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Investigating the effect of sex hormones on sexual dimorphism in the lung tissue microenvironment

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1. Introduction

1.1 Sex differences in lung diseases

Lung diseases are one of the top three global causes of death, in low-, middle-, and high-income countries, according to the World's Health Organization (WHO). Sex differences in the prevalence, manifestation, outcome, morbidity and mortality of all lung diseases are widely acknowledged [1]. This can be easily observed in every day clinical practice, with women having a significantly higher incidence of respiratory symptoms, leading them to visit their General Practitioner [2]. Concerning the recent novel Corona Virus Disease (COVID-19) pandemic, even if the prevalence of COVID-19 is the same between men and women, men seem to have higher risk of worse outcome and mortality [3]. In some lung diseases, female sex is predominantly affected, while in others male is, a bias which is affected by many factors, including age. It is important here to be mentioned that sex refers to the biological effect of sex hormones, chromosomes, reproductive organs etc., while the term gender contains the behavioural aspect of every individual and the common characteristics of social life, that individuals with same gender follow [4]. Both of them have been suggested to have an effect on different diseases [4].

Sex differences are manifested in all obstructive lung diseases, e.g., with regard to asthma (Figure 1). Asthma is an inflammatory disease of the airways, which is characterized by exaggerated airway narrowing and results in expiratory airflow limitation, with main symptoms being wheezing and dyspnoea [5]. It is more prevalent in males until puberty, and then during adulthood there is a clear female predominance [6–8]. Furthermore, the severity of asthma has been suggested to change during both the different phases of the menstrual cycle [9] and pregnancy [10], indicating an effect of the different sex hormones' levels on the disease. In addition to asthma, the other typical example of respiratory obstructive diseases is Chronic Obstructive Pulmonary Disease (COPD). COPD is characterized by persistent respiratory symptoms and airflow limitation, due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases [11]. The prevalence of COPD is now almost equal in men and women [11]. However, there is a significantly higher burden of COPD in women with higher rate of annual moderate or severe exacerbations [12]. Women also develop COPD symptoms in a younger age [13]. In the last report of the Global Initiative for Chronic Obstructive Lung Disease (GOLD), it is clearly mentioned that further research is needed to shed light on the disease's sex bias [11]. Another obstructive lung disease is bronchiectasis, which is defined as an abnormal permanent dilation of the bronchi resulting from inflammation and destructive changes of the bronchial wall, caused by many different underlying diseases [14]. The prevalence of bronchiectasis, not due to Cystic Fibrosis (CF), in

Europe and USA is higher in women, across all ages [15,16]. This can be explained due to the fact that many diseases, which cause bronchiectasis, are more common in women. However, in India, men seem to have higher prevalence of bronchiectasis, as there men have also higher incidence of tuberculosis [15]. CF is an autosomal genetic disorder, caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which affect multiple organs, but mainly the lung [17]. As an autosomal inherent disease, CF prevalence is equal between boys and girls. However, the median survival of women is lower than men [18].

Apart from obstructive diseases, sex differences are also observed in almost all other lung diseases (Figure 1). Interstitial lung diseases (ILD) are a big complex group of disorders with the most common of them being Idiopathic Pulmonary Fibrosis (IPF). IPF is characterized by progressive interstitial fibrosis and IPF prevalence and mortality are higher in men [19]. Sarcoidosis is another well-studied systematic inflammatory autoimmune disease, which most commonly affects lungs by the development of non-caseating granulomas. Regarding this disease, the prevalence is slightly higher in women [20]. The higher incidence of autoimmune diseases seen in women leads also to higher prevalence of ILDs related to connective tissue disorders [21]. A special type of such a disease is Interstitial Pneumonia with Autoimmune Features (IPAF), which also affects more commonly women [22]. Pulmonary Hypertension is the result of many different disorders and the typical finding in this condition is the elevated blood pressure in the pulmonary artery. In this disease, even if prevalence is significantly greater in females, worse disease outcomes and mortality are greater in men, a phenomenon called ‘oestrogen paradox’ [23]. As far as lung cancer is concerned, it seems that women are more susceptible to lung cancer development after smoking, at every level of exposure to cigarette [24,25]. Data from the Center for Disease Control and Prevention of U.S.A (CDC) from 2019 show higher incidence and mortality of lung cancer in men, which is expected as still the prevalence of smoking, in total, is greater in men [26]. Of note, patients who are not smokers and are diagnosed with lung cancer, are more likely to be females [27]. Men smokers seem to manifest more frequently squamous cell carcinoma, while women manifest more often adenocarcinoma [28].

Sex-specific susceptibility to respiratory infections varies among pathogens (Figure 1). Adult women, especially in reproductive age, experience worse outcomes and higher mortality during influenza infections [29], while in infants and children the predominance differs between different countries[30,31]. We now know that men are more susceptible to COVID-19, as the hospitalization, the admission to the Intensive Care Unit, the morbidity and mortality are 1,5 – 2 fold higher in men, even though the incidence of the disease seems to be similar between the two sexes [3,32,33]. Community acquired Pneumonia, which is mainly caused by *Streptococcus pneumoniae*, affects also predominantly men, with worse outcomes of the

disease and higher mortality[34]. Sex differences in the lung diseases mentioned above are presented in Figure 1.

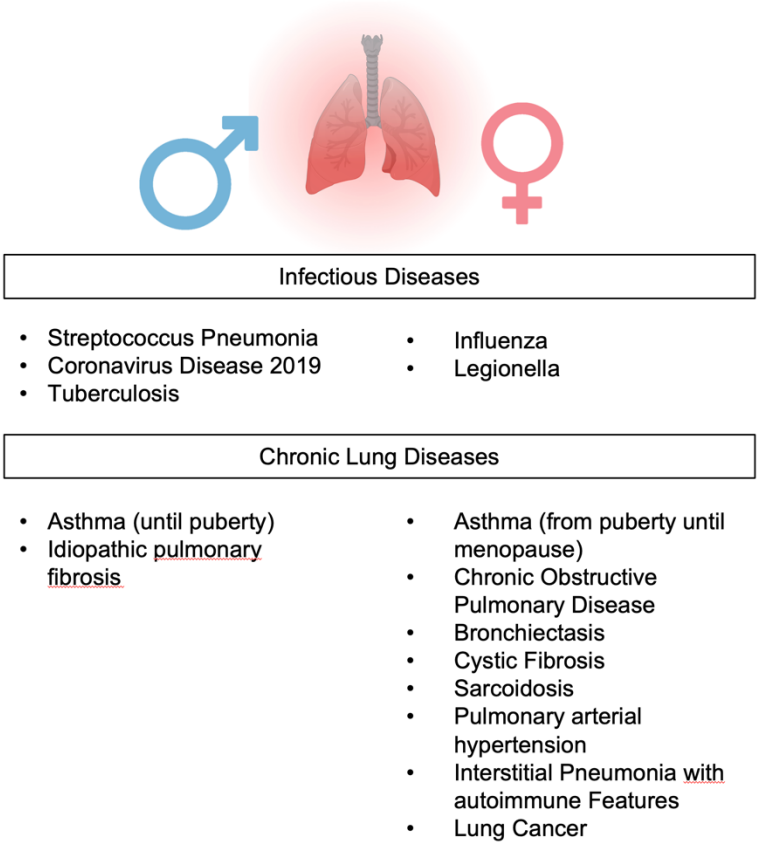

Figure 1 Sex differences in lung diseases.

1.2 Tissue resident immunity in lung health and disease

The lung is one of the largest interfaces of the human body to the external environment, and thus the existence of multiple mechanisms coping with external insults is essential. The lung tissue microenvironment plays a very important role in sustaining homeostasis and it consists of the respiratory epithelium and a network of non-circulating immune cells, which reside in the lung [35]. Specific populations of both adaptive and innate immune cells frame tissue resident immunity and they are able to respond quickly and effectively to any invading pathogens. [36]. These cells “break” the limit between innate and adaptive immunity, having many functions both during the acute phase of the inflammation and later by acting as immune surveillance cells. However, there is increasing evidence that suggests the involvement of disrupted tissue-resident immunity in the development of diseases. [37,38]. Below, I will briefly go through every important lung tissue resident immune cell population separately.

Macrophages

Alveolar (AM) and interstitial (IM) macrophages [37] are the two resident lung-specific macrophages population. AM are the main resident macrophage population in the lung and they are situated inside the alveoli [39]. This means that they are constantly exposed to external stimuli. On the other hand, IM are fewer in number and can be found within the lung tissue. AM have a close interaction with the respiratory epithelium and play a crucial role in eliminating invading pathogens and other particles by engulfing them through phagocytosis [37]. AMs' function has been studied in different lung diseases. The significance of AM becomes evident in viral respiratory infections, as depleting them during influenza or respiratory syncytial virus (RSV) infection results in higher levels of the virus in the lungs and a more severe disease outcome [40–42]. During a viral infection, alveolar macrophages primarily contribute by producing type I IFN, which promotes the recruitment of monocytes to the lungs and helps control viral replication [43]. However, during SARS-CoV-2 infection, AMs seem to contribute to the SARS-CoV-2-associated "cytokine storm" and to the progression to acute respiratory distress syndrome [44,45]. Additionally, during this infection, IFN is produced in lower levels than in other viral infections [46]. As far as bacterial infections are concerned, AMs recognise bacteria through Toll Like Receptors (TLRs) [47] and, through phagocytosis followed by their apoptosis, they eliminate the pathogen . Essential in this process is the function of the AMs' phagosome, which produces reactive oxygen and nitrogen species to damage the pathogen [48]. Also, after bacteria's recognition, AM secrete many pro-inflammatory cytokines [49]. Not only they effectively eliminate the invading pathogen, but also, they play a crucial role in resolving inflammation and promoting tissue repair. They achieve this by releasing anti-inflammatory cytokines like IL-10 and IL-1ra and by removing dead cells and their inflammatory remnants through a process called AM-mediated efferocytosis [50,51]. The facts that IM in lung are significantly increased after influenza infection and that they express MHCII during bacterial pneumonia, indicate a potential role of these cells in coping with the invading pathogens [52]. AMs have been found to play a significant role in asthma. AM depletion worsens allergic airway inflammation (AAI) in mice [53]. It seems that in asthma patients, the more impaired the phagocytotic and efferocytotic capacity of AMs, the more severe the inflammation and exacerbations of the disease become [54]. On the other hand, AMs produce mediators, like CCL17, CCL8, and CCL24, which enhance eosinophilia and type 2 immunity in both mice and patients with asthma, while they also secrete pro-inflammatory cytokines, such as TNF-a, IL-1β, IL-6, IL-8, and IL-17, which promote AAI [55]. Lung-resident macrophages interact also with the lung tumors. Tissue-resident macrophages were found to be linked to the growth of primary tumors in the lungs of mice [56]. In lung cancer, a special type of lung macrophages is detected, the Tumor-associated macrophages (TAMs), which

seem to be the tissue-resident IM, which promote tumor growth in an IL-9-dependent manner [57].

Dendritic Cells (DCs)

Pulmonary DCs are the primary cells responsible for presenting antigens. They initiate adaptive immunity by transporting antigens to the lymph nodes that drain the lungs [39]. The three main lung-resident DC populations seem to be the monocyte-derived DCs (moDCs), plasmacytoid DCs (pDCs), and conventional DCs (cDCs), which can be further divided in CD103+ cDCs (cDC1s) and CD11b+ cDCs (cDC2s) [37,39,58] :

- CD103+ cDCs, also known as cDC1s, transport and present antigens from the alveoli to CD8+ (and CD4+) T cells, which leads to the production of effector CD8+ T cells[59]. In the human lung, the myeloid type 2 DCs serve as the equivalent subset of CD103+ cDCs [CD11c+BDCA-3+(CD141)] [60,61]. Depletion of these cells in mice infected with influenza hampers their ability to clear the virus and worsens the outcome of the disease. This underscores the significance of these cells in maintaining the host's overall homeostasis [62].
- The human equivalent population of CD11b+ mouse DCs (cDC2s) are the myeloid type 1 DCs (CD11c+CD1c+) [60,61]. Interestingly, these are the only dendritic cells accumulating in SARS-CoV-2-infected human lungs [63], possibly due to the fact that they have the ability to boost the activity of CD4+ T cells and activate follicular helper T cells, both of which play a role in promoting effective immune responses against viruses [64].
- moDCs are attracted to the lungs during inflammation, but their presence in the lungs during normal conditions is not yet well understood.
- pDCs play a very important role in antiviral responses, as they are the main cells producing Interferon 1 [65]. Murine pDCs express specific markers, like the plasmacytoid dendritic cell antigen-1 (PDCA-1) [39,58]. Pulmonary human pDCs are characterized as CD11c−BDCA-2+ [60,61]. Bone marrow stromal antigen-2 (BST-2) is a marker expressed by both murine and human pDCs [66].

Despite the common functions of DCs described above, their role differs among different infectious diseases. For instance, in individuals with severe COVID-19, there have been reports of a decrease in the number of DCs, impaired ability to present antigens, and reduced production of IFN I [63,67,68]. Upon bacterial infections, even if DCs sustain their antigen presenting function[36], in mice infected with S. pneumoniae, a deficiency in DCs was associated with limited bacterial spread and lower systemic inflammation, suggesting that pneumococci exploit DC-mediated proteolysis to spread beyond the lungs [69]. Regarding M. tuberculosis, DCs control infection and granuloma formation through bacillus phagocytosis

and induction of cytokine production [70], while infected DCs migrate to lung-draining lymph nodes and trigger adaptive immune responses [71].

DCs take also part in lung inflammation related to asthma. The interaction between allergens and DCs is followed by differentiation of naïve T helper (Th) cells, which in turn trigger ILC2 and Th2 cell accumulation and these two cell populations produce finally type 2 cytokines [72]. Murine pDCs can be categorized into different subgroups depending on the presence of the surface markers CD8α or CD8β, as these markers have been found to either inhibit or promote the development of asthma [73]. DCs have the ability not just to initiate and maintain allergic Th2 immune responses, but also regulate and restrict them [74].

Innate lymphoid cells (ILCs)

ILCs are innate immune cells, which also reside in mucosal organs (tissue-resident cells) [75,76]. They have a common lymphoid origin and lack antigen-specific receptors [77,78]. ILCs guard tissue homeostasis by initiating and maintaining protective immune responses against invading pathogens [79,80]. On the other hand, dysregulated ILCs contribute to the pathogenesis of respiratory diseases [81,82].

ILC1s, ILC2s, ILC3, natural killer (NK) cells, and lymphoid tissue inducer (LTi) cells are the five members of the ILC family. The functions of the ILCs can be compared with the function of T cells, with ILC1s, ILC2s, and ILC3s being functionally analogous to CD4+ T helper (Th)1, Th2, and Th17 cells, respectively, while NK cells are cytotoxic cells functionally similar to CD8+ T cells [77]. A major difference between the human and murine lung regarding ILCs, is that ILC3 reside mainly in the respiratory tract of the former, while ILC2s are the main population in the lungs of the latter [75].

NK cells and ILC1s are referred to as ILC Group 1 and mostly take part in the antiviral and antitumor immunity[83–85]. IL-12, IL-15 or IL-18 trigger the secretion of interferon γ (IFN-γ) from these cells and IFN-γ, in turn, boosts intracellular pathogen elimination and antigen presentation by other immune cells [83,85]. NK cells have an additional cytotoxic function by secreting perforin and granzyme B [86]. NK cells' antiviral function is highlighted by the fact that NK cell depletion causes higher pulmonary virus titers in influenza-infected mice [87,88], while ILC1 and NK cell protective role has been also proven in experiments with RSV [89,90]. Regarding COVID-19, decreased NK, ILC1 and ILC2 numbers have been measured in the blood of COVID-19 patients [91], a finding indicating the migration of ILCs in the lung upon severe infection. Furthermore, NK cells seem to act against M. tuberculosis, since they bind on the pathogen and can produce IFN-γ, thereby having cytotoxic effects [92].

