Sex as a Biological Variable in Allergen-Induced Airway Inflammation

by

Dina Hanaey Dandrawy Mostafa

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements for the degree of

Master of Science

Department of Immunology

University of Manitoba

Winnipeg, Manitoba

Preface

Thesis Summary

Biological sex is a key variable that profoundly impacts disease prevalence, severity and response to therapy. There are marked disparities in immunological-based responses between males and females that can drive sex-bias in the prevalence of chronic inflammatory disease. Allergic asthma is a leading chronic respiratory disease characterized by airway inflammation that results in airway obstruction and difficulty in breathing. Asthma displays age- and sex-related differences in prevalence and disease severity. Asthma incidence and severity are more prominent in boys during childhood, but this trend shifts around puberty, as asthma prevalence and severity becomes more predominant in females in adulthood. Notably, females are more prone to suffer from uncontrolled asthma and corticosteroid-unresponsiveness disease, making it more challenging to manage symptoms. Despite, the recognition of sex-related differences in asthma, sex as a biological variable is excluded in most preclinical murine model studies. In this thesis, I outline molecular mediators that distinguish sex-related differences in allergen-mediated airway inflammation. My findings will be valuable to understand mechanisms that shape sex-disparity in allergic disease processes, and importantly for sex-based personalized drug development efforts in asthma.

To stratify sex-related differences in airway inflammation, I have used a two-week house dust mite (HDM)-challenged murine model in two different mice strains (BALB/c and C57BL/6NJ). As genetic variations influence the inflammatory response which can potentially impact sex-related differences in response to allergen, I have used two different strains of mice. I examined leukocyte accumulation in bronchoalveolar lavage fluid (BALF), serum IgE levels (total and HDM-specific) and profiled the abundance of a panel of 29 different cytokines in BALF and lung tissues in my studies. I identified a sex-specific cytokine biosignature in the lung, both in naïve and HDM-challenged mice which was mice strain dependent. Overall, my results showed that female BALB/c mice predominantly mount a Th17-biased response to inhaled HDM challenge compared to males, whereas C57BL/6NJ female mice display a mixed Th1/Th2-skewed response. Males of both strains show a Th2-slewed response compared to females. This highlights the interplay between genetic variability and sex difference in airway inflammation and the importance of taking the mice strain into consideration when interpreting results.

Proteomics approaches are important in the discovery of novel biomarkers. In this thesis, I focused on outlining sex-specific allergen-mediated protein changes that are commonly enhanced in BALF of both mice and humans. The Mookherjee lab previously conducted unbiased proteomic profiling utilizing liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) to characterize allergen-induced alterations in secreted proteins (secretome) in the lung of female BALB/c mice (in the two-week HDM-challenge mouse model), as well as in a controlled allergen exposure human model. Human exposures were performed by our collaborator Dr. Christopher Carlsten at University of British Columbia. Comparative assessment of the murine and human proteomics datasets uncovered 19 proteins commonly enhanced in both mice and human BALF, in response to allergen challenge. I selected the top 10 protein targets from this 19 common protein biosignature and examined their abundance in BALF from mice and human (both females and males) allergen exposure studies using western blot.

I examined sex-specific changes in allergen-induced selected proteins in BALF obtained from HDM-challenged murine model and from humans exposed to nebulized allergen. Female BALB/c mice showed significantly higher levels of HDM-induced eosinophil peroxidase (EPX), S100A8, S100A9 and properdin, compared to males. Likewise, human female participants showed higher levels of EPX compared to males in BALF following allergen challenge. These results showed that EPX is a biomarker that shows female-bias in both mice and humans following allergen challenge. In contrast to mice data, human male participants demonstrated elevated levels of S100A8 and S100A9 in BALF compared to females. Notably, two allergen-induced proteins in BALF displayed species-specific sex-bias. Female participants showed significantly higher allergen-induced coronin1A/TACO only in human BALF compared to males. While HDM-mediated properdin increase was significantly higher in females compared to males, only in mice BALF and not in humans.

Overall, the findings in this thesis provide the foundation for future work that recognises the importance of sex-disaggregated data and will enable the translation of novel biomarkers from animal models to human studies in a sex-dependent manner.

Acknowledgements

Foremost, I would like to praise and thank God, that bestowed me with countless blessing.

