakes, NJ, USA).

Table 1. Monoclonal antibodies used in flow cytometry assays.

Cell Type	Antibody	Fluorochrome	Clone
In vitro MDSC	CD11b	PE-Cy7	M1/70
	Ly6C	PerCP Cy5.5	AL-21
	Ly6G	APC	1A8
In vitro Treg cell	CD4	BB700	RM4-5
	Foxp3	PE	MF23
In vivo MDSC	CD45	PE-Cy7	30-F11
	CD11b	FITC	M1/70
	Ly6C	PerCP Cy5.5	AL-21
	Ly6G	APC	1A8
In vivo Treg cell	CD45	PE-Cy7	30-F11
	CD4	BB700	RM4-5
	Foxp3	PE	MF23

2.11. M-MDSC Adoptive Transfer

For adoptive transfer, MDSC were generated in the presence or absence of ArtC (10 μM). Subsequently, M-MDSC ($Ly6G^{-}Ly6C^{+}$) were purified using FACS Melody sorting (BD Biosciences, San Jose, CA, USA), and 1×10^5 cells were transferred by intrapharyngeal route to OVA sensitized-mice during the second allergen challenge. Twenty-four hours after the third challenge, animals were euthanized to evaluate lung inflammation.

2.12. Cytokines

IL-4, IL-5, IL-10, and IL-13 levels were determined in BALF and lung homogenates using ELISA kits following the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The limit of detection was 31.2 pg/mL.

2.13. Statistical Analysis

Data were analyzed using GraphPad Prism Version 8.1 (GraphPad Software, Inc., San Diego, CA, USA). Two-group comparisons were analyzed by non-paired t-test, and three or more groups' comparisons were calculated by ANOVA one-way followed by Tukey's test. Correlation analyses were performed following Pearson's correlation coefficient. The histological score was calculated by the Chi-square test. Data were shown as the mean ± standard deviation (SD), and the results were considered significant with a *p*-value less than 0.05.

3. Results

3.1. ArtC Isolation

The chromatographic processes used to obtain pure ArtC were previously reported by Rodrigues and colleagues [24] and yielded approximately 68 mg of purified compound, which was used for running the biological assays. ArtC chemical structure was confirmed by ^{1}H NMR ($CDCl_3$, 300 MHz) δH: 7.72 (d, 1H, J 15.9 Hz, H7), 7.22 (s, 2H, H6 and H2), 6.31 (d, 1H, J 15.9 Hz, H8), 5.33 (t, 2H, J 7.2 Hz H2′, and H2″), 3.37 (d, 4H, J 15.9 Hz, H1′, and H1″), and 1.80 (d, 12H, H5′, H5″, H4′, and H4″). For $C_{19}H_{24}O_3$ $[M + H]^+$: 301.1759 (Figure 1a); found $C_{19}H_{24}O_3$ $[M + H]^+$: 301.1790 (Figure 1b).

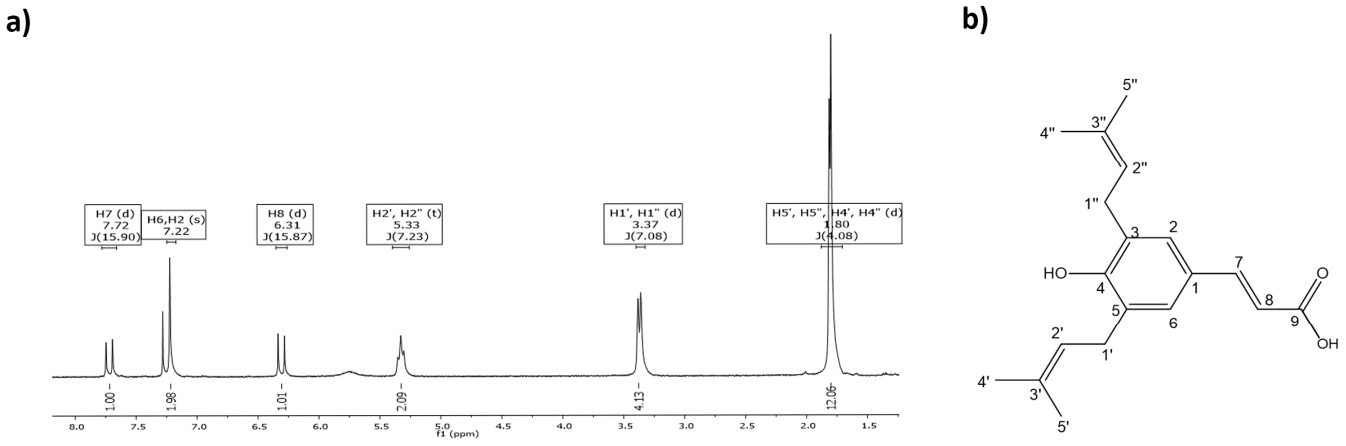

Figure 1. ArtC isolation. (**a**) ^{1}H NMR spectrum of ArtC (CD3OD, 300 MHz); (**b**) chemical structure of ArtC.

3.2. ArtC Attenuates Allergic Airway Inflammation

We and others have previously demonstrated the anti-inflammatory effect of propolis in experimental allergic asthma [8–10,27]. However, a major challenge for the development of immunotherapies employing natural products, such as propolis, is to identify the individual active compounds that display anti-inflammatory activities. In order to do that, OVA-sensitized and challenged mice were treated with ArtC (treated group) or with vehicle (PBS, non-treated group), as depicted in Figure 2a. Following treatment, mice were challenged with the allergen and lung inflammation was evaluated (Figure 2a). No

differences in body weight were detected prior to and after treatment between treated and non-treated groups (Figure 2b).