As stated above, ILC2s are the most prevalent ILC population in the murine lung. ILC2s are identified through flow cytometry in mouse lungs by the expression of several markers, such as IL7Ra, CD25, ST2, CD69, CD90, and CD44, while being lineage negative [80,93]. A

difference between murine and human ILC2s, is that human ILC2s express CD161 instead of CD44 [77]. The transcription factor GATA3 seems to be very important for both murine and human ILC2s, as it regulates the development, maintenance and activation of these cells [75,94]. Upon stimulation by the epithelial alarmins, ILC2s secrete type 2 cytokines, such as IL-5, IL-4 and IL-13 [95–97]. Thus, their contribution to asthma is crucial. Patients with asthma have increased circulating and lung-resident ILC2s [98,99], but mainly the ones with eosinophilic asthma, since neutrophilic asthma is associated with higher amounts of ILC1s and ILC3s [100]. ILC2s [101] promote airway eosinophilia through IL-5 production, and enhance airway hyperresponsiveness, goblet cell hyperplasia and Th2-mediated AAI via IL-13 secretion [81].

ILC3s and LTi cells are activated by retinoic acid-related orphan receptor-γt (RORγt)-mediated signalling and they secrete IL-17, IL-22, GM-CSF, and/or tumor necrosis factor-α (TNFα) thereby inducing Th17-like immune responses [96]. Since they produce IL-22, they maintain and support epithelial barrier integrity and function [96], as IL-22 is an important cytokine for epithelial barrier regeneration [102]. Moreover, ILC3s protect from the recurrence of bacterial pneumonia by secreting IL-22 [103]. ILC3s are widely known for their role in coping with bacterial infections [104]. Upon infection, ILC3s, as mentioned above, secrete IL-17 and IL-22 [104], thereby enhancing the airway epithelial barrier function and promoting immune responses against S. pneumoniae [105]. Also, through the secretion of the same cytokines, ILC3s respond early to M. tuberculosis infection.

Tissue resident memory T cells (TRMs)

TRMs are CD4+ and CD8+ memory T cells, which adhere to peripheral tissue and lack homing receptors [106,107]. Due to this fact, TRMs reside in peripheral tissues for long periods of time. Through DC-mediated activation, effector T cells differentiate to TRMs and subsequently migrate from lymphoid tissues into the lung [108,109]. They offer a rapid response to secondary infections [110] and they take part in antitumor local immunosurveillance [111]. Nevertheless, their dysregulation can also lead to pathogenic immune response [112]. The most well-known surface marker they express (both in mice and in humans) is CD69, which competitively inhibits sphingosine-1-phosphate (S1P) receptor expression thereby impeding S1P-mediated tissue cell escape [113,114]. Increased expression of CD44 and CD103, along with reduced CD62L and CCR7, also suggest a diminished capacity for cell migration and homing [115,116]. As expected, CD4+ and CD8+ TRM cells have common phenotypic characteristics. However, a major lung-specific difference is that CD4+ TRM cells typically display minimal or absent CD103 expression, in contrast with CD8+ TRMs [117,118].

CD4+ TRMs are more abundant than CD8+ TRMs in the lung [117,118]. They are located mainly in the bronchus-associated lymphoid tissue (iBALT), a space constantly exposed to

environmental pathogens [119]. On the other hand, CD8+ TRMs are primarily located within recently formed niches called repair-associated memory depots (RAMDs), which serve as the main hubs for tissue regeneration following injury [120,121].

As far as their function against infections is concerned, many studies have been conducted, regarding influenza, RSV and COVID-19, indicating the existence of viral specific TRMs, both CD4+ and CD8+[122–124]. These cells not only enhance virus clearance and protect from a potential re-infection [125], but also they offer protection against many different pathogens, as a cross-reactive immunity with other viruses with similar epitopes has been described [126]. During bacterial respiratory infections, CD8+ TRMs through IFN-γ secretion and CD4+ TRMs through IL-17 have the ability to attract neutrophils in the infected lung, contributing to pathogen elimination [127]. Notably, the use of antibiotics like clarithromycin has been linked to a decreased population of CD4+ TRM cells in the lungs, leading to a compromised host response during a subsequent S. pneumoniae re-infection. As long as asthma is concerned, a Th2-TRM population has been recognised, which release cytokines that enhance and maintain airway eosinophilia [128].

TRMs play a very important role in immune responses against cancer. Cytotoxic CD8+ T cells with high CD103 expression present increased proliferation and cytotoxicity against cancer cells of non-small cell lung cancer (NSCLC) [129]. The effectiveness of CD8+ T cell cytotoxicity is significantly influenced by CD4+ TRM cells, which can hinder tumor growth by producing IFN-γ or by eliminating tumor cells themselves. [130]. In metastatic lung cancer, TRMs can be found not only in the primary tumor and the contralateral mammary mucosa, but also in the pre-metastatic lung. CXCR6 appears to play a pivotal role in ensuring the retention of TRMs within the primary tumor. This increased TRM retention increases also in the lung the tumor-derived TRM cells and promotes protection against metastasis [131].

γδ-T cells

γδ-T cells are also lung resident cells that respond rapidly to threats, which can harm lung homeostasis by triggering immune responses [132]. In this way, they take part in protection against infectious factors, tumor surveillance and tissue repair [133]. These cells possess both typical adaptive characteristics and the capacity to mount strong innate-like responses. Mucosal and epithelial tissues, such as the skin and the lung, contain high amounts of γδ-T cells, accounting for 8-20% of resident lymphocytes in the lung [134]. Beyond the airway mucosa, lung γδ-T cells can be located in all non-alveolar regions. Notably, Vγ4+ and Vγ1+ γδ-T cells are primarily distributed in parenchymal lung regions [135]. Resident lung γδ-T cells primarily produce IL-17 [132], indicating their role in combatting pathogens and maintaining pulmonary stability [136]. They also produce IL-22, which protects mice from lung fibrosis [137]. Nevertheless, lung γδ-T cells may also play a role in aberrant immune responses and

consequently contribute to the development of diseases, as exemplified by allergic asthma [138].

Regarding the role of γδ-T cells against infections, a study with infant mice infected with influenza A virus, has shown, that IL-33 production by epithelial cells leads to the accumulation of γδ-T cells that produce IL-17A, which, in turn, initiates type 2 immunity and recruits ILC2 and regulatory T (Treg) cells. This recruitment enhances the release of amphiregulin and facilitates tissue repair [139]. In the case of human influenza virus infection, the primary immune response is driven by Vγ9Vδ2-T cells that produce IFN-γ [140]. Interestingly, activated human Vγ9Vδ2-T cells have the capability to eliminate influenza-infected human alveolar epithelial cells and hinder viral replication *in vitro* [141]. Additionally, they can mitigate disease severity in immunodeficient mice infected with human influenza virus strains in humanized models [142]. Furthermore, these cells have been suggested to enhance the efficiency of vaccination, as a study showed that, after RSV immunization followed by infection, higher levels of Vγ4+ γδ-T cells were observed in mouse lungs. These cells demonstrated the ability to produce various pro-inflammatory cytokines such as IFN-γ, TNF, IL-4, and IL-5 when stimulated *ex vivo* [143]. Data from patients with COVID-19, revealed decreased γδ-T cells in the blood [144]. In another study, a shift of γδ-T cells towards an effector-like phenotype with enhanced tissue infiltration capacity was detected in patients who had recovered from COVID-19 infection [145]. Both findings mentioned above indicate the important role of lung-resident γδ-T cells in coping with COVID-19 infection. In S. pneumoniae infection, except for the ability of γδ-T cells to produce IL-17, which was mentioned above, they can also decrease DCs and AMs in the lungs thereby facilitating resolution of inflammation caused by pneumococcal infection. [146].

Many contradictory studies can be found in the literature about the function of γδ-T cells in asthma. On the one hand, γδ-T cell-deficient mice with ovalbumin-induced asthma demonstrated reduced airway eosinophilia, peribronchial lymphocytic infiltration, airway hyperresponsiveness, serum IgE and IL-5 levels compared to wild-type mice [147,148]. On the other hand, IL-17-producing γδ-T cells have been suggested to enhance the resolution of eosinophilic and Th2-mediated airway inflammation [138,149].

1.3 The role of the respiratory epithelial barrier in lung diseases

The respiratory epithelium plays a leading role not only in maintaining the homeostasis of the lung but also in the pathogenesis of respiratory diseases. The fact that the respiratory epithelium is constantly exposed to all the pathogens contained in the inhaled air, justifies this crucial role and highlights the importance of its effective protective functions. The main cellular

components of the respiratory epithelium are the ciliated, the goblet, the club and the basal cells [150]. The composition of human extra- and intrapulmonary airways includes all the cells mentioned above, but in the bronchioles proximal to the alveoli the basal cells are absent. In the mouse lung, goblet cells are generally nearly absent and there are no basal cells in the intrapulmonary airways (mice do not have bronchioles) [150]. The epithelial cells are tightly connected with each other, forming the respiratory epithelial barrier. The "glue" binding these cells strongly together are three structures made of junctional proteins, the apical junctional complexes: adherens junctions (AJ), tight junctions (TJ), and desmosomes [151]. TJ are the most well studied structures in different diseases and they seem to affect significantly the permeability of the respiratory epithelial barrier and thus, we focus on them in the current thesis. TJs seal the membranes of the adjacent cells with three transmembrane proteins: claudins, occludins (OZLN), and junctional adhesion molecules (JAM). A peripheral membrane protein, Zonula Occludin (ZO), binds to these transmembrane proteins and stabilizes them in the cytoskeleton [151].

The main functions of the respiratory epithelium that protect the lung from environmental threats are:

1. the mucus production and the muco-ciliary clearance. On the top layer of the epithelial cells, the "gel like" mucus traps most of the inhaled particles and through the muco-ciliary escalator these are then brushed upwards [152]. Furthermore, the mucus contains also antimicrobial peptides, highly polymeric mucins and neutralizing antibodies which contribute to the elimination of the inhaled pathogens [153].
2. the epithelial-immune crosstalk. During pathogen invasion, the epithelial cells respond by releasing pro-inflammatory cytokines, IFNs, nitric oxide, antimicrobial peptides and extracellular vesicles, "notifying" host immunity about the incoming threat [154]. Interestingly, during viral infections, epithelial cells express various death-associated molecules (TRAIL, caspases etc.), leading to epithelial cell death at an early stage of the infection thereby eliminating both infected cells and replicating viruses.
3. the apical junctional complexes mentioned above [154]. These complexes not only stabilize the epithelial cells, making them able to act properly, but also inhibit bacterial migration from the airways to the bloodstream [155].

In the current thesis, we will focus on the integrity of the respiratory epithelial barrier, namely the integrity of the apical junctional complexes. The alteration of the apical junctional complexes in various lung diseases will be described below.

COPD is also known as the smokers' disease. The increased epithelial permeability and the impaired epithelial barrier function due to smoking have been studied for many years [156]. Decreased Transepithelial Electrical Resistance (TEER), an indication of higher barrier

permeability, and downregulation of OCLN, ZO-1, and ZO-2 have been found in cultured human epithelial bronchial cells exposed to cigarette smoke extract [157,158]. The respiratory epithelium of COPD patients is more vulnerable to barrier dysfunction, as downregulation of the expression of the junctional protein genes has been observed in these patients [159,160]. The increased epithelial barrier permeability in smokers, and especially in COPD patients, contributes to frequent respiratory infections and COPD exacerbations, which lead to the higher morbidity of these patients [161,162].

Data from asthma patients also indicate an impaired function of the respiratory epithelial barrier in this case, as both abnormal staining of the junctional proteins OCLN and ZO-1 in lung biopsies and disruption of these proteins in epithelial cell cultures have been observed [163]. Moreover, adherens junctional proteins α-catenin and E-cadherin are lower expressed in these patients [164]. It seems that inhaled pathogens, namely viruses, allergens and cigarette smoke, impair the function and structure of apical junctional complexes [165–167]. Many airborne allergens have a proteolytic activity, which affects the proteins of the apical junctional complexes. By direct cysteine and serine proteinase activity, they disrupt OCLN, claudin-1 [166], and E-cadherin molecules [168], degrading in this way the apical junctional proteins [169–171]. Of note, E-cadherin deficient mice manifest many asthmatic pathological features, namely loss of ciliated cells, progressive epithelial damage, decreased ZO-1 expression, zones of epithelial denudation, and goblet cell metaplasia. Taking the above into consideration, many researchers suggest that, since the absence of apical junctional proteins occurs in asthma and allergens and viruses can dislocate these proteins, this may be one of the main mechanisms underlying the pathogenesis of asthma [172].

Cystic Fibrosis is characterized by airway surface liquid dehydration and subsequently impaired muco-ciliary clearance due to mutations of the CFTR gene [173], but except for this primary dysfunction, defective apical and gap junctions have also been described. Specifically, cultured human airway epithelial cells mutated for the CFTR presented altered TEER and impaired trafficking of connexin 43 compared to healthy airway epithelial cells, due to impaired function of TJ and gap junctions [174]. Furthermore, another study with cultured human airway epithelial cells derived from CF patients showed impaired protective gap junctions closing upon TNF-α stimulation [175]. These data indicate a disrupted function of the airway epithelial barrier in patients with CF.

Fibrotic lesions, deposition of high amounts of extracellular matrix proteins (mainly collagen) and patchy alveolar epithelium injury are typical in the lungs of IPF patients. The epithelial barrier function is altered also in this disease. Alveolar permeability increases and the function of the AJ is impaired [176]. A functional test, measuring the clearance of aerosolized 99mTc-DTPA, in IPF patients also revealed the increased alveolar permeability [177]. This can be partially justified by the different expression of the TJ components by the alveolar epithelium

in IPF patients compared to healthy individuals, which leads to the dysfunction of the airway epithelial barrier [178].

The integrity of the respiratory epithelial barrier is also disrupted in respiratory infections. In Influenza A virus lung infection, reduced expression of occludins and of junctional adhesion molecules leads to the disruption of the epithelial barrier [165]. Interestingly, a protein of the virus, the non-structural protein 1 (NS1) can interact with TJ proteins, rearrange ZO-1 and destabilize the junctional integrity [179]. Under normal conditions, the airway epithelial barrier inhibits extracellular respiratory bacteria like *Streptococcus pneumoniae* to migrate, but the disruption of the epithelial barrier by the Influenza A virus, enables *S. pneumoniae* migation from the airways to the bloodstream [180,181]. *S. pneumoniae* also harms the airway epithelial barrier, through the pneumococcal cytotoxin, pneumolysin, which disrupts TJ and reduces cilia organization, affecting also the ciliary beating [182,183].

1.4 The role of sex hormones in lung diseases

The effect of sex hormones on some lung diseases seems obvious, as the sex bias of these diseases changes according to the normal shift of the sex hormone concentration in different age stages. For example, during childhood, asthma is more prevalent in boys, while in puberty and adulthood, females are more frequently affected [8].This observation indicates the protective role of testosterone (TES) in asthma. However, in many lung diseases, the effect of sex hormones is not so clear. Moreover, it is still elusive on which molecular pathways the sex hormones act thereby leading to a better or a worse disease outcome and consequently a sex bias, which we observe as clinicians.