This work would not have materialized and been possible without the remarkable support that I have received from my supervisor, colleagues, family, and friends. They all provided me a great deal of support and assistance throughout the writing of this thesis.

Firstly, I would first like to thank my supervisor, Dr. Neeloffer Mookherjee, whose incredible mentorship was invaluable for my development as a scientist and my own personal growth. Your mentorship paved the way to creates a positive, safe, and inclusive lab environment. Your constant feedback and insight motivated me to sharpen my thinking and elevated my work to a higher level. You have provided me substantial amount of time for scientific discussions that allowed me to develop this project. Your constant support during this pandemic and while formulating this thesis was a tremendous help that I truly believe I would not have come this far without your constant encouragement and motivation.

To my colleagues, thank you for all the insights and scientific discussions throughout my graduate degree that helped me produce this research project. Thank you to helping me practice my presentations, engaging with my countless questions as I was being trained as a scientist and editing my documents. Most importantly, thank you for creating a wonderful lab environment that has been a blessing during the hard times of the pandemic.

To my committee members, Dr. Keijzer and Dr. Arsenio, thank you for all the guidance and feedback that helped me throughout my degree.

To my parents, Hanaey and Nabila, and my sister and brother, Aya, and Mohammed, thank you for always believing in me and your endless support that made me the person I am today, the never-ending support and always believing in me.

Dedication

For my family who are my home, especially my father and mother, who have struggled to give me everything they never had.

For my supervisor, Dr. Mookherjee, who truly believed in me.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AHR	Airway hyperresponsiveness
APCs	Antigen-presenting cells
AR	Androgen receptors
AREG	Epidermal growth factor amphiregulin
ART	Androgen replacement treatment
ASM	Airway smooth muscle
BALF	Bronchoalveolar lavage fluid
BCA	Bicinchoninic acid
BCR	B cell receptor
BMDCs	Bone-marrow derived DCs
C	Cysteine
CD	Cluster of differentiation
CD40L	CD40 ligand
CD5L	CD5 antigen-like protein
CIHR	Canadian Institutes of Health Research
CLRs	C-type lectin receptors
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus infectious disease 2019
CVB3	Coxsackievirus B3
CXCR3	C-X-C chemokine receptor 3
DC	Dendritic cells
DHT	Dihydrotestosterone
E1	Estrogen
E2	Estradiol
E3	Estriol
EAE	Experimental autoimmune encephalitis
ECM	Extracellular matrix

ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
ELISA	Enzyme-linked immunosorbent assay
EPX	Eosinophil peroxidase
ERα	Estrogen receptor alpha
ERα−/−	Estrogen receptor alpha knockout
ERβ	Estrogen receptor beta
ERE	Estrogen responsive elements
FCG	Four core genotypes
FDA	Food and Drug Administration
FOXP3	Forkhead-box-P3
GNLY	Granulysin
GPCR	G-protein-coupled receptors
GPI-PLD	Glycosylphosphatidylinositol-specific phospholipase D
GPR30	G protein-coupled receptor 30
GR	Glucocorticoid receptor
GZMA	Granzyme A
HDM	House dust mite
hnRNPU	heterogeneous nuclear ribonucleoprotein U
IAV	Influenza A virus
IFN	Interferons
Ig	Immunoglobulin
iNOS	Inducible nitric oxide synthase
ILs	Interleukins
IP-10	Interferon gamma-induced protein 10
IPF	Idiopathic pulmonary fibrosis
IL-1RA	IL-1 receptor antagonist
IL-2RG	IL-2 receptor subunit gamma
IL-12Rβ2	IL-12 receptor β2
IL13RA2	IL-13 receptor subunit alpha 2
ILC2	Group 2 innate lymphoid cells

IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IRF5	IFN regulatory factor 5
ISGs	Interferon stimulated genes
KC	Keratinocyte chemoattractant
LBP	Lipopolysaccharide binding protein
LC–MS/MS	Liquid chromatography—tandem mass spectrometry
LPS	Lipopolysaccharide
LTβ	lymphotoxin β
LTB4	Leukotriene B4
MAMPs	Microbe-associated molecular patterns
MBP	Major basic protein
MCP-1	Monocyte chemoattractant protein-1
MF-ISGs	IFN-responsive genes in macrophages
MHC	Major histocompatibility complexes
MIP-1a	Macrophage inflammatory protein-1a
miRNAs	microRNAs
MMP-9	Matrix metalloproteinase 9
MSD	Meso Scale Discovery
NE	Neutrophil elastase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of Health
NK	Natural Killer
NLR	NOD-like receptor
NLRP12	Nod-like receptor pyrin domain containing 12
NO	Nitric oxide
OVA	Ovalbumin
OX40L	OX40 ligand
P4	Progesterone
PAMPs	Pathogen-associated molecular patterns
PAS	Periodic acid schiff
PBMCs	Peripheral blood mononuclear cells

pDCs	Plasmacytoid dendritic cells
PMA	Phorbol 12-myristate 13-acetate
PR	Progesterone receptor
PRRs	Pattern recognition receptors
RIG-I	Retinoic acid inducible gene I
RLRs	Retinoic acid inducible gene I like receptors
ROS	Reactive oxygen species
RT	Room temperature
SABV	Sex as a biological variable
SAGER	Sex and Gender Equity in Research
SOCS1	Suppressor of cytokine signaling
SLE	Systemic lupus erythematosus
T2	Type 2
TB	Tuberculosis
TFH	Follicular helper T cells
TJs	Tight junctions
TLRs	Toll-like receptors
TMB	3,3’,5,5’-tetramethylbenzidine
TNF	Tumour necrosis factor
Treg	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
XSCID	X-linked severe combined immunodeficiency

Copyright and License Information

Figure 1. Illustration summarizing differential immunological-based responses between males and females in innate & adaptive immunity

Fischinger S, Boudreau CM, Butler AL, Streeck H, Alter G. Sex differences in vaccine-induced humoral immunity. Semin Immunopathol. 2019 Mar;41(2):239-249. This figure was modified with BioRender.com.

Figure 5. Anatomy of the murine lung.

Sato S, Bartolák-Suki E, Parameswaran H, Hamakawa H, Suki B. Scale dependence of structure-function relationship in the emphysematous mouse lung. Front Physiol. 2015 May 12;6:146.

Chapter 1: General Introduction

1.1 Immune System

1.1.1 General Overview

The immune system is an intricate network of specialized cells and molecules which has evolved in complexity to deal with a universe of pathogenic microbes and other danger that threaten normal host functions employing an array of diverse mechanisms [1, 2]. A primary function of the immune system is to mobilize a response to an invading pathogen, toxin or substances that can enter through mucosal surfaces [3]. Fundamentally, the immune system has the ability to discriminate between the body's own cells and pathogens (self from non-self) by detecting structural features (antigens) on the surface of the pathogen that distinguishes it from host cells [1, 2, 4, 5]. This distinction between self and non-self is central to eliminating pathogens and other 'foreign' antigens without damaging the host's own tissues. Further, the immune system can recognize and clear dead and faulty cells in the body of the host [1, 2, 4, 5]. The immune system responds to pathogens and other antigens with secretion of molecular factors and proteins such as cytokines and chemokines, which promote inflammation as the first line of defence, including facilitating leukocyte recruitment to the site of infection and inducing other antimicrobial functions [5].

The ongoing global pandemic of coronavirus infectious disease 2019 (COVID-19) has raised public awareness regarding the vital role of the immune system in fighting infections, as recovery from COVID-19 infection is strongly associated with eliciting an efficient immune response [6, 7]. Although the process of inflammation is essential for infection clearance and host defense mechanisms, this process needs to be regulated. At times, the severity of infections correlate with activation of various inflammatory pathways that cause hyperinflammation and cytokine storm, which results in tissue damage and multi-organ failure [8–10]. Dysregulation of inflammation resulting in persistent and amplified inflammation also leads to chronic inflammatory disorders [10, 11]. Thus, understanding the specific cellular and molecular processes involved in the initiation and regulation of immune responses is an essential step for disease management and development of effective new therapies.