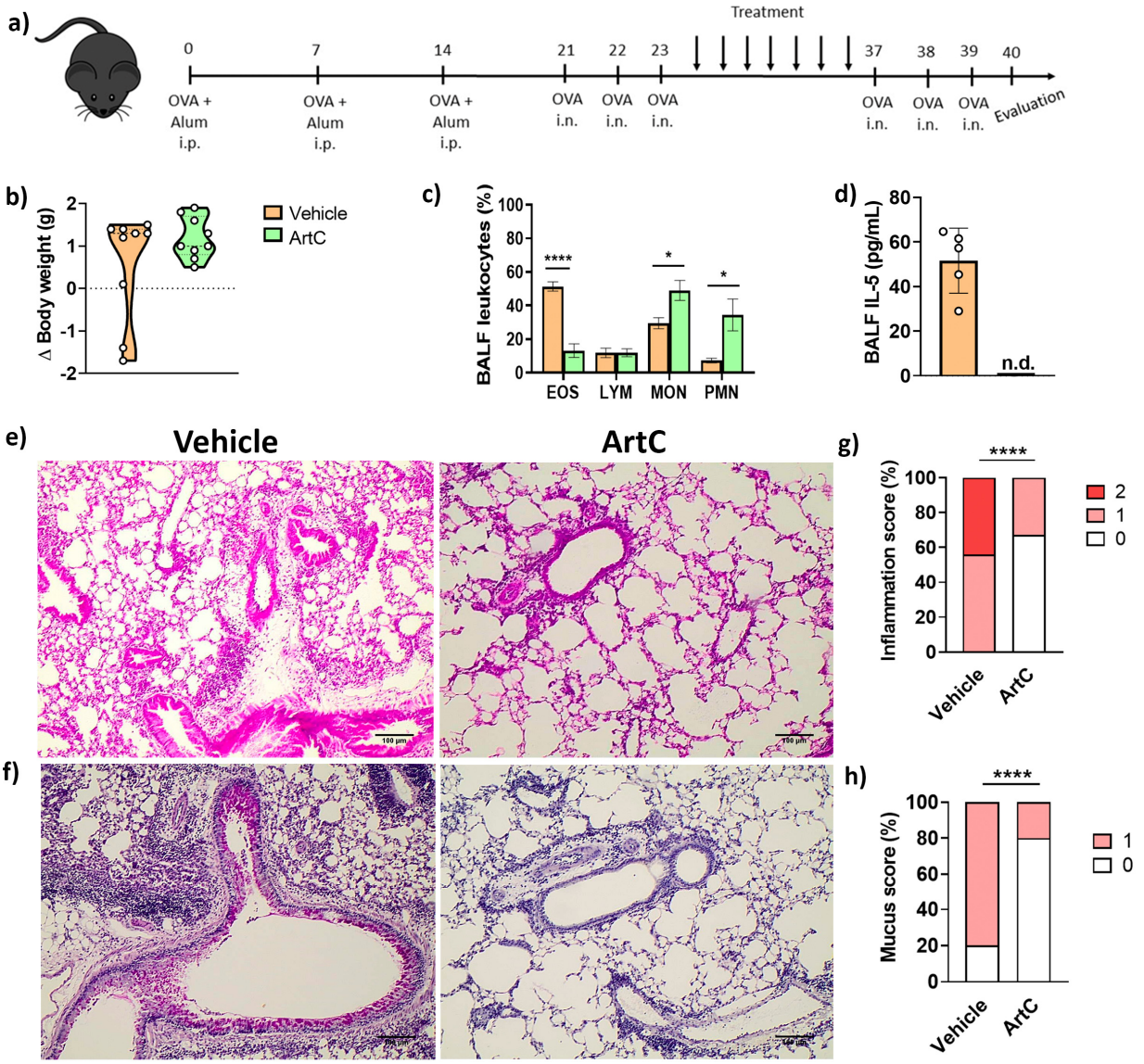

Figure 2. ArtC reduces allergic airway inflammation. (**a**) Experimental design; (**b**) body weight variation of mice exposed to the allergen before and after treatment with ArtC; (**c**) frequency of eosinophils (EOS), lymphocytes (LYM), mononuclear cells (MON) and polymorphonuclear cells (PMN) in the BALF; (**d**) IL-5 in the BALF (n.d.: not detected); (**e**) representative images of lung inflammation (magnification 200×, scale bar 100 μM) and (**f**) mucus production (magnification 200×, scale bar 100 μM); (**g**) lung inflammation score (0—without inflammation; 1—mild; 2—moderate to severe inflammation); (**h**) mucus score (0—without mucus secretion; 1—mild). Data are representative of two independent experiments (n = 4–5/group/experiment), except for (**d**) (one representative experiment), and expressed by mean ± SD. * $p < 0.05$ and **** $p < 0.0001$.

There was a significant reduction in the frequency of eosinophils and IL-5 levels along with an increase in mono and polymorphonuclear cells in the BALF of ArtC-treated animals compared with that of non-treated allergen-exposed mice (Figure 2c,d). No difference was found in IL-4 concentrations in lung homogenates and no IL-4 was detected in the BALF comparing groups treated or not treated with ArtC, previously exposed to OVA (vehicle group and ArtC group). IL-13 concentrations were also similar in the BALF and in the lung homogenates between both groups (data not shown). The histopathological analysis clearly showed a significant decrease in the perivascular and peribronchial cellular infiltrates

(Figure 2e) and mucus secretion (Figure 2f) in lungs of ArtC-treated mice compared to those of animals in the vehicle-treated group. Inflammation and mucus scores were evaluated and a reduction in both parameters was observed following ArtC treatment, whereas two represented the highest level of inflammation and one the highest level of mucus production, respectively (Figure 2g,h).