Based on the shifting sex bias that is observed in asthma in different stages of life, TES is considered protective, while female sex hormones are related to negative outcomes [184]. Two well-studied mechanisms have been proposed to explain the positive effect of androgens on asthma, namely their effect on airway smooth muscles and on the asthmatic inflammatory response. Airway hyperresponsiveness is a common feature of asthma [185] that leads to airway narrowing and excessive airway obstruction. Many studies indicate that TES and its metabolites enhance airway smooth muscle relaxation by acting on many ion channels like the L-type voltage dependent Ca2+ channels (L-VDCCs) and thus, reduce airway hyperresponsiveness. Regarding inflammatory asthmatic response, strong evidence supports that male sex steroids attenuate ILC2-, Th2 cell- and IL-17A-mediated responses, namely the most important mediators in asthma pathogenesis. Furthermore, they inhibit leukotriene biosynthesis, which are also cytokines that enhance asthmatic inflammatory responses. Many studies have tried to shed light on the effect of female sex steroids on asthma. It seems that estrogens, overall, affect asthma negatively [186]. Different studies indicate a contradicting

role of estrogens in airway hyperresponsiveness in asthma, as some studies show that they enhance airway smooth muscle relaxation, while others show the opposite [187]. However, clearer to us is the role of estrogens in asthma-related airway inflammation with most studies pointing out increased allergic inflammation through estrogen receptor (ER) A signalling. Specifically, estrogens have been proposed to trigger higher IgE levels, higher amount of eosinophils, DCs and Th2 cells and greater production of IL-17A. Interestingly, fluctuating levels of estrogens and progesterone in the menstrual cycle lead to the perimenstrual asthma (PMA) [184], which is the cyclical worsening of asthma during the luteal phase and/or during the first days of menstruation [188].

Current evidence indicates that women are more susceptible to COPD than men. In accordance to this, androgens have been proposed to have an alleviating effect on COPD and estrogens to increase the disease burden. It is important to mention here, that the influence of sex hormones and especially of estrogens in COPD is poorly explored. A meta-analysis showed that the lower the TES levels are in men, the worse the disease outcome is and also that COPD male patients have lower levels of TES compared to healthy male individuals [189]. Another study demonstrated that testosterone replacement therapy led the COPD patients to less hospitalization rates and slowed down the progression of the disease [190]. An animal study showed lower eosinophil recruitment in androgen receptor knockout mice upon COPD induction, which was mediated by a M2 polarization of the alveolar macrophages [191]. Evidence about the effect of estrogens on COPD is provided by epidemiological studies, which indicate that COPD exacerbations are increased after female hormone replacement therapy [192].

A male bias has been identified in the case of pulmonary fibrosis (PF). Studies with mouse models indicate that androgens worsen lung fibrosis and injury [193,194]. Specifically, a study focusing on bleomycin-induced PF in mice showed that lung function is restored in male mice after castration, while fibrosis is aggravated after 5α-Dihydrotestosterone supplementation [193]. Regarding the effect of estrogens on PF, reported results seem to be discrepant. In different animal models of bleomycin-induced PF, ovariectomy was associated with less inflammation or fibrosis in one study [194], while it led to disease exacerbation in another [195]. In human samples, assessment of ER expression in IPF patients suggests that estrogens may contribute to disease pathogenesis [196].

Of note, the role of estrogens in lung cancer has been extensively studied and their function in favor of the cancer cells has led to the use of aromatase inhibitors as lung cancer therapy [197]. Specifically, many studies provide evidence that estrogens contribute to the proliferation and the progression of lung cancer, as human lung cancer cells express the ERβ [198] and high expression of this receptor in lung cancer cells is related to worse disease outcomes [199]. The effect of androgens on lung cancer cells is not as well understood as the one of estrogens.

Epidemiological studies report an association between high TES serum levels and lung cancer progression[200], while the use of 5α reductase inhibitors is related to better survival outcomes [201]. Enhanced lung cancer cell proliferation after exposure to androgens has also been shown *in vitro* [202].

Sex hormones also play a role in lung infection manifestation and progression, as seen also in the case of SARS-CoV-2. Men >60 years old and/or with comorbidities like diabetes, obesity and COPD have the highest risk to suffer from severe COVID 19-related outcomes [203]. Interestingly all these risk exacerbating conditions are related to lower levels of TES [204]. Of note, male patients with SARS-CoV-2 pneumonia who were admitted in the Intensive Care Unit or died had lower levels of total and calculated free TES [205]. Due to the higher COVID-19 burden observed in patients with low TES levels, the use of testosterone has been proposed to inhibit the progression of SARS-CoV-2 pneumonia [206]. Estrogens seem to have also a similar effect with TES on coronavirus lung disease, as either after the use of an ER antagonist or after ovariectomy, female mice infected with SARS-Cov-2 presented higher morbidity and mortality [207]. Moreover, administration of estrogens has also been proposed as an approach against SARS-CoV-2 pneumonia, especially for women in menopause [208].

1.5 Hypothesis and objectives of the current thesis

The majority of lung diseases are manifested with a clear sex bias and sex hormones seem to be one of the most important factors underlying these sex differences, since they can affect lung disease manifestation and outcome. However, the molecular targets of the sex hormones in this context remain elusive. The two main compartments of the lung tissue microenvironment, namely the lung resident immune cells and the airway epithelial barrier, play a crucial role in sustaining homeostasis. Of note, dysfunction of these compartments has been implicated in the pathogenesis of all common lung diseases. Our hypothesis is that sex differences in lung resident immunity and the airway epithelial barrier, likely mediated by sex hormones, underline the sexual dimorphism of respiratory diseases.

In order to address this hypothesis, the aim of the present dissertation was to:

1. detect sex differences in the frequency of innate and adaptive resident immune cells in the lungs of adult naïve male and female mice.
2. identify the effect of sex hormones on lung resident immune cells and on the integrity of the respiratory epithelial barrier, using hormone manipulation approaches in male and female mice.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

All standard chemicals, for buffer, solution or medium preparation, used for the experiments of the current thesis are listed in Table 1.

Table 1: Chemicals

Chemical Name	Company
Citrate buffer	DCS, Hamburg, Germany
Collagenase D	Roche Diagnostics GmbH, Mannheim, Germany
CompBeads (anti-rat/anti-hamster/anti-mouse Ig κ and negative control compensation particles)	BD Bioscience, Heidelberg, Germany
SPHERO™ AccuCount Particles (Counting Beads)	Spherotech TM, Lakeforest, Illinois, USA
DNase I	Sigma-Aldrich Chemie GmbH, Munich, Germany
Ethylenediaminetetraacetic acid solution (EDTA)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Bioethanol 99%	TH Geyer GnbH, Renningen, Germany
Fetal calf serum (FCS)	Gibco/Thermo Fisher Scientific, Waltham, MA, USA
Formaldehyde solution	Sigma-Aldrich Chemie GmbH, Munich, Germany
Isoflurane 100%	Sedana Medical AB, Danderyd, Schweden
Normal rat serum (NRS)	Jachson ImmunoResearch Laboratories, West Grove, PA, USA
Mowiol 4-88	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Paraffin	DCS, Hamburg, Germany
Paraformaldehyde (PFA)	Biochemica, Billingham, UK
Percoll	Cytiva, Upsala, Sweden
T-M 30 days Testosterone Implants	Belma technologies, Liege, Belgium

Triton-X-100	Merck Millipore, Darmstadt, Germany
Xylene	Greyer, Renningen, Germany
Xylol replacement medium [Xylolersatzmedium (XEM)] HS200	DiaTec Diagnostics GmbH, Dortmund, Germany

2.1.2 Media, buffers and solutions

Table 2: Media, buffers and solutions

Name	**Composition**	**Company**
Blocking solution (immunofluorescence)	0.1% Triton-X-100 in 1x PBS 10% FCS 3% BSA	
Citrate buffer (pH=6)	1L H2O 2.1g Sodium Citrate	
Dulbecco's Phosphate Buffered Saline (DPBS)		Gibco, Waltham, MA, USA
FACS buffer	Dulbecco's Phosphate-Buffered Saline (PBS) 0.5% EDTA 1% FCS 10% Counting beads solution	Gibco/ThermoFisher Scientific, Waltham, MA, USA
FC Block solution	2,5% TruStain Fcx 5% NRS 92,5% PBS	
RPMI Medium		Gibco, Waltham, MA, USA

2.1.3 Plastic and other materials

Consumables used for all the conducted experiments are listed in Table 3.

Table 3: Plastic and other materials

Name	Company
Cell strainer (40µm nylon)	Falcon Brand Products, Corning, NY, USA
Tissue-Tek III Blue biopsy cassettes	Sakura Finetek Europe
Embedding cassettes “Macro”	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Standard microscope slides	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
BD Venflon needle protected intravenous cannula 20G, 1x32mm	BD, Becton, Dickinson and Company, Sweden
BD Micro-fine+ Insulin syringes U100 0,3x8 mm	BD, Becton, Dickinson and Company, Sweden
Syringes (1ml, 5ml, 10 ml)	B. Braun Melsungen AG, Hessen, Germany
Polystyrene, round-bottom FACS tube (5 ml)	Falcon Brand Products, Corning, NY, USA
Non-absorbable surgical sutures 2-0 50cm	Resorba, Nürnberg, Germany
Syringe needle 27G x ½’’ 0,4x12mm	B. Braun Melsungen AG, Hessen, Germany
Syringe needle 26G x ½’’ 0.45 x 12mm	B. Braun Melsungen AG, Hessen, Germany
Syringe needle 21G x ½’’ 0.80 x 40mm	B. Braun Melsungen AG, Hessen, Germany
Safe-Lock Tubes 1.5 mL	Eppendorf, Hamburg , Germany
50 mL plastic tubes	Greiner Bio-One GmbH, Frickenhausen, Germany
10 mL plastic tubes	Greiner Bio-One GmbH, Frickenhausen, Germany

2.1.4 Antibodies

Antibodies used in flow cytometry and immunofluorescence are listed in Tables 4 and 5

Table 4: Antibodies used for flow cytometry

Conjugated Fluorochrome	Antigen	Dilution	Company
AF700	CD45	1:400	BioLegend, San Diego, CA, USA
AF488 (FITC)	Ly-6g	1:400	BioLegend, San Diego, CA, USA
APC	CD11c	1:200	BD Bioscience, Heidelberg, Germany
APC	TCDγδ	1:200	eBioscience, San Diego, CA, USA
APC-Cy7	CD11b	1:200	BD Bioscience, Heidelberg, Germany
APC-Cy7	CD44	1:400	BioLegend, San Diego, CA, USA
BUV 737	CD4	1:800	BD Bioscience, Heidelberg, Germany
BV421	CD19	1:400	BioLegend, San Diego, CA, USA
BV421	CD69	1:200	BioLegend, San Diego, CA, USA
BV510	CD3	1:200	BioLegend, San Diego, CA, USA
BV510	CD19	1:400	BioLegend, San Diego, CA, USA
BV510	MHC II	1:600	BioLegend, San Diego, CA, USA
eFluor 506 (BV510)	Fixable Viability Dye	1:1000	eBioscience, San Diego, CA, USA
BV650	MHCII	1:800	BioLegend, San Diego, CA, USA
BV650	CD8	1:800	BioLegend, San Diego, CA, USA
BV711	NK-1.1	1:300	BioLegend, San Diego, CA, USA
BV785	CD103	1:100	BioLegend, San Diego, CA, USA
FITC	CD3	1:200	BD Bioscience, Heidelberg, Germany
PE	Siglec-F	1:50	BD Bioscience, Heidelberg, Germany

PE-Texas Red	F4/80	1:200	Invitrogen Carlsbad, CA, USA
PE-Cy7	CD45	1:400	BioLegend, San Diego, CA, USA
PerCP-Cy5.5	Ly-6c	1:200	BioLegend, San Diego, CA, USA
PerCP-Cy5.5	CD62L	1:400	BioLegend, San Diego, CA, USA

Table 5: Antibodies for immunofluorescence

Antigen	Origin	Dilution	Company
Anti-ZO-1	Rabbit polyclonal	1:100	Invitrogen, ThermoFisher Scient
anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 568	Donkey polyclonal	1:1000	ThermoFisher Scientific, Waltham, MA, USA
Hoechst 33258		1:5000	Sigma-Aldrich Chemie GmbH, Munich, Germany

2.1.5 Equipment and instruments

Experiments were conducted using standard laboratory equipment. Special instruments used are listed in Table 6.

Table 6: Instruments

Name	Company
Flow cytometer LSR Fortessa	BD Bioscience, Heidelberg, Germany
Microtome SM2010R	Leica, Wetzlar, Germany
Rotina 380 centrifuge	Hettich, Tuttlingen, Germany
SP8 LIGHTNING confocal microscope	Leica, Wetzlar, Germany
ThermoMixer comfort	Eppendorf, Hamburg, Germany

2.1.6 Softwares

Software used for data acquisition and analysis is found in Table 7.

Table 7: Softwares

Name	Company
GraphPad Prism version 8	GraphPad Software, La Jolla, CA, USA
FlowJo version 9.9.5	TreeStar, Ashland, OR, USA
FACS DivaTM Software version 8.0.1	BD Bioscience, Heidelberg, Germany
ImageJ	National Institutes of Health, Bethesda, Maryland, USA
LasX	Leica, Microsystems

2.1.7 Mice

Adult male and female C57BL/6 mice were purchased from Charles River and kept in the animal facility of the University Medical Centre Hamburg-Eppendorf in a 12-hour light/dark circle with ad libitum access to food and water. All animal studies were designed in accordance with institutional guidelines and approved a priori by the respective German authorities (Behörde für Gesundheit and Verbraucherschutz Hamburg; approval number: N20/29).

2.2 Methods

2.2.1 Ovariectomy, Castration and sham operation

Ovariectomy

Adult female mice were anaesthetized with isoflurane inhalation in an isoflurane chamber. Isoflurane was administered in conjunction with pure oxygen (3–4%) for anesthesia induction in mice. After immobilization of the mice, they were placed in a prone position and connected to an isoflurane nose cone. Eye lubricant was frequently used in order to prevent corneal drying and damage. -Mouse hair was shaved off the flank area (between the last rib and above the pelvis). Skin was disinfected. An incision in the skin on the right side was made. The musculature was separated using curved tip scissors. The ovarian fat pad was pulled out of the incision. Using the tweezers hemostatic, the region below the ovary was tightly clamped. Using a sterile thread, two knots were made in order to delimitate the area, which was removed, and then the ovary was removed. The same procedure was repeated for the left side. The peritoneum was closed with absorbable sutures and the skin was closed with surgical wound clips. The animals were daily observed for any inflammatory signs at the surgical wound and were examined for any behavior suggesting pain or distress.

Castration

Adult male mice were anaesthetized with isoflurane inhalation in an isoflurane chamber. Isoflurane was administered in conjunction with pure oxygen (3–4%) for anesthesia induction in mice. After immobilization of the mice, they were placed in a prone position, and connected to an isoflurane nose cone. Eye lubricant was frequently used in order to prevent corneal drying and damage. Mouse hair was shaved off the flank area (between the last rib and above the pelvis). Skin was disinfected. A vertical incision through the skin in the midline of the lower abdomen, approximately 1.5 cm anterior to the penis was made. Then a small incision (<1 cm) through the peritoneum was made. The cut edge of the peritoneum was gripped and lifted up in order to expose the peritoneal cavity beneath. From inside the peritoneal cavity the right testicular fat pad was gripped. The fat pad was pulled through the opening in the peritoneum and skin and the testis came out as well. We used a cautery pen to cut through the fat pad that was holding the testis and also the testicular artery was cut with the cautery pen. Then the testis and fat pad were removed. The same procedure was repeated for the left testicle. The peritoneum was closed with absorbable sutures and the skin was closed with surgical wound clips. The animals were daily observed for any inflammatory signs at the surgical wound and were examined for any behavior suggesting pain or distress.