An emerging component of immune function is the influence of biological sex. Biological sex is defined by sex chromosomes (XX and XY), reproductive organs (testes and ovaries) and gonadal sex steroids (including androgens, estrogens, and progestogens). There is a disparity in manifestation and pathogenesis of many widespread diseases between males and females, which results in sex-specific outcomes from autoimmune and infectious diseases as well as sex differences in response to therapies. Thus, there are significant gaps in our understanding of sex-biased immune responses and precise mechanisms mediating these differential responses. This chapter will provide an overview of key components of the immune system, airway inflammation and asthma (which is the focus of this thesis), and sex-related differences in immune response and asthma.

1.1.2 Innate & Adaptive Immunity

The immune system utilises two lines of defenses, innate and adaptive immunity, to efficiently detect and eliminate pathogens [2, 4]. The innate arm of the immune system acts as a first line of defense that has two major functions. Firstly, it acts as a physical barrier to prevent invasion of pathogenic microorganisms through mucosal tissue, skin and chemical barrier (including, pH, enzyme, antimicrobial peptides and complement system) [12]. Secondly, the innate immune system eliminate invading pathogens that penetrated mucosal barriers through phagocytosis and cytotoxicity mechanisms [12–14]. Additionally, the innate immune system is crucial to the activation of adaptive immunity through secretion of cytokines and antigen presentation to T and B cells [14]. Innate immunity elicits rapid responses to invading pathogens mediated by innate immune cells including dendritic cells (DC), neutrophils, macrophages, monocytes, and dendritic cells [15–18]. The majority of these innate immune cells express various pattern recognition receptors (PRRs), including toll-like receptors (TLRs), NOD-like receptors (NLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) and C-type lectin receptors (CLRs) [12].

The PRRs can recognize and detect pathogen-associated molecular patterns (PAMPs), or microbe-associated molecular patterns (MAMPs) expressed on the pathogen's surface [12]. This induces innate immune cells to produce inflammatory cytokines, chemokines, and other molecules, including reactive oxygen species (ROS) for pathogen clearance [2, 4].

The secretion of proinflammatory cytokines promote inflammation that further facilitates leukocyte recruitment [5].

Innate immunity has a key role in the induction and consequent direction of adaptive immunity, mainly through antigen-presenting cells (APCs). APCs process and present foreign antigens on their surface via major histocompatibility complexes (MHC) for recognition by lymphocytes such as T cells [4, 5]. The classical APCs include dendritic cells, macrophages, and B cells. The interaction of APCs with T cells bridges innate and adaptive immune responses and influences the differentiation of T cells [2]. The adaptive immune response generally takes days or even weeks to allow for more specificity to pathogens and establishment of immune memory to provide the host with long-term protection [18]. There are two types of adaptive responses: the cell-mediated immune responses mediated by T cells, and the humoral immune response which is mediated by activated B-cells and production of antibodies [15]. Naïve T cells can be classified as CD4+ or CD8+ depending on expression of cluster of differentiation (CD) surface molecules CD4 or CD8, which impact whether T cell receptors (TCRs) will engage with an MHC II or an MHC I molecule on APCs [5, 15]. Naïve CD4+ TCR bind to antigen-embedded MHC II molecules on APCs and are activated to become T helper (Th) cells. These can further differentiate into various Th subsets based on the presence of specific cytokines and the expression of distinctive lineage-defining transcription factors [16]. The main Th subsets identified so far include; Th1, Th2, and Th17 cells, regulatory T (Treg) cells that are essential to maintain self-tolerance (unresponsiveness to self antigen) and follicular helper T cells (TFH) that aid B cells for antibody production [16] . Cytokines released by the various Th cells can amplify the immune response. Each Th subset secretes signature effector cytokines that specialize in mediating responses to different types of pathogens, for example IL-4 secreting Th2 cells combat extracellular parasites while IFN-γ producing Th1 cells target intracellular pathogens [16, 19].

Contrary to CD4+ T cells, naïve CD8+ TCR bind to the antigen-embedded MHC I molecules on APCs and become cytotoxic T cells , that can eliminate pathogens and infected cells [15, 16]. Cytotoxic T-cells can eliminate infected cells through the release of cytotoxic granules including perforin, and granzymes which causes the formation of pores in the target cell membrane that allow for cytotoxic granules to infiltrate inside the infected cells and ultimately resulting in apoptosis of infected cells [20].