Collectively, these results show that the in vivo treatment with ArtC reduced allergic airway inflammation in a model of OVA-induced asthma.

3.3. ArtC Induces Treg Cells Differentiation In Vitro, but Not In Vivo

Because we previously reported that propolis increases the differentiation of regulatory (Treg) T cells [10], we first investigated whether purified ArtC would induce Treg cells in vitro. Treg cells were differentiated from $CD4^+CD62L^+$ naive T lymphocytes purified from spleen and lymph nodes and further cultured in the presence of recombinant IL-2 and TGF-β. Figure 3a depicts the gate strategy used to evaluate Treg cells expressing the transcription factor Foxp3. ArtC was not toxic at concentrations up to 10 μM (Figure 3b). The addition of ArtC in the latter non-toxic concentration (10 μM) increased the differentiation of Treg cells compared with those cells cultured with IL-2 and TGF-β only (CTL) (Figure 3c,d). We next evaluated the frequency of Treg cells in the lungs of ArtC-treated mice according to the gate strategy represented in Figure 3e. ArtC treatment neither affected the frequency of $Foxp3^+$ Treg cells (Figure 3f) nor the production of IL-10 (data not shown) in the lungs of treated mice, indicating that although ArtC induced $Foxp3^+$ Treg cells differentiation in vitro, this outcome was not observed in lungs of mice exposed to the allergen in vivo.

3.4. ArtC Augments M-MDSC Frequency In Vitro and In Vivo

Propolis treatment was shown to increase the differentiation of MDSC [10] and we found a significant increase in the frequencies of mononuclear and polymorphonuclear cells in the BALF of animals treated with ArtC (Figure 2c). Next, we assessed the ability of ArtC to induce the differentiation of MDSC in vitro. MDSC is a heterogeneous population of immature myeloid cells that expands in pathologic situations and exhibits suppressive functions that regulate the immune response [19,20]. The two subsets of MDSC, granulocytic (PMN-MDSC: $Ly6G^+Ly6C^{int}$) and monocytic (M-MDSC: $Ly6G^-Ly6C^+$) were characterized as depicted in Figure 4a. ArtC was not toxic at concentrations up to 20 μM (Figure 4b), and therefore, for these experiments we used the same concentration of ArtC employed in Treg cell cultures (10 μM) (Figure 3b). The presence of ArtC increased the frequency of M-MDSC and reduced the frequency of PMN-MDSC compared to control cells (bone marrow cells cultured with IL-6 plus GM-GSF only) (Figure 4c–e).

We also evaluated the frequency of $Ly6G^-Ly6C^+$ (M-MDSC) and $Ly6G^+Ly6C^{int}$ (PMN-MDSC) in the lungs of mice exposed to the allergen, treated or not treated with ArtC. We used the gate strategy depicted in Figure 5a to characterize subsets of MDSC in the lungs. ArtC-treated mice exhibited an increase in the frequency (Figure 5b,c) and in the numbers (Figure 5d) of $Ly6G^-Ly6C^+$ cells but neither in the frequency (Figure 5b,e) nor in the number (Figure 5f) of $Ly6G^+Ly6C^{int}$ cells in the lungs. We performed a correlation analysis and found a negative correlation between the frequencies of pulmonary eosinophils and $Ly6G^-Ly6C^+$ cells in vehicle-treated mice, while no significant correlation was observed in the ArtC-treated group (Figure 5g). These findings suggest that $Ly6G^-Ly6C^+$ cells negatively regulate eosinophilic inflammation during allergic asthma.

3.5. M-MDSC Suppress the Allergic Airway Inflammation

To confirm the role of M-MDSC differentiated in the presence of ArtC in down-regulating allergic pulmonary inflammation, we next adoptively transferred sorted M-MDSC generated in vitro from bone marrow in the presence or absence of ArtC. On the second day of OVA challenge, sorted M-MDSC were transferred by intrapharyngeal route

(Figure 6a). A representative photomicrography of sorted differentiated M-MDSC before cell transfer is shown in Figure 6b.