Sham operation

Adult male and female mice were anaesthetized with isoflurane inhalation in an isoflurane chamber. Isoflurane was administered in conjunction with pure oxygen (3–4%) for anesthesia induction in mice. After immobilization of the mice, they were placed in a prone position and were connected to an isoflurane nose cone. Eye lubricant was frequently used in order to prevent corneal drying and damage. Mouse hair was shaved off the flank area (between the last rib and above the pelvis). Skin was disinfected. A vertical incision through the skin in the midline of the lower abdomen was made. Then a small incision through the peritoneum was made. Then the peritoneum was closed with absorbable sutures and the skin was closed with surgical wound clips. The animals were daily observed for any inflammatory signs at the surgical wound and were examined for any behavior suggesting pain or distress.

2.2.2 Testosterone implants in female mice

Adult female C57BL/6 mice were anaesthetized with isoflurane inhalation in an isoflurane chamber. Isoflurane was administered in conjunction with pure oxygen (3–4%) for anesthesia induction in mice. After immobilization of the mice, they were placed in a prone position and connected to an isoflurane nose cone. Eye lubricant was frequently used in order to prevent corneal drying and damage. Mouse hair was shaved off the flank area. Skin was disinfected. Using a special injector, we injected and implanted the testosterone implants subcutaneously. The animals were daily observed for any inflammatory signs at the surgical wound and were examined for any behavior suggesting pain or distress. These implants were designed to release daily doses of testosterone in order to achieve physiological plasma concentration in mice (for male mice). The implants released from 51.9 to 154.5 µg/24hr testosterone resulting in testosterone plasma concentrations of 0.9-3.7 ng/ml.

2.2.3 *In vivo* CD45 cell staining

In order to distinguish $CD45^+$ cells circulating in the bloodstream from $CD45^+$ cells residing in the lung parenchyma, we injected intravenously 5 µg (diluted in 100 µl PBS) of an AF-700 conjugated anti-CD45 antibody into the right retro-orbital sinus of isoflurane-anaesthetized mice and euthanized them 3 minutes later by cervical dislocation [209]. For *in vitro* CD45 staining, a PE-Cy7 conjugated anti-CD45 antibody was used. Using flow cytometry, the lung resident CD45 cell population was later identified as PE-Cy7 positive and AF-700 negative.

2.2.4 Tissue collection

Adult mice were anaesthetized with isoflurane inhalation. After the procedure described above in order to distinguish the resident CD45+ cells, mice were sacrificed by cervical dislocation. A large incision was made up to the neck of the mouse and blood was collected from the abdominal vena cava. Then, perfusion of the lungs was performed via the right ventricle of the heart, to remove the blood from the lungs. For flow cytometry, lungs were carefully collected, cleaned from all the surrounding tissues and placed in complete RPMI. To isolate lung tissues for histology, the trachea was exposed. A small cut on the trachea was made and the lungs were inflated through the trachea with 1ml 4 % PFA dissolved in 1x PBS. The trachea was then ligated, and then the lungs were carefully removed and cleaned from the surrounding tissues. Isolated lungs were placed in embedding white cassettes and subsequently in tubes containing a large volume of 4% PFA and kept at 4 °C overnight.

2.2.5 Single cell isolation from mouse organs

To perform flow cytometry, single-cell suspensions of lungs was generated. The collected in complete RPMI lungs, were minced and digested using 10 µl collagenase D (working concentration: 2 mg/ml) and 2 µl DNase I (10 U/µl) dissolved in 1x PBS. Following incubation at 37 °C for 30 min, the digested lung tissues were passed through a 40-µm cell strainer and diluted in 40 ml 1% FCS dissolved in 1 x PBS. After centrifugation at 450g for 8 min at 4 °C, the cell pellet was resuspended in4ml 40% Percoll solution and was slowly added on the top of a 67% Percoll solution. After centrifugation at 400g for 30 min at 20 °C, 1 ml of the middle phase containing the immune cells was taken and resuspended in 30ml 1% FCS dissolved in 1 x PBS. After centrifugation at 450g for 8 min at 4 °C, the cell pellet was dissolved in 1ml 1% FCS dissolved in 1 x PBS.

2.2.6 Flow cytometry

Extracellular staining

To conduct flow cytometry for two different panels, single cells isolated with the method described in paragraph 2.2.5 were resuspended in 1mL PBS and divided in two equal parts. Then they were centrifuged at 450g for 8 min at 4 °C. To block unspecific binding, cells were incubated with rat anti-mouse CD16/CD32 Mouse Fragment Crystallizable Block (1:200) and normal rat serum (1:100) in 50 µl FACS buffer for 15 min at 4 °C. Subsequently, the cells were incubated with the respective antibodies for extracellular staining for 30 min at 4 °C in the dark. To identify dead cells, the fixable dead/life stain eFluor 506 (1:1000) was added to the antibody

mix. Next, the cells were washed with PBS (450 g, 8 min, 4 °C) to remove unbound antibodies and they were dissolved in 200 µl FACS buffer, containing 20.000 counting beads. Then the samples were directly used for flow cytometry. To determine the optimal concentration for each antibody, titration was performed prior to staining. Data were acquired using a BD LSR/Fortessa II flow cytometer and analyzed using FlowJo software.

Data analysis

The data acquired with the BD LSR/Fortessa II flow cytometer, were analysed for both panels. Regarding the first panel mainly focusing on the innate resident immunity (+ B cells), firstly cell populations were distinguished based on their size using the forward scatter (FCS) and their granularity with the side scatter (SSC). Next, after excluding doublets and dead cells with the help of the dead/life stain, living single cells were detected. Using the marker CD3 in the same fluorescence channel as the dead/life staining, we excluded all T cells from the analysis of this panel. Subsequently, CD45+ resident cells were distinguished from the CD45+ circulating cells as described in paragraph 2.2.3. Resident cell populations (CD45 in vitro+, CD45 in vivo-) were characterized as follows [58,210,211]:

- B cells: $CD45_{in\ vitro}^{+}CD45_{in\ vivo}^{-}CD3^{-}CD19^{+}$
- NK cells: $CD45_{in\ vitro}^{+}CD45_{in\ vivo}^{-}CD3^{-}CD19^{-}NK1.1^{+}$
- Neutrophils: $CD45_{in\ vitro}^{+}CD45_{in\ vivo}^{-}CD3^{-}CD19^{-}NK1.1^{-}Ly6G^{+}CD11b^{+}$
- Alveolar Macrophages: $CD45_{in\ vitro}^{+}CD45_{in\ vivo}^{-}CD3^{-}CD19^{-}NK1.1^{-}Ly6G^{-}SiglecF^{+}CD11c^{+}$
- Eosinophils: $CD45_{in\ vitro}^{+}CD45_{in\ vivo}^{-}CD3^{-}CD19^{-}NK1.1^{-}Ly6G^{-}SiglecF^{+}CD11c^{-}CD11b^{+}$
- Interstitial Macrophages: $CD45_{in\ vitro}^{+}CD45_{in\ vivo}^{-}CD3^{-}CD19^{-}NK1.1^{-}Ly6G^{-}SiglecF^{-}F4/80^{+}MHCII^{+}$
- Infiltrating monocytes: $CD45_{in\ vitro}^{+}CD45_{in\ vivo}^{-}CD3^{-}CD19^{-}NK1.1^{-}Ly6G^{-}SiglecF^{-}F4/80^{+}MHCII^{-}$
- CD103+ DCs: $CD45_{in\ vitro}^{+}CD45_{in\ vivo}^{-}CD3^{-}CD19^{-}NK1.1^{-}Ly6G^{-}SiglecF^{-}F4/80^{+}MHCII^{+}CD11b^{-}CD103^{+}$
- CD11b+ DCs: $CD45_{in\ vitro}^{+}CD45_{in\ vivo}^{-}CD3^{-}CD19^{-}NK1.1^{-}Ly6G^{-}SiglecF^{-}F4/80^{+}MHCII^{+}CD11b^{+}CD103^{-}$
- pDCs: $CD45_{in\ vitro}^{+}CD45_{in\ vivo}^{-}CD3^{-}CD19^{-}NK1.1^{-}Ly6G^{-}SiglecF^{-}F4/80^{+}MHCII^{+}CD11b^{+}CD103^{-}Ly6C^{+}$

The gating strategy for the populations mentioned above is presented in Figure 2.

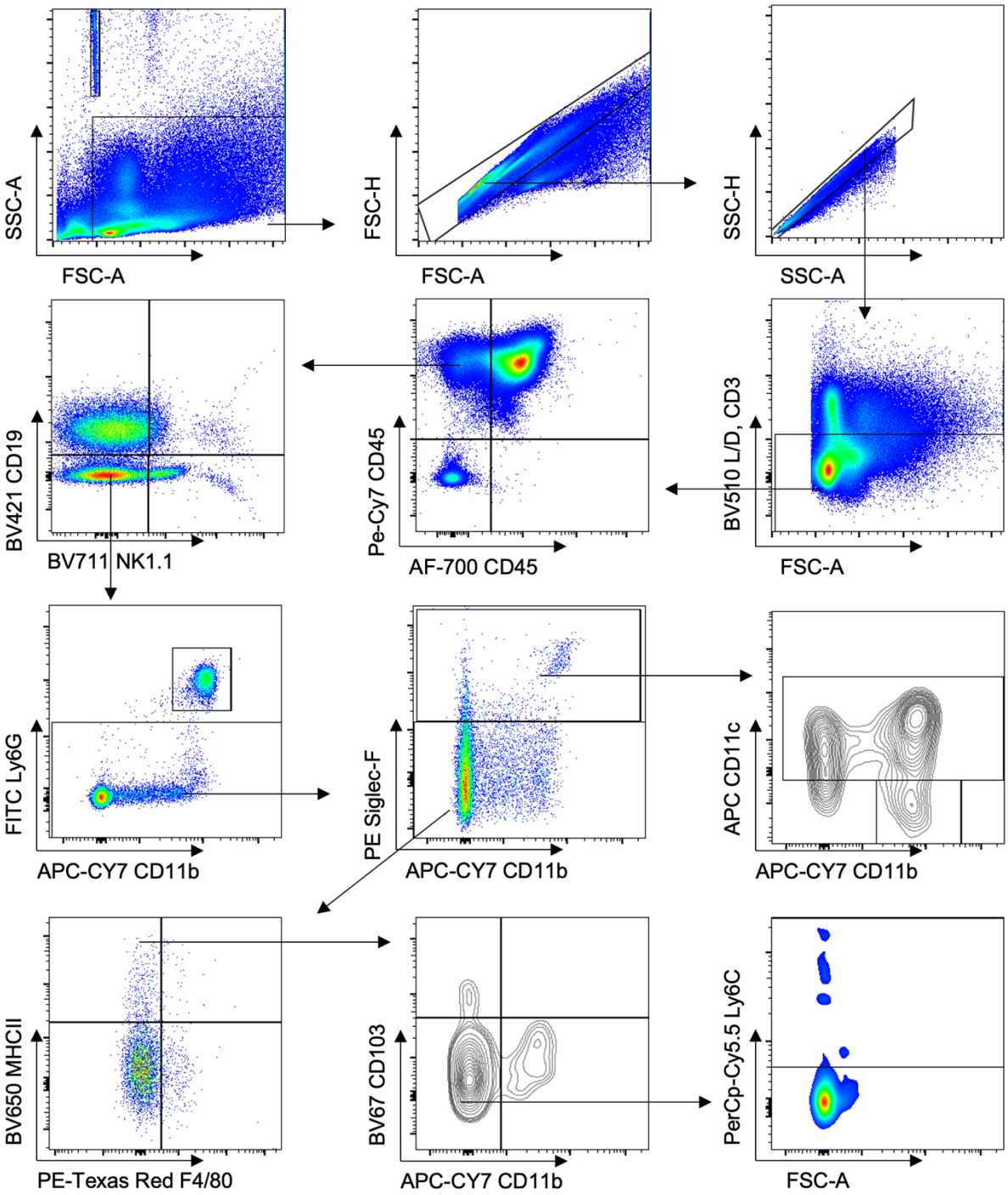

Figure 2. Gating strategy for the panel mainly focusing on the lung resident innate immunity

Regarding the panel targeting the adaptive resident immunity, cell populations were firstly distinguished based on their size using the forward scatter (FCS) and their granularity with the side scatter (SSC). Next, after excluding doublets and dead cells with the help of the dead/life stain, living single cells were detected. In the same fluorescence channel with the dead/life

staining, we used the markers CD19 and MHCII to exclude B cells and some innate cells from the analysis of this panel. Subsequently, CD45+ resident cells were distinguished from the CD45+ circulating cells as described in paragraph 2.2.3. Resident cell populations (CD45 in vitro+, CD45 in vivo-) were characterized as follows [212]:

- γδ T cells: $CD45_{in\ vitro}^{+}CD45_{in\ vivo}^{-}CD19^{-}MHCII^{-}CD62L^{-}CD3^{+}TCR\ \gamma\delta^{+}$
- CD4 TRM: $CD45_{in\ vitro}^{+}CD45_{in\ vivo}^{-}CD19^{-}MHCII^{-}CD62L^{-}CD3^{+}TCR\ \gamma\delta^{-}CD4^{+}CD8^{-}CD69^{+}CD44^{+}$
- CD8 TRM CD44+: $CD45_{in\ vitro}^{+}CD45_{in\ vivo}^{-}CD19^{-}MHCII^{-}CD62L^{-}CD3^{+}TCR\ \gamma\delta^{-}CD4^{-}CD8^{+}CD69^{+}CD44^{+}$
- CD8 TRM CD103+: $CD45_{in\ vitro}^{+}CD45_{in\ vivo}^{-}CD19^{-}MHCII^{-}CD62L^{-}CD3^{+}TCR\ \gamma\delta^{-}CD4^{-}CD8^{+}CD69^{+}CD103^{+}$

The gating strategy for the populations mentioned above is presented in Figure 3.

Compensation of spectral overlay

Before sample acquisition, compensation of spectral overlay of the fluorochromes used in each panel was performed as described previously [213]. Specifically, each antibody was coupled with anti-mouse/rat/hamster Ig Kappa (κ) beads based on the host in which the antibody was generated. The b eads were incubated with the respective antibody for 20 min at RT in the dark, then washed with FACS buffer and centrifuged (450 g, 5 min, 4 °C). Antibody volumes were determined empirically and varied from 0.1 to 1.25 µl per sample. For a single stained cell viability sample, 1x 10^6 spleen cells, half of which were killed at 70 °C for 5 min before the incubation with the antibody, were used. To detect auto-fluorescence, unstained beads and cells were used. The measurement of the samples was performed using the LSR/Fortessa II flow cytometer in the Compensation Setup mode of the FACS Diva software and the compensation values were automatically calculated. To further correct compensation post measurement, if needed, “fluorescence minus one” (FMO) samples were measured. FMO samples are cells stained with all fluorescent antibodies in the respective panel except for one antibody.