B cells are well-recognised for the production of antibodies but they also play a role in antigen presentation and cytokine production which shapes the response of other immune cells such as T cells [5]. Antibodies are classified into five different classes including Immunoglobulin (Ig)A, IgD, IgM, IgG, and IgE. Each of these has specific functions in the immune responses, for example IgE is associated with allergies and anaphylaxis [2, 5]. Antibody secretion is initiated upon exposure to antigen that is recognized by B cell receptor (BCR) that has a unique specificity for each antigen epitope and facilitate the internalization of antigens and subsequent presentation of the resulting peptides on Class II MHC molecule [2]. The binding of BCR with the antigen in combination with costimulatory signals can stimulate B-cell differentiation into antibody producing plasma cells and memory B cells [21]. B cells also present antigen to T cells, the interaction of BCR and TCR plays a critical role in contributing to B-cell activation with the help of Th cells through cytokine production that aid in B cell proliferation and directing the type of antibody secreted [21, 22]. In certain circumstances, some antigenic epitopes can directly activate B cells without assistance from T cells [5, 22]. However, antibodies generated through microbial antigens alone have reduced functional versatility compared to those stimulated without T cells assistance [22].

1.1.3 Cytokines & Chemokines

Cytokines are low molecular weight secreted proteins (~5–70 kDa) that can act in a paracrine fashion on another cell and/or in an autocrine fashion on the same cell [17]. Therefore, cytokines are considered potent signalling molecules that play a crucial role in mediating immune responses in both health and disease [23]. The main function of cytokines is to regulate local and systemic inflammation, but these molecules can also orchestrate a range of processes including cellular proliferation, apoptosis, hematopoiesis, metabolism, chemotaxis, and tissue repair [23].

There are different types of cytokines; including various interleukins (ILs), chemokines, lymphokines, interferons (IFN) and tumour necrosis factor (TNF). Cytokines are synthesized and secreted by a number of immune cells such as T cells, neutrophils, macrophages, B cells and mast cells, as well as other cell types such as endothelial cells, fibroblasts, epithelial cells and various stromal cells [24]. In order for a cytokine to induce an effect on a target cell, these bind to a specific cytokine receptor on the cell surface [25].

Cytokine receptors are membrane glycoproteins that constitute of several units and the binding of cytokines to these receptors induce a cell signaling cascade that impacts cell function [25].

Chemokines are a family of heparin-binding cytokines that guide the migration and recruitment of cells in a process known as chemotaxis [26, 27]. Thus, they play a pivotal role in guiding immune cells to sites where they are needed to facilitate biological processes such as inflammation [26, 27]. Additionally, chemokines are involved in activation of adhesion molecules to allow leukocyte extravasation [28, 29]. Thus, chemokines are key drivers of the process of inflammation. Chemokines are classified into 4 different families, which are defined based on the arrangement of the conserved cysteine (C) residues, CXC, CX3C, CC and C type chemokines [26, 30]. Chemokines are the only subset of cytokines that act on the superfamily of G-protein-coupled receptors (GPCR) on target cells [31]. Key chemokines that regulate migration and infiltration of macrophages, neutrophils and lymphocytes include CXCL10 (also known as Interferon gamma-induced protein 10 (IP-10)) , CCL2 (monocyte chemoattractant protein-1 (MCP-1)) and CCL3 (also known as Macrophage inflammatory protein-1α (MIP-1α) [26, 30, 31]. Cytokines that trigger or promote inflammatory responses through contributing to the activation, differentiation, and proliferation of immune cells are known as pro-inflammatory cytokines, whereas those that are critical for resolution of inflammation are classified as anti-inflammatory cytokines [32]. Some of the major inflammatory cytokines include IFN-γ, IL-1, IL-4, IL-8, TNF-α and IL-6, while potent anti-inflammatory cytokines include IL-10 and IL-1 receptor antagonist (IL-1RA) [32]. Elevated concentrations of cytokines and chemokines are implicated in the pathogenesis of a range of diseases particularly their intense involvement in amplifying inflammation [17]. This is highlighted in cases of COVID-19, poor prognosis is associated with hyperproduction of proinflammatory cytokines, such as IL-6, IL-1, IL-12, IFN-γ, and TNF-α [33].