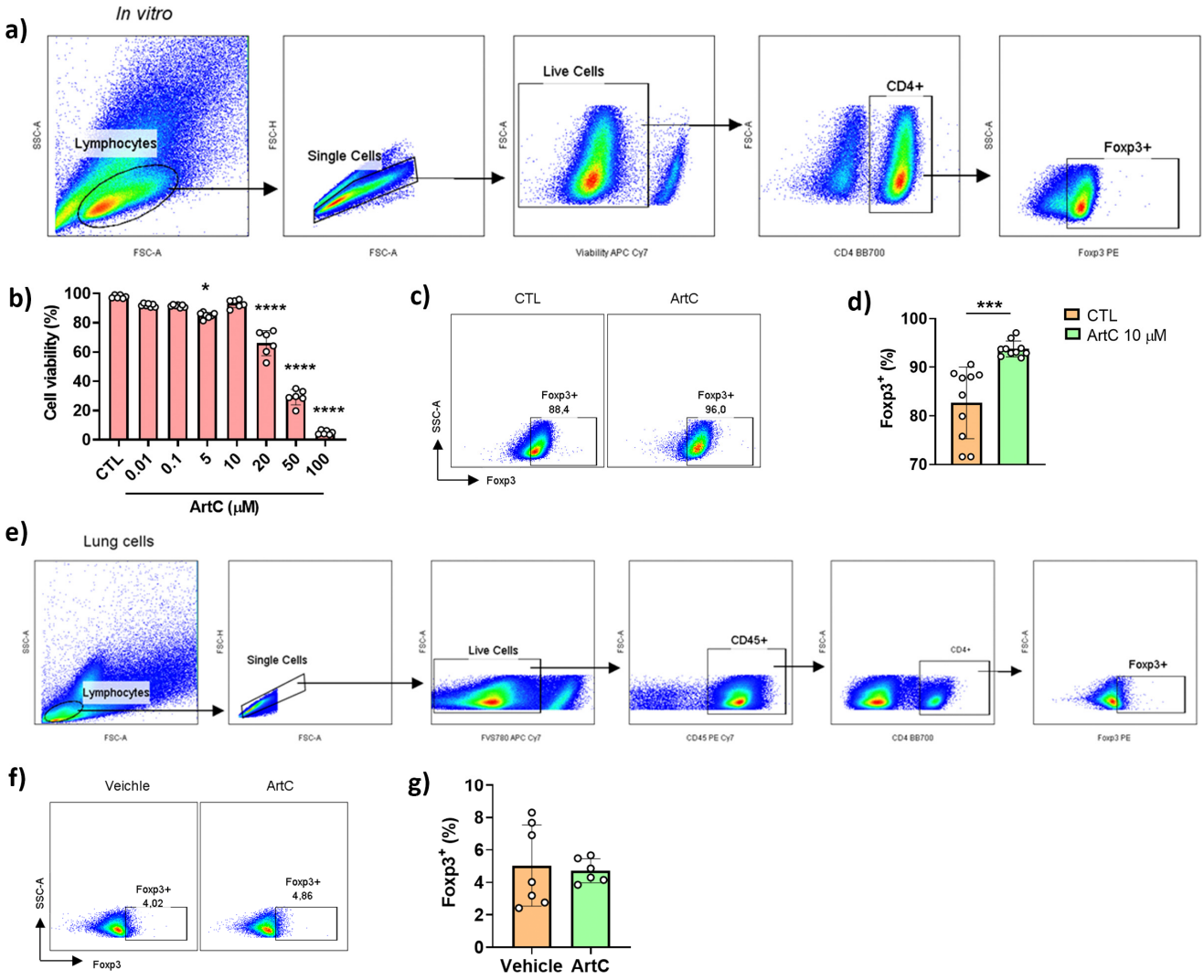

Figure 3. ArtC induces Treg cells differentiation in vitro. (**a**) Gate strategy for Treg cell ($CD4^+Foxp3^+$) characterization in vitro by flow cytometry; (**b**) frequency of live cells ($FVS780^-$) in culture of $CD4^+$ T cells stimulated with ArtC versus control (CTL); (**c**) representative dot plot of Treg cells generated in vitro in the presence or absence of ArtC; (**d**) percentage of Treg cells generated in vitro. Data are representative of three independent experiments (n = 3–4/group/experiment) and expressed by mean ± SD; (**e**) gating strategy for Treg cell characterization in vivo; (**f**) representative dot plot of Treg cell in the lungs of mice exposed to the allergen and treated or not (vehicle) with ArtC; (**g**) frequency of Treg cells in the lungs. Data are representative of two independent experiments (n = 4–5/group/experiment) and expressed by mean ± SD. * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$.

A significant reduction in both the frequency and number of eosinophils in the BALF of challenged mice was observed following ArtC-treated M-MDSC transfer (Figure 6c,d). Although a similar trend was observed following transfer of non-treated M-MDSC, the results were not significant (Figure 6c,d). An increase in the frequency, but not number, of monocytic cells was also observed following transfer of ArtC-treated M-MDSC (Figure 6c,d). In addition, we observed a reduction in IL-4 (Figure 6e), but not in IL-5 levels (Figure 6f) in the lungs homogenates after the transfer of ArtC-treated M-MDSC. IL-13 levels in the BALF and lungs homogenates are not changed between three groups (data not shown). Similarity, we did not observe any change in IL-10 levels in the lungs (data not shown). IL-4, IL-5 and IL-10 levels are not detected in the BALF. Flow cytome-

try analysis showed that intrapharyngeal transfer of sorted M-MDSC increased that cell population in the lung tissue compared with the lungs of mice that did not receive cell transfer (Figure 6g–i). Histological analysis revealed that transfer of both M-MDSC groups significantly decreased pulmonary infiltrates (Figure 6j) and mucus secretion (Figure 6k). However, the inflammation and mucus score (Figure 6l,m) were significantly lower in the lungs of mice transferred with ArtC-treated M-MDSC in comparison to those that received non-treated M-MDSC. These results confirm that ArtC treatment of M-MDSC increases their capacity in reducing eosinophil infiltration, inflammation, and mucus secretion in the lungs during airway allergic inflammation.