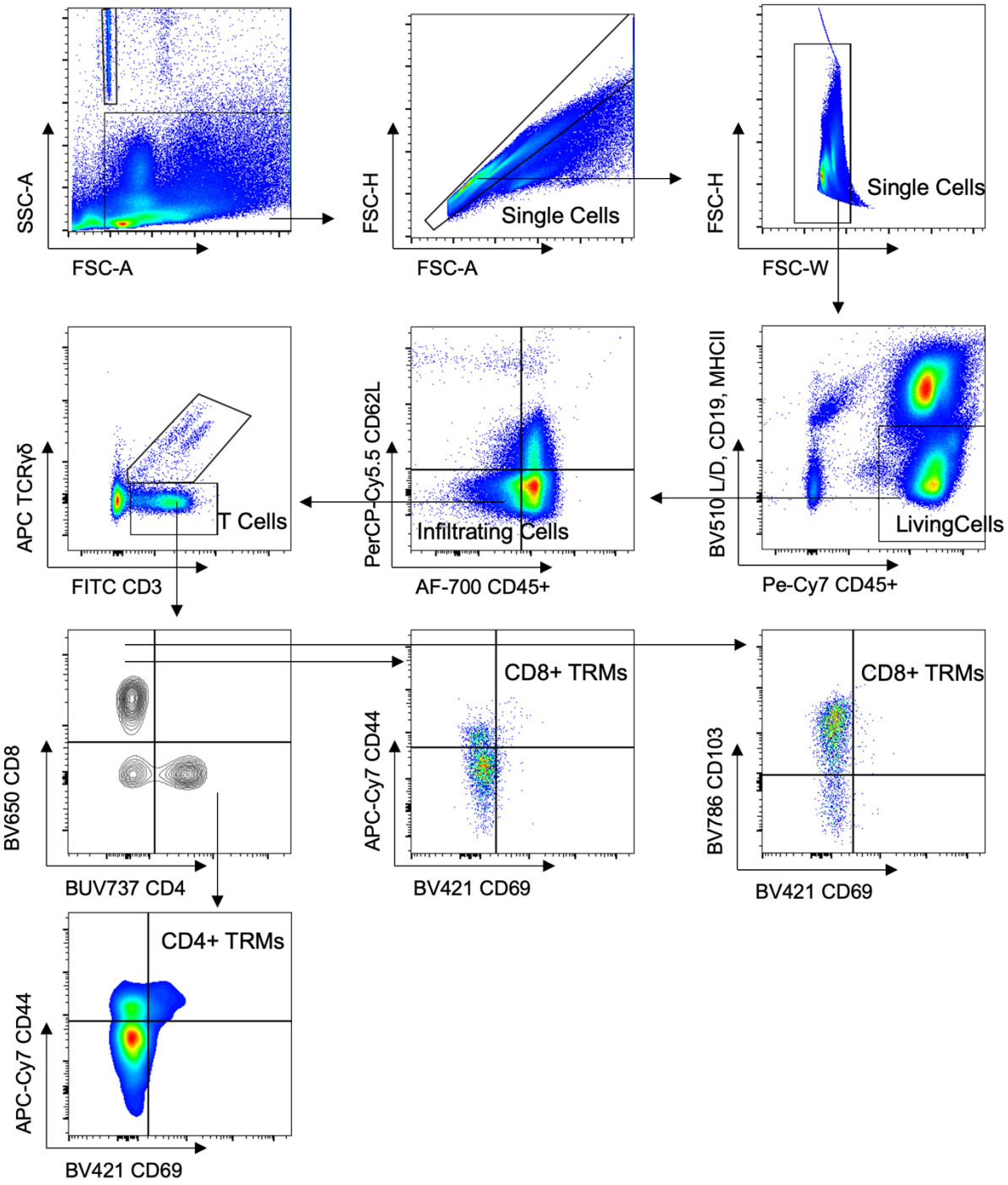

Figure 3. Gating strategy for the panel mainly focusing on lung resident adaptive immunity.

2.2.7 Histology

Lungs were collected, as described in 2.2.4, and were put in embedding white cassettes. They were transferred in 1x PBS, following overnight fixation in 4% PFA. We used our established protocol for histology of paraffin embedded tissues [213]. For histological sectioning, the lungs were subsequently embedded in paraffin. To this end, the tissues were first dehydrated by immersion in increasing concentrations of ethanol resulting in water and formalin removal. Next, the lungs were cleared from ethanol by xylene, which allows paraffin infiltration thereby resulting in embedding the tissues in a paraffin block. The paraffin-embedded tissue blocks were then cooled down to -12 °C for 30 minutes before they were sectioned at 4µm using the microtome SM2010R. The sections were mounted on glass slides and finally dried overnight at 37 °C.

Dehydration and infiltration were performed according to the following protocol:

- Ethanol 70 % for 1 h
- Ethanol 80 % for 1 h
- Ethanol 90 % for 1 h
- Ethanol 95 % for 1 h
- Ethanol 100 % for 1 h
- Ethanol 100 % for 1.5 h
- Xylene I for 1 h
- Xylene II for 1 h
- Paraffin type 3, 58 °C for 1 h
- Paraffin type 3, 58° C for 1 h
- Paraffin type 3, 58 °C for 1 h

Before staining, deparaffinization and rehydration of the slides were performed following the established protocol:

- Xylene, 3 x 5 min
- Ethanol 100 %,2 x 5 min
- Ethanol 96 % for 2 min
- Ethanol 90 % for 2 min
- Ethanol 80% for 2 min
- Ethanol 70% for 2 min
- Washing in ddH2O for 5 min

2.2.8 Immunofluorescence staining

For immunofluorescent staining, a rabbit polyclonal anti- ZO-1 (Zonula occludens-1) antibody was used. The primary antibody was then detected by a secondary donkey polyclonal anti-rabbit antibody containing the Alexa Fluor 568 dye. For immunofluorescent staining we used the established in our lab protocol [213] No primary antibody was applied on sections that served as isotype controls. Prior to staining, heat-induced retrieval of epitopes was performed. Specifically, the deparaffinized lung tissue sections were placed in a microwaveable vessel containing 0.1 M sodium citrate buffer (pH 6.0) and boiled in a microwave (900W for 2 min and then 250 W for 7 min). The citrate-based solution allows the retrieval of antigens and enhances staining intensity by breaking the protein cross-links that are generated due to formalin fixation and mask the antigenic sites in the tissue thereby. After antigen retrieval and cooling down of the slides, immunofluorescence was performed following the established protocol:

First day

- Washing with 1x PBS for 2 min
- Cold 4% PFA for 10 min
- Washing with 1x PBS, 3 x 2 min
- 0.3% Triton-X-100 dissolved in 1x PBS for 5 min
- Washing with 1x PBS, 3 x 2 min
- Blocking solution at RT for 1h
- Incubation with primary antibody diluted (1/100) in blocking solution at 4°C, overnight

Second day

- Washing with 1x PBS, 3 x 2 min
- Incubation with secondary antibody diluted (1/1000) in blocking solution at RT for 1h
- Washing with 1x PBS, 3 x 2 min
- Incubation with Hoechst 33258 diluted (1/5000) in 1x PBS at RT for 5 min
- Washing with 1x PBS, 3 x 2 min
- Mounting of slides with Mowiol

2.2.9 Histological analysis

The integrity of the tight junctions in the bronchial epithelium was assessed by immunofluorescent staining of lung sections with the anti-ZO-1 antibody as described above. The slides were observed and images were captured with the Sp8 Leica Confocal Microscope. ZO-1, also known as tight junction protein-1, is located on the cytoplasmic membrane surface of the intercellular tight junctions and mediates signal transduction via cell-cell junctions. Ten airways from two levels of the lung parenchyma were examined for each mouse. The

abundance of ZO-1+ cells in each airway indicated the tight junction integrity of the bronchial epithelial barrier and was scored as follows:

- 0: 100% ZO-1+ cells
- 1: 80-95% ZO-1+ cells
- 2: 50-80% ZO-1+ cells
- 3: 25-50% ZO-1+ cells
- 4: 5-25% ZO-1+ cells
- 5: <5% ZO-1+ cells

2.2.10 Statistics

Animals were allocated to different groups by alternation. The results shown represent mean ± SEM. Comparisons between two groups were performed using student's t-test or Mann-Whitney test, depending on the normality of distribution. P values were considered statistically significant when <0.05 (*: p≤0.05; **: p≤0.01; ***: p≤0.001; ****: p≤0.0001). All statistical analyses were done and plots were created using GraphPad Prism versions 8.0 and 9.0.

3. Results

3.1 Sex differences in lung resident immunity in naïve mice

To identify potential sex differences in lung resident immunity, we used age-matched adult male (n= 4) and female (n= 5) naïve C57BL/6 mice and performed an intravascular staining protocol, which allowed us to distinguish, through flow cytometry, the resident (infiltrating) from the circulating immune cells [209,214], as described above (Figure 4a, b). Interestingly, significant differences in both the innate and adaptive lung resident immunity were observed between male and female mice (Figure 4c-j). Regarding the innate resident cells, male mice presented higher frequencies of alveolar macrophages and infiltrating monocytes residing in their lungs compared to the female ones (Figure 4d, 4e). On the other hand, female lungs had more $CD103^{+}$ DCs and pDCs compared to the male ones (Figure 4f, 4g). As far as the lung resident adaptive resident immunity is concerned, female mice had significantly higher frequencies of CD4+ TRMs and slightly higher frequencies of CD8+TRMs, a trend that did not reach levels of significance (Figure 4i, 4j).

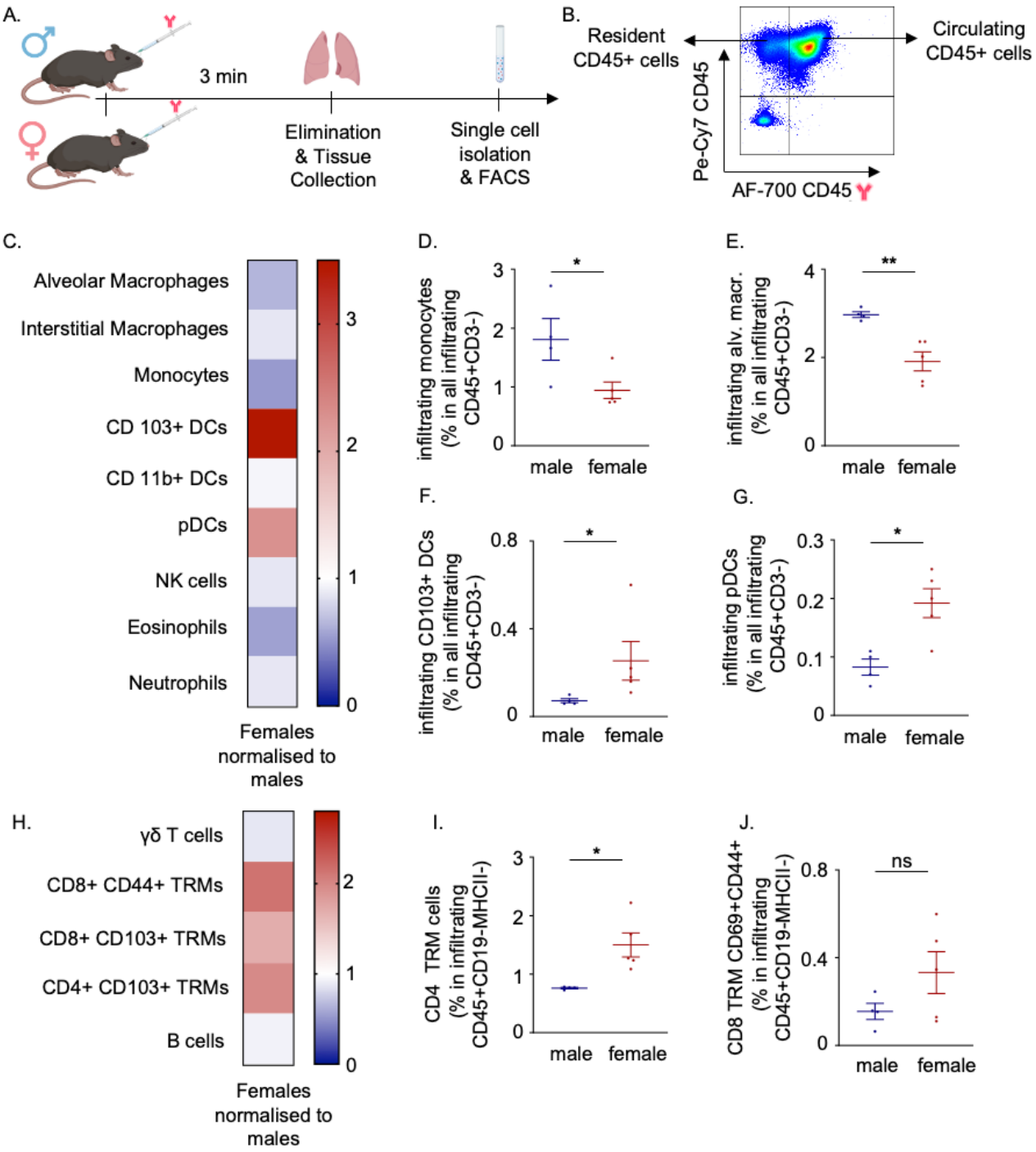

Figure 4. Sex differences in the innate and adaptive resident immunity of the murine naïve lungs (A) Graphic description of the experimental setup. Created with BioRender.com. **(B)** Gating for distinguishing the infiltrating (resident) CD45+ cells from the circulating ones. **(C)** Heatmap depicting the frequency of innate infiltrating immune cells in the female lung normalised to the frequency of innate infiltrating immune cells in the male lung. Resident $CD45^{+}CD3^{-}$ cells are the maternal population to which all frequencies are expressed. **(D-G)** Frequency (%) of infiltrating (d) monocytes, (e) alveolar macrophages, (f) $CD103^{+}$ DCs, and (g) pDCs, in all infiltrating $CD45^{+}CD3^{-}$ cells in male and female mouse lungs. **(H)** Heatmap depicting the frequency of adaptive infiltrating immune cells in the female lung normalised to

the frequency of adaptive infiltrating immune cells in the male lung. **(I-J)** Frequency (%) of infiltrating (i) $CD4^+$ TRMs, (j) $CD8^+CD69^+CD44^+$ TRMs, in all infiltrating $CD45^+CD19^-MHCII^-$ cells. Data are shown as mean ± SEM. *: p≤ 0.05, **: p≤ 0.01 as assessed by Mann-Whitney test. Non-significant differences (p > 0.05) are stated as ns.

3.2 The effect of sex hormones on lung resident immunity

Our next objective was to study the effect of sex hormones on lung resident immune cells. To this end, we conducted castration and ovariectomy in male and female mice respectively. Firstly, adult male C57BL/6 mice (n=5) underwent castration, while control male mice were sham operated (n=5), as described above. 14 days after the operation, we sacrificed the mice, following the protocol for the distinction of resident from the circulating $CD45^+$ immune cells, as described above (Figure 5a). Of note, castration resulted in a reduction of most of the innate lung resident immune cell populations (Figure 5b). Specifically, significantly decreased infiltrating monocytes and interstitial macrophages were found in the lungs of castrated mice. Interestingly, lower alveolar macrophages were also found after castration, a difference that did not reach levels of significance though (Figure 5c-e). Infiltrating NK cells were also decreased after castration (Figure 5f). Moreover, lower frequencies of $CD11b^+$ DCs, $CD103^+$ DCs and pDCs were also observed after castration (Figure 5g-i). Regarding adaptive immunity, castration resulted in a higher frequency of infiltrating B cells (Figure 5k). Additionally, castrated male mice presented higher frequencies of infiltrating CD8+ T cells and a non-statistically significant trend of higher CD8+ TRM, in their lungs (Figure 5j).

The next step was to perform ovariectomy to female C57BL/6 mice (n=5). Sham operated female mice served as controls (n=5). 14 days after the operation, the mice were sacrificed and the same protocol for distinguishing the resident immune cells was followed (Figure 6a). No statistically significant differences were observed after ovariectomy both in the innate and the adaptive lung resident immunity (Figure 6b-6h).