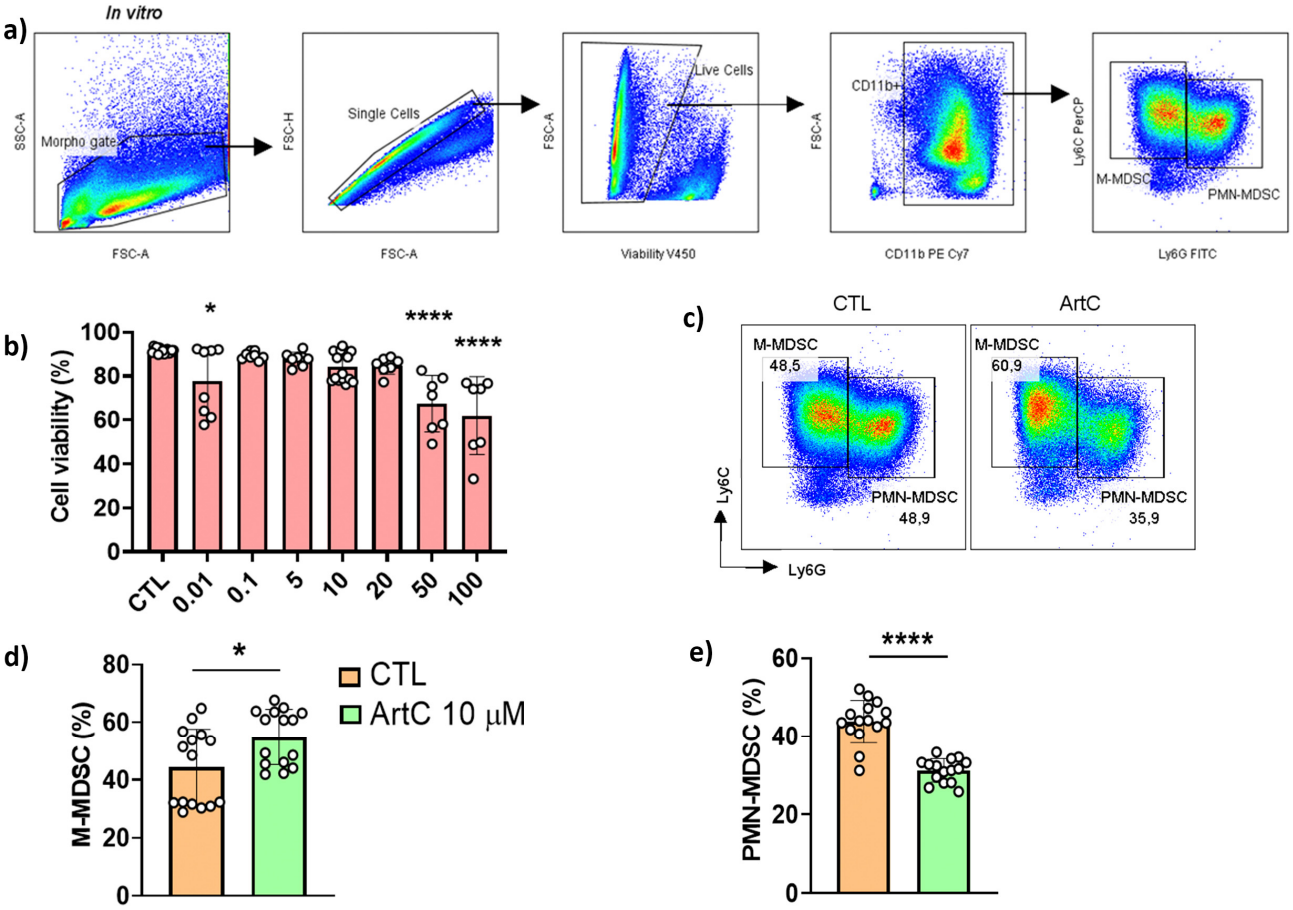

Figure 4. ArtC induces M-MDSC differentiation in vitro. (**a**) Gate strategy for M- (Ly6G^{-}Ly6C^{+}) and PMN-MDSC (Ly6G^{+}Ly6C^{int}) characterization in vitro; (**b**) frequency of live cells (FVS780−) in culture of bone marrow cells stimulated with rIL-6, GM-CSF and ArtC versus control (CTL); (**c**) representative dot plot of M- and PMN-MDSC generated in vitro in the presence of ArtC (10 μM); (**d**) percentage of M-MDSC, and (**e**) PMN-MDSC. Data are representative of four independent experiments (n = 3–4/group/experiment) and expressed by mean ± SD. * $p < 0.05$ and **** $p < 0.0001$.

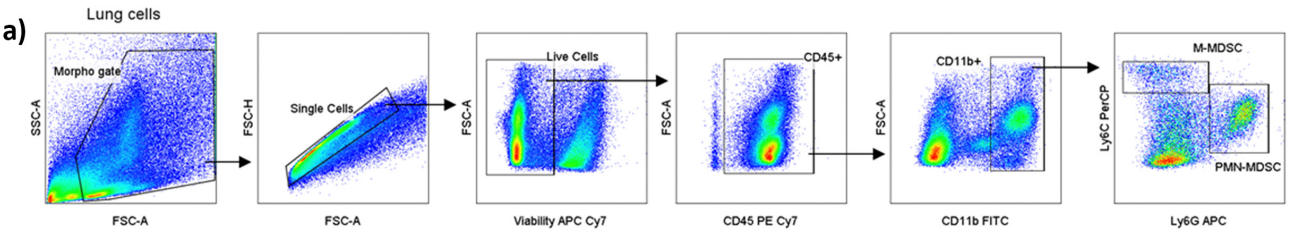

Figure 5. *Cont.*

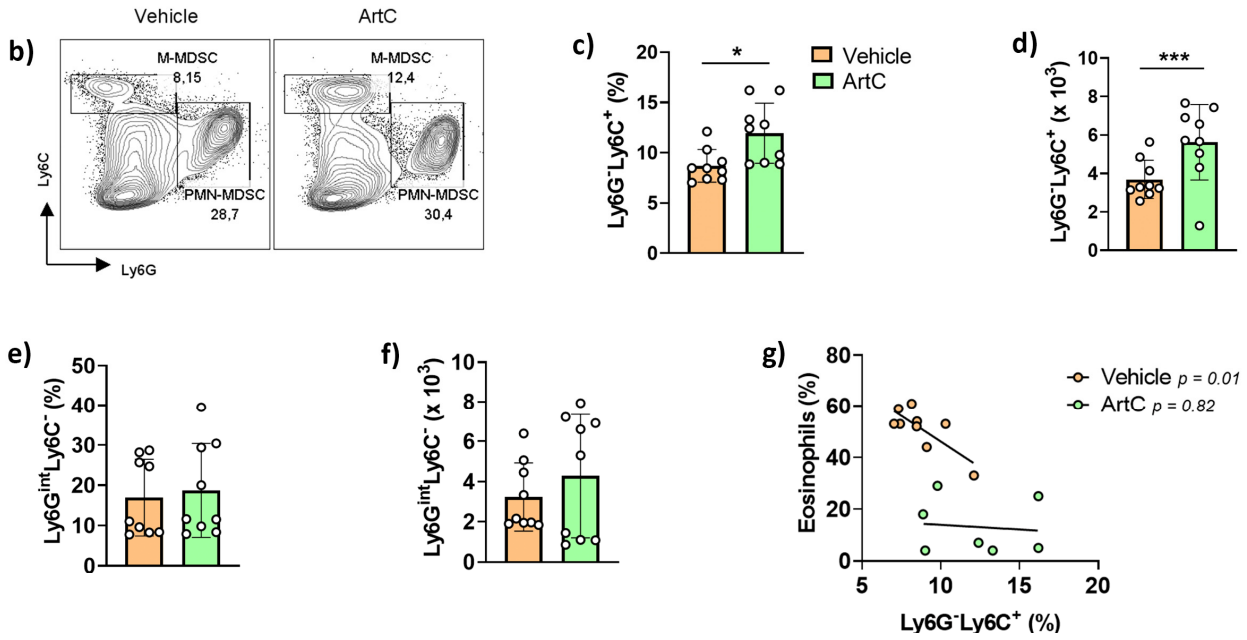

Figure 5. ArtC induces M-MDSC in the lungs of mice exposed to the allergen. (**a**) Gate strategy for $Ly6G^{-}Ly6C^{+}$ (M-MDSC) and $Ly6G^{+}Ly6C^{int}$ (PMN-MDSC) characterization in vivo; (**b**) representative dot plot of $Ly6G^{-}Ly6C^{+}$ and $Ly6G^{+}Ly6C^{int}$ in the lungs of mice exposed to the allergen treated with or not (Vehicle) with ArtC; (**c**) frequency, and (**d**) absolute number of $Ly6G^{-}Ly6C^{+}$ in the lungs; (**e**) frequency, and (**f**) absolute number of $Ly6G^{+}Ly6C^{int}$ in the lungs; (**g**) correlation between BALF eosinophils and lung $Ly6G^{-}Ly6C^{+}$. Data are representative of two independent experiments (n = 4–5/group/experiment) and expressed by mean ± SD. * $p < 0.05$ and *** $p < 0.001$.