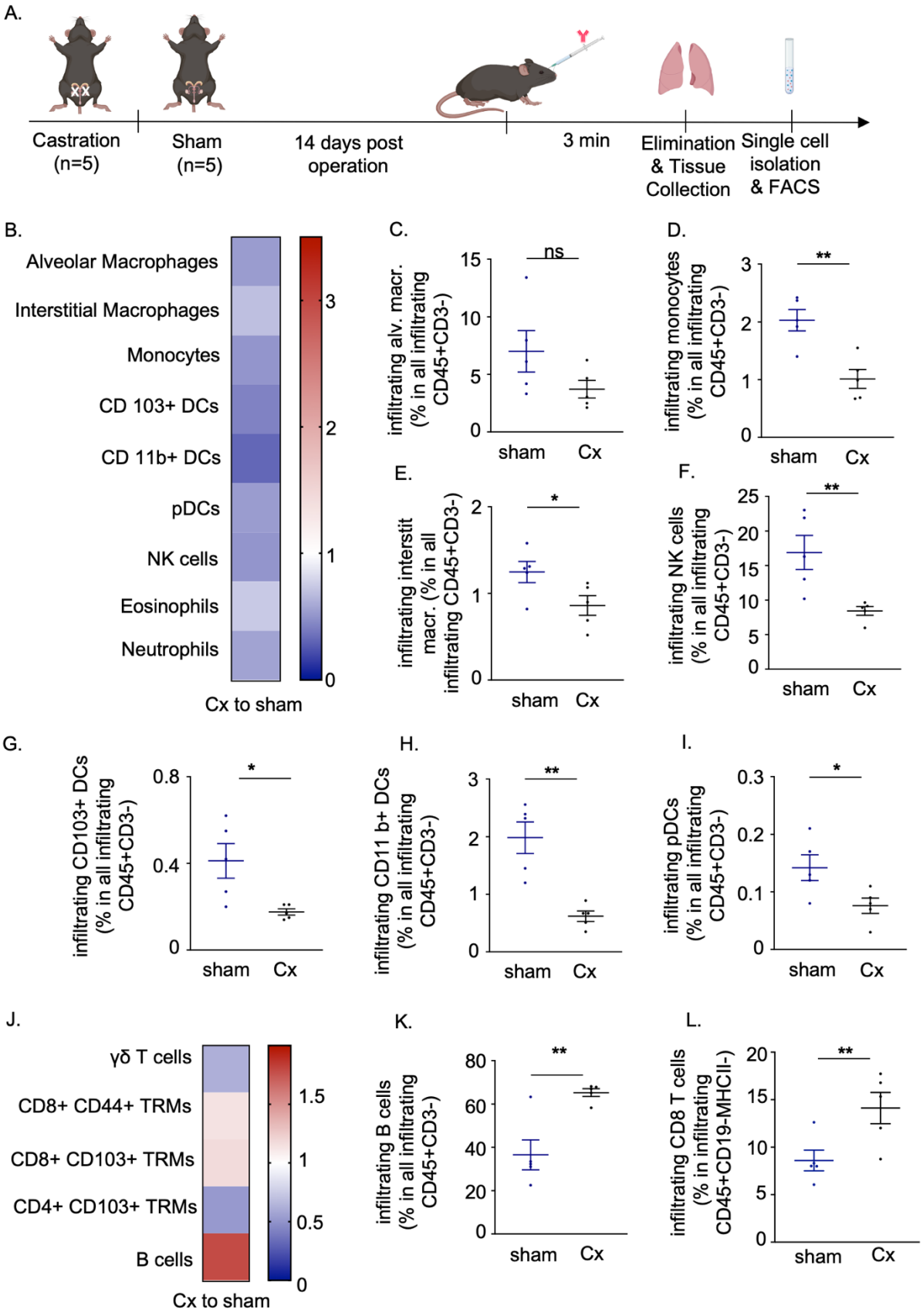

Figure 5. The influence of castration on lung resident immunity of male mice.

(A) Graphic description of the experimental setup. Created with BioRender.com. **(B)** Heatmap depicting the frequency of innate infiltrating immune cells in the lungs of castrated (Cx) male

mice normalised to the frequency of innate infiltrating immune cells in the lungs of sham operated male mice. Resident $CD45^+CD3^-$ cells are the maternal population to which all frequencies are expressed. **(C-I)** Frequency (%) of infiltrating (c) alveolar macrophages, (d) monocytes, (e) interstitial macrophages, (f) NK cells, (g) $CD103^+$ DCs, (h) $CD11b^+$ DCs, and (i) pDCs, in all infiltrating $CD45^+CD3^-$ cells. **(J)** Heatmap depicting the frequency of adaptive infiltrating immune cells in the lungs of castrated male mice normalised to the frequency of adaptive infiltrating immune cells in the lungs of sham operated male mice. **(K)** Frequency (%) of infiltrating B cells in all infiltrating $CD45^+CD3^-$ cells. **(L)** Frequency (%) of infiltrating $CD8^+$ cells in all infiltrating $CD45^+CD19^-MHCII^-$ cells. Data are shown as mean ± SEM. *: $p \leq 0.05$, **: $p \leq 0.01$ as assessed by Mann-Whitney test. Non-significant differences ($p > 0.05$) are stated as ns.

3.3 The effect of sex hormones on the airway epithelial barrier

Lung resident microenvironment consists not only of the lung resident immune cells, but also of the respiratory epithelium. Thus, we studied also the effect of sex hormones on the integrity of the airway epithelial barrier. Firstly, we investigated the influence of androgens using castration. 30 days after post operation, we sacrificed the mice, collected the lungs and after embedding them in paraffin and staining them with a fluorescent anti-ZO1 antibody, we assessed the integrity of the tight junctions in the airway epithelium (Figure 7a, b). No significant differences were observed in the tight junction disruption between castrated and sham operated male mice. (Figure 7c).

As a next step, female adult C57BL/6 mice were ovariectomised or sham operated and 30 days post operation, the same procedure was followed in order to study the effect of ovariectomy on tight junction integrity (Figure 7 d, e). Also here, no statistically significant difference in the integrity of the tight junctions could be observed between the groups.

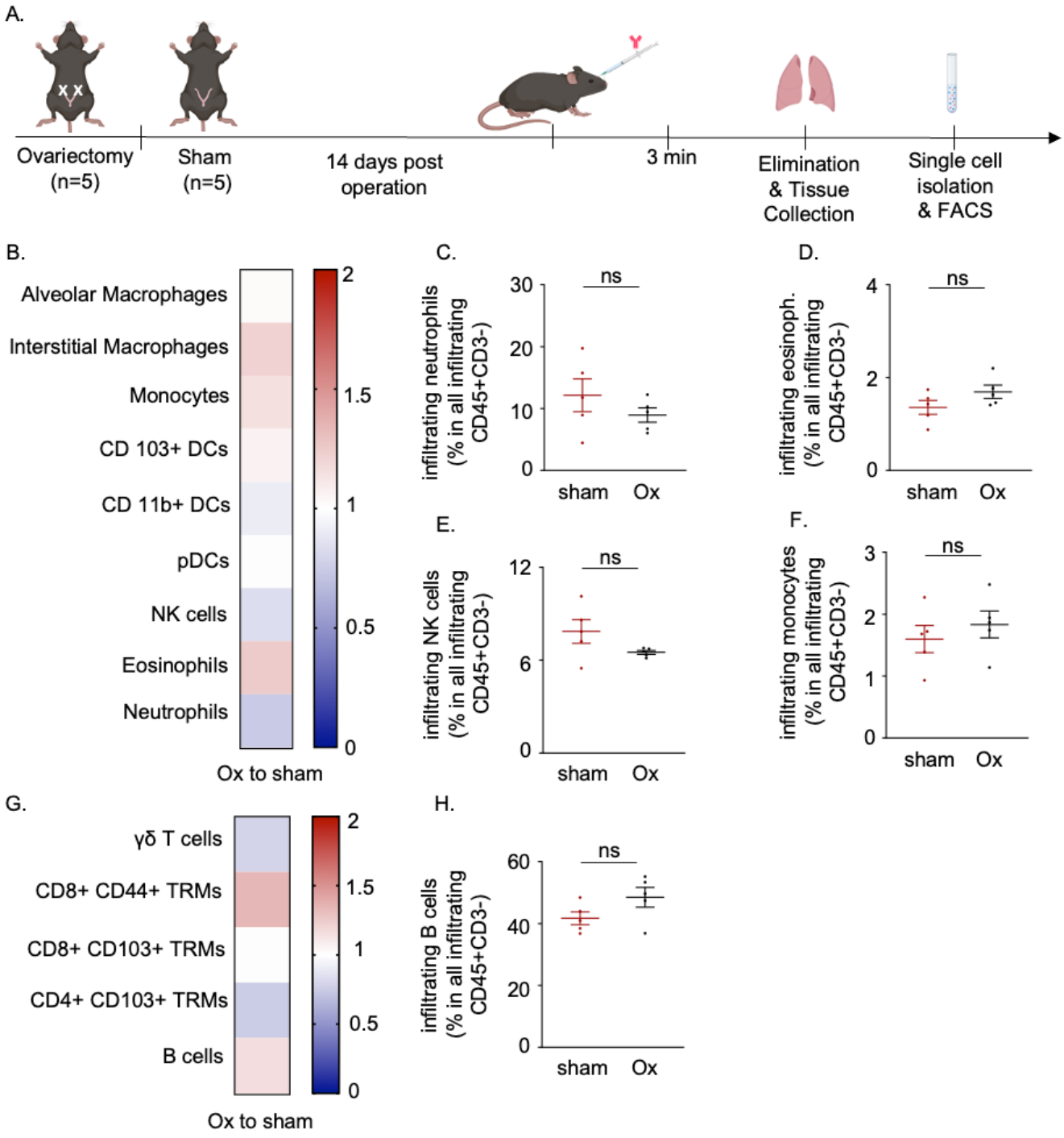

Figure 6. The influence of ovariectomy on lung resident immunity of female mice.

(A) Graphic description of the experimental setup. Created with BioRender.com. **(B)** Heatmap depicting the frequency of innate infiltrating immune cells in the lungs of female ovariectomised (Ox) mice normalised to the frequency of innate infiltrating immune cells in the lungs of female sham operated mice. Resident $CD45^{+}CD3^{-}$ cells are the maternal population to which all frequencies are expressed. **(C-F)** Frequency (%) of infiltrating (c) neutrophils, (d) eosinophils, (e) NK cells, (f) monocytes, in all infiltrating $CD45^{+}CD3^{-}$ cells. **(G)** Heatmap depicting the frequency of adaptive infiltrating immune cells in the lungs of female ovariectomised mice,

normalised to the frequency of adaptive infiltrating immune cells in the lungs of female sham operated mice. **(H)** Frequency (%) of infiltrating B cells in all infiltrating $CD45^{+}CD3^{-}$ cells. Data are shown as mean ± SEM. Non-significant differences ($p > 0.05$) are stated as ns, as assessed by Mann-Whitney test.

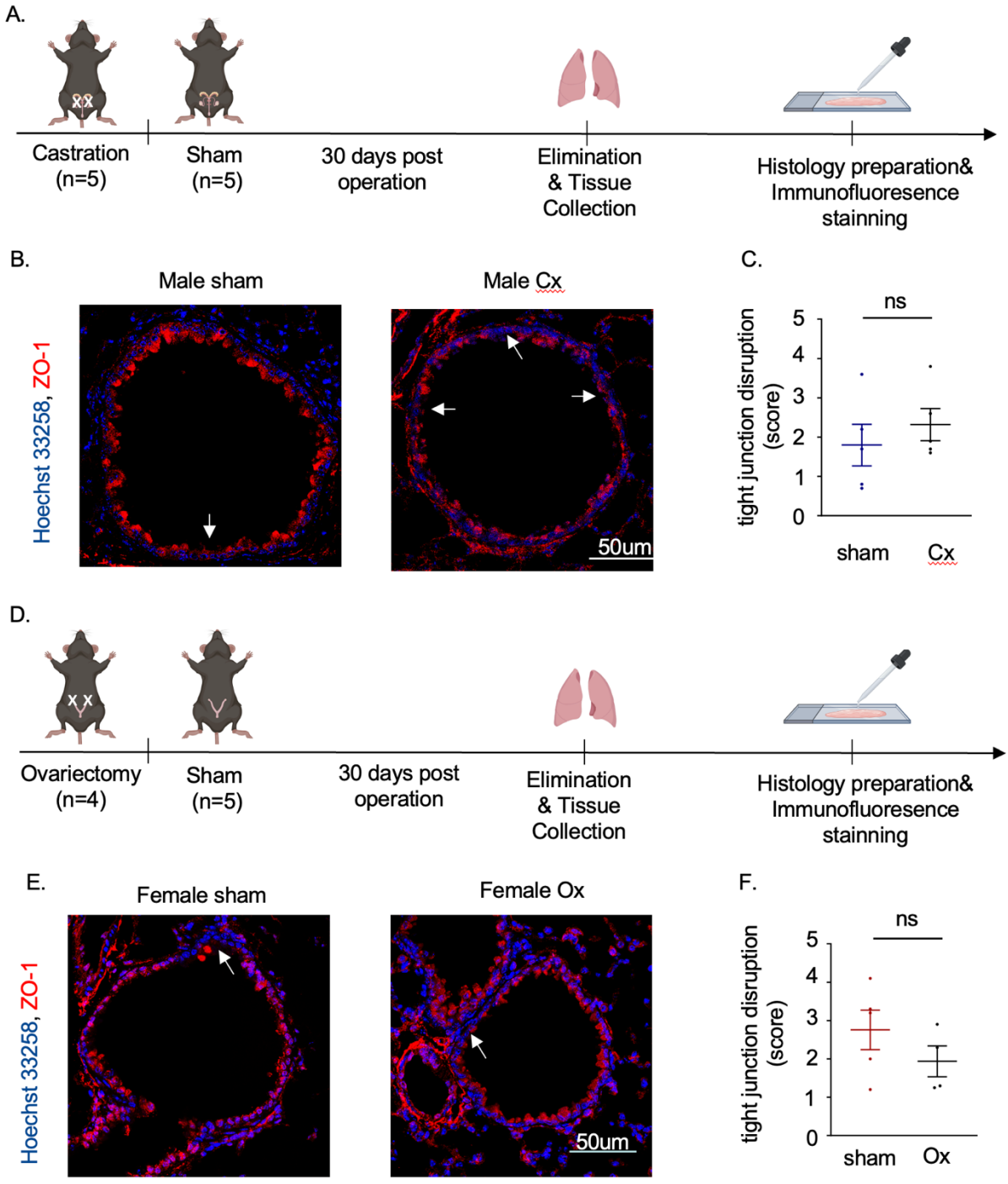

Figure 7 The influence of sex hormones on the airway epithelial barrier. (A) Graphic description of the experimental setup. Created with BioRender.com. **(B)** Representative images of lung sections from sham operated and castrated (Cx) male mice, after

immunofluorescence staining with anti-ZO-1 (red; tight junctions) and Hoechst 33258 (blue; nuclei). Arrows indicate lesions in tight junction barrier within the bronchial epithelium. **(C)** Semiquantative analysis of tight junction disruption in the lungs of sham operated and castrated (Cx) male mice. **(D)** Graphic description of the experimental setup. Created with BioRender.com **(E)** Representative images of lung sections from sham operated and ovariectomised (Ox) female mice, after immunofluorescence staining with anti-ZO-1 (red; tight junctions) and Hoechst 33258 (blue; nuclei). Arrows indicate lesions in tight junction barrier within the bronchial epithelium. **(F)** Semiquantative analysis of tight junction disruption in the lungs of sham and ovariectomised (Ox) female mice. Data are shown as mean ± SEM. Non-significant differences ($p > 0.05$) are stated as ns, as assessed by Mann-Whitney test.

3.4 Effect of testosterone administration on lung resident immunity of female mice

Since castration, and thus testosterone manipulation, strongly affected lung resident immunity, we administered testosterone to female mice to test the effect of such a supplementation on lung resident immunity. Testosterone implants were implanted to 5 adult C57BL/6 female mice, while 5 untreated age-matched female mice served as controls (Figure 8a). 14 days after the implantation, the mice were sacrificed, following the protocol for the distinction of the resident from the circulating $CD45^+$ immune cells, as described above (Figure 8a). Upon administration of testosterone, some lung resident innate immune cell populations were increased, while others were decreased (Figure 8b-8f). Specifically, the frequency of neutrophilssignificantly increased upon testosterone administration (Figure 8e). Regarding adaptive resident immunity, all examined cell populations were reduced after testosterone administration (Figure 8g). Of note, the only statistically significant reduction was found in infiltrating B cells (Figure 8h).