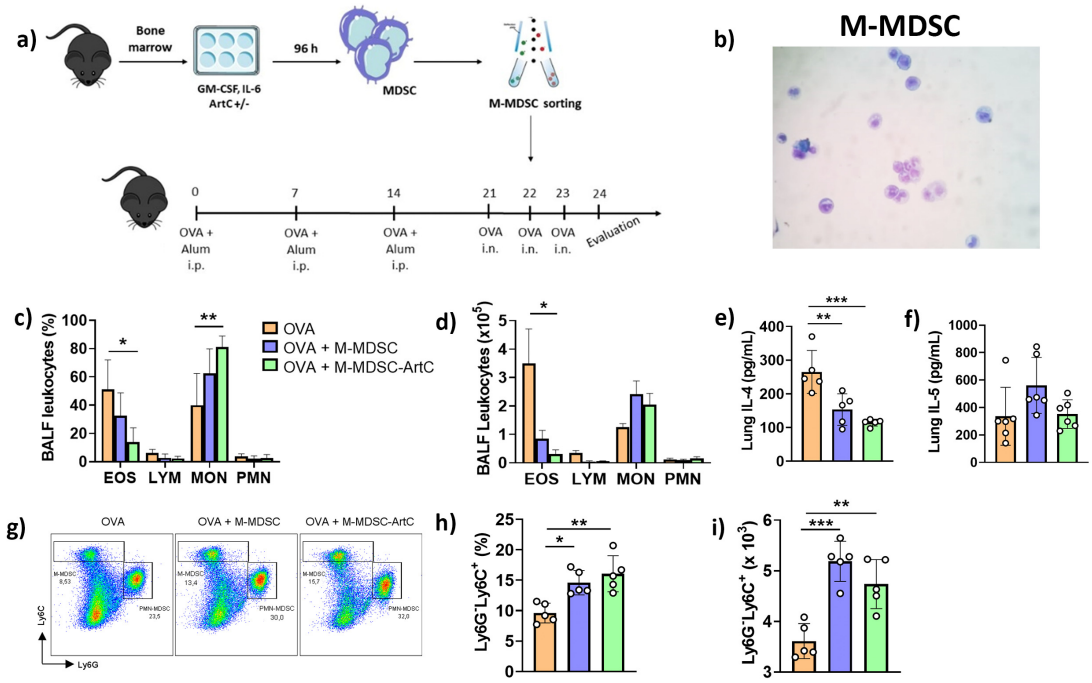

Figure 6. *Cont.*

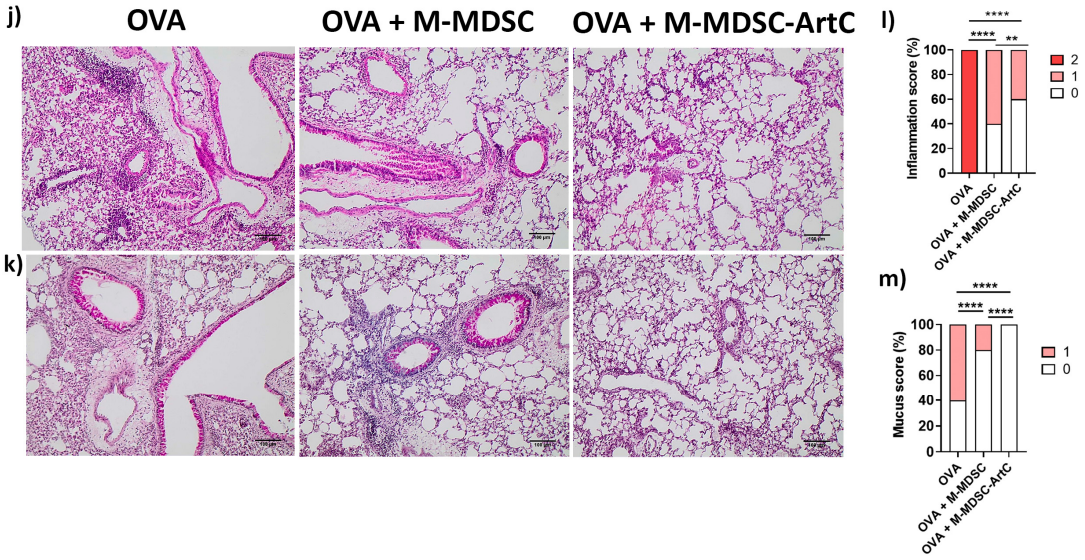

Figure 6. ArtC-induced M-MDSC reduces the allergic airway inflammation in mice. (**a**) Experimental design; (**b**) sorted M-MDSC, before cell transfer; (**c**) frequency and (**d**) number of eosinophils (EOS), lymphocytes (LYM), mononuclear cells (MON) and polymorphonuclear cells (PMN) in the BALF post cell transfer; (**e**) IL-4 and (**f**) IL-5 levels in the lungs; (**g**) representative dot plot of M- and PMN-MDSC in the lungs of post cell transfer; (**h**) percentage and (**i**) number of M-MDSC in the lungs; (**j**) representative images of lung inflammation (magnification 200×, scale bar 100 μM) and (**k**) mucus production (magnification 200×, scale bar 100 μM); (**l**) Lung inflammation score (0—without inflammation; 1—mild; 2—moderate to severe inflammation); (**m**) Mucus score (0—without mucus secretion; 1—mild). Data are representative of two independent experiments (n = 5–6/group/experiment), except for (**e**–**h**) (one representative experiment), and expressed by mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

4. Discussion

In this study, we show for the first time that ArtC, a major component of green propolis [15], induced M-MDSC in vitro differentiation and augmented the frequency of lung M-MDSC ($CD11b^{+}Ly6G^{-}Ly6C^{+}$) in an already established experimental model of asthma. The reduction in eosinophilic recruitment, pulmonary inflammation and mucus secretion, and the increase in M-MDSC frequency encourage the use of ArtC as a target molecule to be investigated as an adjuvant therapy for allergic asthma, which has not been previously pursued.

MDSC were primarily described in the context of tumors. Researchers observed myeloid hyperplasia in the tumor microenvironment, and later they identified that those cells displayed an immunosuppressive role [28]. Since then, the role of MDSC in tumors and other diseases has been explored. In the context of allergic asthma, MDSC can directly suppress the function of effector T (Teff) cells or induce Treg cells expansion in the lungs [29]. Cao and coworkers showed that PMN-MDSC reduced the allergic airway inflammation by inhibiting cytokine production and type 2 innate lymphoid cell (ILC2) function in a model of papain-induced lung inflammation [30]. Moreover, a previous study of our group showed that the treatment of animals exposed to allergen with green propolis induced accumulation of PMN-MDSC in lungs [10]. However, the role of M-MDSC in asthma and other allergic disorders was not known.

ArtC have been reported as an anti-inflammatory molecule [16,17,31]. Because our group previously showed that propolis treatment during experimental asthma induced Treg cells and MDSC, we considered it reasonable to investigate whether the mechanism of ArtC action would be the same as that of propolis. Although ArtC induced Treg cell differentiation in vitro, in vivo treatment with the compound failed to induce any increase in that population in the lungs of animals exposed to the allergen different from our group reported: an increase in Treg cells and PMN-MDSC in the lungs of mice exposed to the allergen and treated with propolis [10]. However, here we found an increase in M-MDSC

in the lungs of mice treated with ArtC. These findings show that propolis and ArtC might play anti-inflammatory action through different mechanisms.