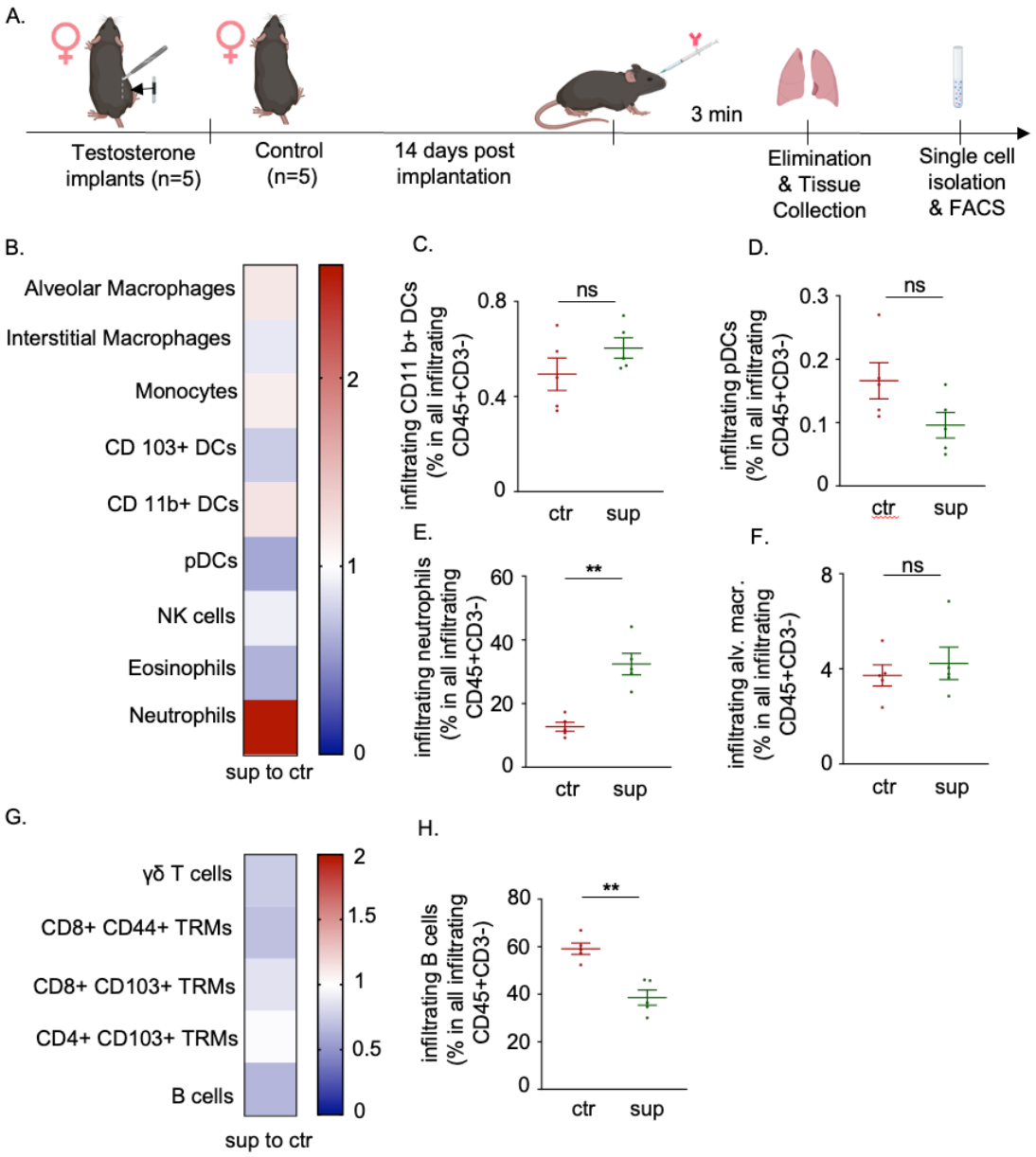

Figure 8. The effect of testosterone on lung resident immunity of female mice.

(A) Graphic description of the experimental setup. Created with BioRender.com. **(B)** Heatmap depicting the frequency of innate infiltrating immune cells in the lungs of female testosterone-treated mice normalised to control female mice. (**C-F)** Frequency (%) of infiltrating (c) $CD11b^{+}$ DCs (d) pDCs, (e) neutrophils, and (f) alveolar macrophages in all infiltrating $CD45^{+}CD3^{-}$ cells. **(G)** Heatmap depicting the frequency of adaptive infiltrating immune cells in the lung of female testosterone-treated mice normalised to control female mice. **(H)** Frequency (%) of infiltrating B cells in all infiltrating $CD45^{+}CD3^{-}$ cells. Data are shown as mean ± SEM. **: p≤ 0.01, as assessed by Mann-Whitney test. Non-significant differences (p > 0.05) are stated as ns.

4. Discussion

Sex differences in respiratory diseases are largely recognized [215], while previous evidence, mainly originating from epidemiological studies, identify sex hormones as the main contributors to the sex bias observed in most cases [216]. Here, in order to better understand the sexual dimorphism of respiratory diseases, we examined the sex differences in lung resident immunity and in the airway epithelial barrier in healthy adult mice and the influence of sex hormones on these two important components of the lung microenvironment. The fact that most lung resident immune cell populations express the ERα and/or Erβ, the progesterone receptor (PR) and the androgen receptor (AR), both in murine and in human lungs, supports our hypothesis [217]. The same receptors are expressed by both the bronchial and the alveolar epithelium [218,219]. Our results revealed higher resident macrophages in the male lung and a significant effect of testosterone on some of the here examined cells, while the higher frequencies of DCs and CD4+ TRM, found in female lung, could not be explained by an estrogen-mediated effect. Our first results indicated higher frequencies of infiltrating monocytes and alveolar macrophages in the mouse male lungs. On the other hand, mouse female lungs exhibited higher frequencies of $CD103^+$ DCs, pDCs and $CD4^+$ TRMs. In accordance to this finding, females are characterized by a greater DC activation and inflammatory response and exhibit a higher $CD4^+$ cell count and $CD4^+/CD8^+$ ratio [220–224]. Males are also known to have a higher count of NK cells [220], with our results showing only a non-significant trend of higher lung residing NK cells in male mice. A similar weak trend was found for the infiltrating neutrophils in the male lung, with other studies demonstrating higher circulating neutrophils in male mice [225].

Despite the fact that sex differences in innate immunity have been previously described [226], most of the studies conducted until now focused on circulating immune cell populations and did not examine potential sex-specific differences in lung resident immunity. To our knowledge, our study is the first one focusing on sex differences in lung resident immunity, which uses an established method for detecting tissue resident immune cells, namely the *in vivo* intravenous immune cell labeling [209]. A previous study focusing on sex differences in immune resident cells of pleural and peritoneal cavities [227] conducted a basic immunophenotyping of $F4/80^+$, $CD4^+$, $CD8^+$ T and B cells without using any of the proposed techniques for distinguishing the circulating from the resident immune cells [214], and found higher numbers of CD3+ T cells in the pleural cavity of female mice.

The analysis of the lungs from castrated and sham operated male mice yielded a striking result for most of the innate lung resident immune cell populations. Specifically, we observed a reduction in the frequencies of all resident macrophages, monocytes, DCs and NK cells. Regarding lung resident monocytes and macrophages, no effect of TES in the recruitment and numbers of these cells in the lung has been reported before. As mentioned above, there is no

available study in the literature investigating specifically the lung resident immunity, so our results can only be compared with data regarding mainly circulating cells. Studies working with blood samples have shown that androgens lead to decreased production of cytokines and other mediators by these cells, indicating a potential immunosuppressive effect. Specifically, after TES exposure, TLR-mediated responses of human monocyte-derived macrophages and murine peritoneal macrophages were decreased [228,229], while synthesis of TNF, iNOS and NO by these cells was also reduced [230]. Moreover, TES reduced the production of IL-1β, IL-6, and TNF-α by human macrophages and human monocytes and enhanced the expression of IL-10 in both human and murine macrophages [230,231]. It has been found that TES also reduces NK cell activity in murine spleen, which is the opposite effect of what we found in the lung. The influence of TES on DCs is not well studied, it is however known that 5α-DHT reduces the stimulation of T-cell cytokine secretion (IL-4, IL-10, and IL-13) by bone marrow-derived dendritic cells, *in vitro* [232]. In accordance to our findings, neutrophils are intensively diminished after genetic AR depletion in mice, while androgens stimulate the proliferation of neutrophils via the modulation of granulocyte colony-stimulating factor (G-CSF) [233].

The strong increasing effect of castration on lung infiltrating B cells, that we observed here, agrees with literature showing that castration increases the number of B cells in the spleen and in the bone marrow [234,235]. Cross-sectional studies have also demonstrated that men with androgen deficiencies have higher blood $CD4^+$ and $CD8^+$ T cell levels [236,237]. Castration in mice has also been shown to increase the numbers of these cells [238]. In line with these findings, we here observed a significantly higher frequency of infiltrating $CD8^+$ T cells in the lung after castration.

In spite of the fact that no statistically significant effects on lung resident immune cells were observed after ovariectomy in female mice, we observed some non-significant trends. We need to clarify here that the model of ovariectomy can be sometimes confusing in finding interpretation, as the higher amounts of both main female sex hormones, namely estrogens and progesterone, are produced in the ovaries in different phases of the menstrual cycle and these hormones can have opposite effects on the targeted cells [239]. Data derived from blood samples have shown that estrogens augment neutrophils in blood [240,241] and reduce eosinophils and monocytes [242][243]. We have to mention that most of the studies about different lung diseases show that estrogens lead to higher production of cytokines by macrophages, but progesterone has exactly the opposite effect [242]. The same happens with the effect of estrogens and progesterone on DCs as the former are considered to enhance DC activation and consequently cytokine production, and the latter to have again the opposite effect [244], with no clear information about the effect of female sex hormones on DC numbers or frequencies. Progesterone also has been shown to increase the number of NK cells [245]. As far as the adaptive immunity is concerned, estrogens have been suggested to inhibit B-cell

lymphopoiesis by diminishing the precursors, pro-B cell, pre-B cell, and mature B cells from the bone marrow [246]. Also, in this study the same effect is shown, as an increasing trend of B cells after ovariectomy is observed. Studies with rats have shown that ovariectomy leads to an increased percentage of CD4-CD8+ cells in the thymus [247,248]**.** We here observed no strong effect of ovariectomy on all studied lung resident innate cell populations, even though most of the non-significant trends were in line with what described above.

Taken together, we can conclude that sex hormones account for some of the here observed sex differences in lung resident immunity, but not for all. Higher frequencies of lung resident macrophages and monocytes in males in combination with their reduction after castration demonstrate that androgens might underline the sex differences seen in these lung residing cell populations. However, testosterone supplementation of female mice resulted in only minor augmentation of the lung resident macrophages and monocytes, indicating an effect of androgens on these cells that can probably be counteracted by other factors in the female lung. Very interesting was the finding of higher resident pDCs and $CD103^+$ DCs in the female lung together with the non-significant trend of lower pDCs and $CD103^+$ DCs in the lungs of testosterone-supplemented females. These results could be interpreted as a suppressive effect of androgens on these cells, even though castration led to lower frequencies of these two cell populations, highlighting again that additional factors to sex hormones may affect lung resident immunity. Regarding $CD11b^+$ DCs, no sex differences were observed, but a strong reduction and an increasing trend of these cells was observed after castration in males and after testosterone supplementation in females, respectively. Thus, the effect of androgens on $CD11b^+$ DCs is similar to the one on monocytes, which is rational due to their common myeloid origin.

Increased TRM cells were found in female mouse lungs. $CD4^+$ TRMs were not significantly affected upon sex hormone manipulation, but the slightly increased CD8+ TRMs in the lungs of castrated males and their decrease in the lungs of testosterone-treated females indicate a likely suppressive androgen effect on CD8+ TRM cells. Of note, androgens were found to profoundly affect infiltrating neutrophils and B cells residing in the mouse lungs. After castration, neutrophils were decreased and B cells were significantly increased, while the opposite effect was observed after testosterone supplementation in female mice. Consequently, androgens seem to regulate negatively the lung resident B cells and positively the neutrophils residing in the lung, in accordance to other studies focusing on the circulating counterparts of these immune cell populations.

The difference between sex and gender was explained above, and we need here to highlight that gender aspects were out of the aims of the current study. We focused on the effect of sex hormones on the establishment of sex differences in the lung tissue microenvironment, but besides hormones, genetic and environmental factors are able to modulate also the tissue

microenvironment in a sex-specific way [242]. By using age-matched mice of the same strain, which are kept under standardized common conditions, the effect of environmental and age-related factors can be minimized. Nevertheless, the difference in sex chromosomes between male (XY) and female organisms (XX), might also –at least partially- account for the observed sexual dimorphism in lung resident immunity. In humans, the X chromosome contains more than 1,100 genes (approximately 5% of the human genome), including some genes essentially involved in the function of the immune system, coding proteins like IL-receptors and TLR [249]. On the other hand, the Y chromosome contains only around 100 genes, including some genes that regulate immune responses. Both in males and females only one X chromosome is active, since in females a X chromosome inactivation takes place in order to balance the expression of X-linked genes between the sexes. However, 3% of the genes in the murine and 15% in the human X chromosome escape the inactivation thereby leading to a “mosaic function” of some genes, that varies among tissues [250]. It has been supported that this mosaicism offers to females an advantage of more rapid and effective immune responses compared to males [226]. The most representative example, supporting the effect of sex chromosomes on immunity, is that men with Klinefelter syndrome (bearing three sex chromosomes, XXY) exhibit higher B and $CD4^+$ T cell numbers as well as increased $CD4^+/CD8^+$ T cell ratios compared to XY males [251], an immune phenotype similar to females. Interestingly, many of these differences are reversed by testosterone therapy [251], indicating the mixed effect of both sex chromosomes and sex hormones on immunity. Such a mixed effect could likely explain our findings showing that sex hormone manipulation alone cannot reverse or induce the sexual dimorphism seen in lung resident immunity.

As far as the airway epithelium is concerned, sex hormone manipulation had no effect on the integrity of the airway epithelial barrier. However, ovariectomy was associated with slightly enhanced tight junction integrity, a finding suggesting an improved barrier function upon lower estrogen levels. In line with this, a study focusing on the function of the epithelial barrier *in vitro* showed that higher concentrations of 17-b-estradiol resulted in decreased occludin levels and increased paracellular permeability [252]. The fact that no strong effect could be here observed can be at least partially explained by the fact that no challenge capable of severely disrupting the integrity of the airway epithelial barrier was used.

Here, we highlight the sexual dimorphism of the local lung tissue microenvironment and especially of lung resident immunity in healthy mice. Given the important role of the lung resident immunity and the airway epithelial barrier not only in the protection from lung diseases, but also in their pathogenesis [160,214], such sex differences may differentially shape disease susceptibility, manifestation and outcome in male and female individuals. Importantly, sex hormones seem to impact these sex differences by acting on lung resident immune cells and shaping mounted responses. Further investigation is needed to reveal the disease-specific role

of the local lung tissue microenvironment and the exact mechanistic pathways determining the sex bias seen in such a context. Finding ways to more efficiently treat the more susceptible to every lung disease sex can substantially improve life quality and disease prognosis.

5. Summary

Sex differences in prevalence, morbidity and mortality of lung diseases are well established. For example, adult women are more susceptible to influenza infection and chronic obstructive pulmonary disease. On the other hand, adult men experience worse outcomes in pneumococcal pneumonia and squamous cell carcinoma. Although sex hormones have been proposed to account for such sex differences, their exact impact on local tissue microenvironment, lung function and thus, the pathogenesis of lung diseases has not been fully clarified. Major compartments of the lung tissue microenvironment are the respiratory epithelium and the lung resident immunity. Given the fact that hormone receptors, including estrogen and androgen receptors, are expressed both on most immune cell subtypes and on the respiratory epithelium, sex hormones may affect lung tissue resident immunity in a sex-specific manner, subsequently leading to a sex-specific manifestation of respiratory immune diseases.

Our aim was to investigate sex differences in lung tissue microenvironment, including lung resident immunity and the respiratory epithelium, and identify the potential role of sex hormones in this context.

To this aim, firstly, through flow cytometry of cell isolated from the lung of male and female adult C57BL/6 mice, we characterized the sexual dimorphism of tissue resident immunity in the naïve lung. Our next step was to investigate the impact of sex hormones on lung tissue resident immunity. Male and female mice underwent castration or ovariectomy, respectively and after 14 days the mice were sacrificed and their lungs were collected for immune characterization with flow cytometry or histological assessment of the integrity of the respiratory epithelial barrier. Finally, assessment of lung resident immunity was also performed upon testosterone supplementation of female mice.

Our results indicate that males have increased alveolar macrophages and lung residing monocytes, while females have more lung resident $CD103^+$ DCs, pDCs and CD4+ TRM cells. Castration resulted in a reduction of lung resident DCs, infiltrating monocytes, interstitial macrophages and lung resident NK cells, while infiltrating B and CD8+ cells in the lung were intensively augmented. In line with these findings, B cells were also significantly decreased and neutrophils increased after testosterone supplementation of female mice. No profound alterations in lung resident immunity were observed after ovariectomy.

This study indicates the existence of sex differences in the lung microenvironment which are likely mediated by sex hormones. These findings shed light into the sex-specific manifestation of respiratory immune diseases and could set the basis for novel personalized therapeutic approaches in this context.

5. Zusammenfassung

Geschlechtsspezifische Unterschiede spiegeln sich in der Prävalenz, Morbidität und Mortalität von Lungenkrankheiten wider. Beispielsweise sind adulte Frauen anfälliger für Influenza-Infektionen und chronisch-obstruktive Lungenerkrankungen. Wohingegen adulte Männer ein schlechterer Outcome bei einer Pneumokokken-Pneumonien und dem Plattenepithelkarzinom haben. Obwohl die Hypothese besteht, dass Geschlechtshormone für diese Geschlechtsunterschiede verantwortlich sind, ist ihre genaue Auswirkung auf die lokale Mikroumgebung des Gewebes, die Lungenfunktion und damit die Pathogenese von Lungenkrankheiten noch nicht vollständig geklärt. Die wichtigsten Kompartimente der Mikroumgebung des Lungengewebes sind das Atmungsepithel und die residenten Immunzellen der Lunge. Hormonrezeptoren, einschließlich Östrogen- und Androgenrezeptoren, sind auf den meisten Immunzellsubtypen sowie auf dem respiratorischen Epithel exprimiert, wodurch Sexualhormone die lungengewebsresidente Immunität auf geschlechtsspezifische Weise beeinflussen können, was zu einer geschlechtsspezifischen Manifestation von Lungenimmunerkrankungen führt.
Unser Ziel war es, die Geschlechtsunterschiede in der Mikroumgebung des Lungengewebes, einschließlich der lungenresidenten Immunität und des Atmungsepithels, zu untersuchen und die mögliche Rolle der Geschlechtshormone in diesem Zusammenhang zu ermitteln.
Zu diesem Zweck haben wir zunächst den Geschlechtsdimorphismus der gewebeeigenen Immunität in gesunden Lungen, die von männlichen und weiblichen erwachsenen C57BL/6-Mäusen entnommen wurden, mittels Durchflusszytometrie charakterisiert. In einem nächsten Schritt untersuchten wir die Auswirkungen der Geschlechtshormone auf die Immunität des Lungengewebes. Hierfür wurden männliche und weibliche Mäuse kastriert oder ovarektomiert. Nach 14 Tagen wurden die Mäuse getötet und ihre Lungen für die Immuncharakterisierung mittels Durchflusszytometrie oder die histologische Beurteilung der Integrität der Atemwegsepithelbarriere entnommen. Schließlich wurde die lungenresidente Immunität auch nach einer Testosterongabe weiblicher Mäuse untersucht.
Unsere Ergebnisse zeigen, dass die männlichen Mäuse vermehrt alveoläre Makrophagen und lungenresidente Monozyten aufweisen, während weibliche Mäuse mehr lungenresidente CD103+ Dendritic Cells (DCs), pDCs und CD4+ TRM-Zellen haben. Die Kastration führte zu einer Verringerung der lungenansässigen DCs, der infiltrierenden Monozyten, der interstitiellen Makrophagen und der lungenansässigen NK-Zellen, während die infiltrierenden B- und CD8+-Zellen in der Lunge stark vermehrt waren. In Übereinstimmung mit diesen Ergebnissen waren nach der Testosteron-Supplementierung von weiblichen Mäusen auch die B-Zellen signifikant verringert und die Neutrophilen erhöht. Die Ovarektomie resultierte in keiner tiefgreifenden Veränderungen der lungeneigenen Immunität.
Diese Studie deutet auf Geschlechtsunterschiede in der Mikroumgebung der Lunge hin, die wahrscheinlich durch Sexualhormone vermittelt werden. Diese Ergebnisse geben Aufschluss über die geschlechtsspezifische Ausprägung von Immunkrankheiten der Atemwege und könnten die Grundlage für neue personalisierte therapeutische Ansätze in diesem Zusammenhang bilden.

6. Abbrevations

5α-DHT	5α-dihydrotestosterone
AAI	Allergic airway inflammation
AJ	Adherens junctions
AM	Alveolar macrophages
AR	Androgen receptor
BALT	Bronchus-associated lymphoid tissue
BST-2.	Bone marrow stromal antigen-2
CCL	C-C motif ligand
CCR	C-C chemokine receptors
CD	Cluster of Differentiation
CDC	Center for Disease Control and Prevention
cDCs	Conventional Dendritic Cells
CF	Cystic Fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
COPD	Chronic Obstructive Pulmonary Disease
COVID-19	Corona Virus Disease 2019
CXCR	CXC chemokine receptor
DCs	Dendritic Cells
EDTA	Ethylenediaminetetraacetic acid solution
ER	Estrogen receptor
FCS	Fetal calf serum
FSC	Forward scatter
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
IFN	Interferon
IL	Interleukin
ILCs	Innate lymphoid cells
ILD	Interstitial lung diseases
IM	Interstitial macrophages
iNOS	Inducible nitric oxide synthase
IPAF	Interstitial Pneumonia with Autoimmune Features
IPF	Idiopathic Pulmonary Fibrosis
JAM	Junctional adhesion molecules
L-VDCCs	L-type voltage dependent Ca2+ channels
LTi	Lymphoid tissue inducer cells
M. tuberculosis	Mycobacterium tuberculosis
MHCII	Major histocompatibility complex
moDCs	Monocyte-derived Dendritic Cells
NK	Natural killer cells
NO	Nitric oxide
NRS	Normal rat serum
NS1	Non-structural protein 1
NSCLC	Non-small cell lung cancer
OZLN	Occludins
PDCA-1	Plasmacytoid dendritic cell antigen-1
pDCs	Plasmacytoid Dendritic Cells
PFA	Paraformaldehyde
PMA	Perimenstrual asthma
PR	Progesterone Receptor
RAMDs	Repair-associated memory depots
RORγt	Retinoic acid-related orphan receptor-γt
RSV	Respiratory syncytial virus
S. pneumoniae	Streptococcus pneumoniae
S1P	Sphingosine-1-phosphate receptor

SARS-CoV-2	Severe acute respiratory syndrome coronavirus type 2
SSC	Side scatter
TAMs.	Tumor-associated macrophages
TEER	Transepithelial Electrical Resistance
TES	Testosterone
Th	T helper cells
TJ	Tight junctions
TLR	Toll Like Receptor
TNF-a	Tumor Necrosis Factor a
Treg	Regulatory T cell
TRMs	Tissue resident memory cells
TSLP	Thymic stromal lymphoprotein
WHO	World's Health Organization
ZO	Zonula Occludin

7. References

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9. Curriculum Vitae

General information

- Date of Birth: 02.04.1997
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University Studies

Faculty of Medicine, School of Health Sciences, University of Thessaly
Entry September 2015 – Graduation July 2021
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Career

- August 2020- July 2021. Practical year of medical studies in University Hospital of Larissa, University of Thessaly, Greece
- October 2021. Medical approbation, from the Panhellenic Medical Association
- January 2022-Present. Scientific Collaborator in Laboratory for experimental feto-maternal medicine, University Medical Center Eppendorf-Hamburg (UKE)

Research activity

Published Papers

- D.E. Zazara, **I. Belios**, J. Lücke , Zhang T, A.D. Giannou. **Tissue-resident immunity in the lung: a first-line defense at the environmental interface. Semin Immunopathol. 2022**.
- Zhang T*, Wahib R*, Zazara DE, Lücke J, Shiri MA, Kempski J, Zhao L, Agalioti T, Machicote AP, Giannou O, **Belios I**, Jia R, Tintelnot J, Seese H, Grass JK, Mercanoglu B, Stern L, Scognamiglio P, Fard-Aghaie M, Seeger P, Wakker J, Kemper M, Brunswig B, Duprée A, Lykoudis PM , Pikouli A, Giorgakis E, Stringa P, Lausada N, Gentilini MV, Gondolesi GE, Bachmann K, Busch P, Grotelüschen R, Maroulis IC, Arck PC, Nakano R, Thomson AW, Ghadban T, Tachezy M, Melling N, Achilles E, Nickel F, Hackert T, Mann O, Izbicki JR, Li J, Gagliani N, Huber S#, Giannou AD#. **CD4+ T cell-derived IL-22 enhances liver metastasis by promoting angiogenesis. Oncoimmunology 2023.**
- Giannou AD, Kempski J, Zhang T, Lücke J, Shiri AM, Zazara DE, **Belios I**, Machicote A, Seeger P, Agalioti T, Tintelnot J, Sagebiel A, Tomczak M, Bauditz L, Bedke T, Kocheise L, Mercanoglu B, Fard-Aghaie M, Giorgakis E, Lykoudis PM, Pikouli A, Grass JK, Wahib R, Bardenhagen J, Brunswig B, Heumann A, Ghadban T, Duprée A, Tachezy M, Melling N, Arck PC, Stringa P, Gentilini MV, Gondolesi GE, Nakano R, Thomson AW, Perez D, Li J, Mann O, Izbicki JR, Gagliani N, Maroulis IC, Huber S. **IL-22BP controls the progression of liver metastasis in colorectal cancer. Front Oncol. 2023**
- P. Tasoudis, C. Arvaniti, A. Adamou, **I. Belios**, J. Stone, N. Horick, D. Sagris, G. Dalekos, G. Ntaios **Interleukin-6 inhibitors reduce mortality in COVID-19: An individual patient data meta-analysis from randomized controlled trials. European Journal of Internal Medicine 2022**
- A. Adamou, S. Giannopoulos, C. Arvaniti, **I. Belios**, D. Dalampira, G. Eleftheriadis, T. Zinoviou, P. Kassas, G. Vavougios, C. Hatzoglou, K. Gourgoulianis, S. Zarogiannis. **Self-reported risk of Obstructive Sleep Apnea syndrome, and awareness about**

it in the community of 4 insular complexes comprising 41 Greek Islands. Sleep Science 2022

- A. Samouilidis, E. Beltsios, G. Mavrovounis, A. Adamou**, I. Belios**, A. Hadjivasilis, I. Pantazopoulos, A. Agouridis. **The use of antenatal dexamethasone in late preterm and term pregnancies to improve neonatal morbidity and mortality: A systematic review and meta-analysis. Cureus 2022**

Papers Submitted- Under Review

- **I. Belios,** T. Zhang, J. Oh, W. Jungraithmayr, S. Huber, P.C. Arck, A.D. Giannou* & D.E. Zazara*. **Sexual dimorphism of lung-resident immunity is modulated via testosterone-dependent pathways. European Journal of immunology**
- Zazara, O. Giannou, S. Schepanski, M. Pagenkemper, A.D. Giannou, **I. Belios**, M. Pincus, A.C. Muntau, K.Hecher, A. Diemert, MD & P.C. Arck. **Fetal lung growth predicts the risk for early-life respiratory infections and childhood asthma. World Journal of pediatrics**

Posters presentations in Conferences

- Adamou, S. Giannopoulos, C. Arvaniti, **I. Belios**, D. Dalampira, G. Eleftheriadis, T. Zinoviou, P. Kassas, G. Vavougios, C. Hatzoglou, K. Gourgoulianis, S. Zarogiannis. **Cross sectional study of the risk for OSAS in the general population of 20 Greek Islands and assessment of the OSAS awareness.** European Respiratory Society International Conference 2018, Paris, France.
- **I. Belios**, G. Eleftheriadis, T. Zinoviou, D. Dalampira, S. Giannopoulos, C. Arvaniti, A. Adamou, G. Vavougios, S. Zarogiannis. **Bioinformatic analysis of AQP1 molecular mimicry and implications for autoimmune diseases.** European Conference on Computational Biology 2018, Athens, Greece.
- P. Tasoudis, C. Arvaniti, A. Adamou, **I. Belios**, J. Stone, N. Horick, D. Sagris, G. Dalekos, G. Ntaios. **Survival analysis of IL-6 inhibitors versus standard of care for COVID-19: a meta-analysis of individual patient data from randomized control trials.** 20th European Congress of Internal Medicine (ECIM 2022) March 2022, Malaga, Spain
- D.E. Zazara & **I. Belios,** P.C. Arck **Dissecting the role of local tissue microenvironment in sex-specific susceptibility to respiratory infections.** 2nd Biennial Meeting Sex Differences in the Immune System, Dublin
- D.E. Zazara, **I. Belios**, T. Zhang, A.D. Giannou, P.C. Arck **Sexual dimorphism of lung-resident innate immunity is modulated via testosterone-dependent pathways.** Cell Symposia: Myeloid cells: From development to function and dysfunction June, 2023, Shanghai, China

Oral presentation in Conferences

- **I. Belios**, C. Urbschat, P. Wiemers, D. Zazara, P. Arck. **Developmental origin of sex-specific differences in lung-resident immunity.** World Congress of the International Society for Immunology of Reproduction (ISIR) 2023, Hamburg, Germany

- **I. Belios**, M. Spohn, M. Albrecht, N. Ledée, A. Wieczorek, A. Diemert, D. Zazara, P. Arck. **Vertical transfer of maternal pathogen-specific antibodies in successive pregnancies and infection risk in infancy.** World Congress of the International Society for Immunology of Reproduction (ISIR) 2023, Hamburg, Germany
- **I. Belios,** Christopher Urbschat, Pauline Wiemers, Anastasios D. Giannou, Dimitra E. Zazara, Petra C. Arck. **Developmental origin of sex-specific differences in lung-resident immunity and respiratory infections.** Joint meeting of American Physiological Society and Hellenic Society of Physiology 2023, Academy of Athens, Athens, Greece.
- Adamou, S. Giannopoulos, C. Arvaniti, **I. Belios**, D. Dalampira, G. Eleftheriadis, T. Zinoviou, P. Kassas, G. Vavougios, C. Hatzoglou, K. Gourgoulianis, S. Zarogiannis. **Cross sectional study of the risk for OSAS in the general population of 20 Greek Islands and assessment of the OSAS awareness.**Annual Panhellenic Respiratory Conference of Hellenic Thoracic Society 2018, Athens, Greece

OTHER SCIENTIFIC ACTIVITIES

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