MDSC produce IL-10, TGF-β, and NO [22,32] and expresses specific markers related to their suppressive functions such as PD-L1 (Programmed Cell Death Ligand 1), *iNOS* (inducible Nitric Oxide Synthase), and Arg1 (Arginase 1) [23,33]. In vitro studies showed that blocking of Arg1 and iNOS increased $CD8^+$ T and B cells proliferation [34,35], suggesting that these mediators were responsible for suppressor functions of MDSC.

In allergic asthma, Cloots and coworkers showed that mice genetically deficient for Arg1 displayed a reduction in *Il4*, *Il5*, *Il13*, *Ccl2*, and *Ccl11*, all Th2-related genes [36], suggesting that Arg1 may regulate type 2 inflammation. Additionally, Arg1 expression is involved in airway fibrosis and bronchial hyper-reactivity [37]. Treatment with ArtC in vitro induced the expression of Arg1 in M-MDSC (data not shown). However, animals exposed to the allergen and treated with ArtC exhibited a reduction in Arg1 expression in lung M-MDSC (data not shown). Besides the reduction in Arg1 expression, ArtC-treated mice also displayed increased expression of PD-L1, although not significant, on M-MDSC in lungs (data not shown). PD-L1 is a co-inhibitory molecule expressed by several cell types. PD-L1 binds PD-1 receptor expressed in activated T lymphocytes and induces cell death and immune suppression [38]. Thus, the PD-L1-PD-1 interaction is also a possible mechanism by which ArtC-induced M-MDSC could negatively modulate Th2 cells in allergic asthma.

Although M-MDSC transfer experiments confirmed the mechanism of action of ArtC inducing anti-inflammatory action in asthma, it remains to be investigated the exact mechanism by which M-MDSC reduces the eosinophilic influx and pulmonary inflammation in animals exposed to the allergen. These mechanisms might require Arg1, PD-L1, or other receptors or molecules that deserve further investigation.

Previous studies reported that after the adoptive transfer of MDSC to mice exposed to the allergen, those MDSC migrate to the lungs and suppress inflammation by a mechanism dependent on TGF-β production [39,40]. Moreover, PMN-MDSC transfer was shown to inhibit lung inflammation, Th2-cytokine production, and ILC2 function by a mechanism dependent on COX1 expression [30]. Similarly, COX1 can be critical to PMN-MDSC expansion and function since COX1 knockout mice and aspirin-treated mice did not develop decreased lung inflammation after adoptive transfer [41]. However, to our knowledge, this is the first study that shows the anti-inflammatory potential of ArtC, a purified natural compound from propolis, in the context of allergic airway inflammation. Thus, ArtC can be selected as a target for adjuvant therapy for allergic asthma, based on its capacity to reduce eosinophilic influx, pulmonary inflammation, and mucus secretion via enhancement of M-MDSC anti-inflammatory functions.

Author Contributions: Conceptualization: N.S.M., T.F.d.C.F.-S. and V.L.D.B.; formal analysis: N.S.M. and D.M.R.; funding acquisition: V.L.D.B. and J.K.B.; investigation: N.S.M., T.F.d.C.F.-S., G.F.C., M.M.M.B., L.N.Z.R., D.L.C.; methodology: N.S.M., T.F.d.C.F.-S., M.M.M.B., D.M.R., D.L.C., J.K.B. and V.L.D.B.; project administration: V.L.D.B.; resources: J.I.H., L.N.Z.R., J.K.B. and V.L.D.B.; supervision: T.F.d.C.F.-S. and V.L.D.B.; validation: N.S.M., T.F.d.C.F.-S. and V.L.D.B.; writing—original draft: N.S.M., T.F.d.C.F.-S. and V.L.D.B.; writing—review and editing: D.L.C. and V.L.D.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the National Council to Control Animal Experimentation (CONCEA, Brazil) and was approved by the local Committee on Ethics in the Use of Animals (protocol number 216/2019).

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Conflicts of Interest: The authors declare no conflict of interest.

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A U T O R I Z A Ç Ã O

A CEUA-FMRP autoriza a execução do projeto intitulado: ***"Papel da IL-22 e da S100A9 na comorbidade asma e pneumonia"***, registrado com o número do protocolo **245/2019**, sob a responsabilidade da **Profa. Dra. Vania Luiza Deperon Bonato Martins,** envolvendo a produção, manutenção ou utilização de animais pertencentes ao *filo Chordata, subfilo Vertebrata* (exceto humanos) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794 de 8 de outubro de 2008, do Decreto nº 6.899 de 15 de julho de 2009 e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA). O Protocolo foi **APROVADO** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo, em reunião de 27 de abril de 2020.

Colaboradores: Mèdéton Mahoussi Michaël Boko
Thais Fernanda de Campos Fraga da Silva
Ana Flávia Gembre Pignata

Lembramos da obrigatoriedade do Relatório Final, em modelo da CEUA, para emissão do Certificado, como disposto nas Resoluções Normativas do CONCEA.

Finalidade		() Ensino (X) Pesquisa Científica		
Vigência da autorização		27/04/2020 a 06/06/2024		
Espécie/Linhagem	Nº de Animais	Peso/Idade	Sexo	Origem
Camundongo / IL-22 KO	115	16g / 45 dias	Fêmea	Centro de Criação de Camundongos Especiais
Camundongo/ C57Bl 6	235	20g / 45 dias	Fêmea	Serviço de Biotério
Camundongo / MRP-14 (S100A9 KO)	115	16g / 45 dias	Fêmea	Centro de Criação de Camundongos Especiais*/

Ribeirão Preto, 27 de abril de 2020

Profa. Dra. Katiuchia Uzzun Sales
Coordenadora da CEUA–FMRP–USP