Cytokines are sometimes used as biomarkers to evaluate disease progression and monitor the efficacy of treatment in a variety of inflammatory conditions [17]. Overall, cytokines and chemokines are critical effector immune molecule involved in the resolution and pathophysiology of disease (infectious and other chronic disease), as well as for maintaining immune homeostasis. It is thus critical to understand the functions of each cytokine within the inflammatory milieu to better understand mechanisms related to regulation of inflammation, and immune homeostasis, which is critical for drug development for chronic and infectious disease.

1.1.4 Inflammation

Inflammation is initiated following a breach in physical barrier either by an injury or invading pathogens [2, 4, 5]. Inflammation is characterized by edema or swelling, heat, redness and pain at the site of injury or infection [4]. These classic signs of inflammation reflect changes in local blood flow and vascular permeability [34]. The heat and redness during inflammation is a result of an increase in vascular diameter which also results in slower blood flow [15, 34]. There is an increase in vascular permeability which results in fluid from blood accumulating in tissue causing edema and pain [34]. The main function of inflammation is to trigger an immune response, wherein vascular changes promote circulating leukocytes to migrate to the site of damage to destroy pathogens, clean up cell debris, and begin the process of tissue repair [15, 35]. As discussed above, numerous inflammatory mediators released from innate immune cells such as pro-inflammatory cytokines initiate the process of inflammation. Also, other mediators such as histamine and prostaglandins secreted from mast cells, and complement proteins can promote inflammation [13, 35]. The resolution of inflammatory responses is critical to maintain immune homeostasis and to prevent tissue damage associated with chronic inflammation [2, 5, 34, 35].

Inflammation is a double-edged sword. Even though, inflammatory processes are crucial for pathogen clearance and repair of damaged tissue, it remains the primary feature of many disorders including atherosclerosis, autoimmune diseases, pulmonary fibrosis, asthma and many other pathologies [2, 13, 34, 35]. This is primarily when inflammatory responses are uncontrolled, remain amplified and persistent, thus becoming chronic and resulting in various pathologies [2, 5, 35]. As my thesis focuses on airway inflammation, innate immune cells in the lungs and their role in the context of allergic asthma will be discussed in more detail in section 1.3.

1.2 Sex as a Biological Variable (SABV)

1.2.1 General Overview

Sex and gender can influence both health and disease, yet they are distinct concepts which are often mistakenly used interchangeably [36–38]. However, their influence is often indistinguishably linked and it is often challenging to separate the effects of sex and gender. Unfortunately, there is a misconception of using the terms sex and gender interchangeably in many publications despite fundamental distinctions between the two [39]. Sex is a biological variable that is encoded in the DNA that defines certain characteristics such as reproductive organs and physiology [39, 41]. Conversely, gender refers to the socially constructed roles, behaviors, expressions, and how individuals identify themselves as boys, girls, women, men, and gender diverse individuals [36, 40]. Gender is not binary but rather flexible as it encompasses how individuals perceive themselves, act and express it [36, 40]. Often it is challenging to separate the effects of sex and gender as they influence each other. For example, sex can impact health by influencing behaviour as testosterone tend to induce a risk-seeking behaviour that is strongly associated with neglecting personal health [39, 41].

Every cell in the body has a sex which indicate that females and males are different down to the molecular and cellular level which essentially can influence cell biology of tissues, clinical characteristics, as well as disease outcomes [36, 39]. This is evident in a study by Deasy and colleagues that demonstrated muscle stem cell taken from male mice have diminished regeneration compared to female mice when transplanted into mice with diseased muscle [42]. These findings emphasize the importance of reporting the sex of the cell line used in the study. Furthermore, failing to account for sex in *in vitro* and *in vivo* studies can lead to spurious results. A detailed report published in 2001 by the Institute of Medicine Committee highlights the importance of inclusion of sex in biomedical research and animal work due to its profound impact on biological and biochemical processes, thus strongly recommending the integration of sex in biomedical research [39]. Incorporation of sex as a biological variable will not only enhance research in various ways but is a good research practice that ensures reproducibility of findings.

There is a need for a set of guidelines implemented internationally to promote a systemic approach in reporting sex and gender findings across multiple research disciplines. Sex and Gender Equity in Research (SAGER) guidelines provide detailed procedures to correctly report sex and gender findings, analyses of sex and gender data and correct interpretation of results [43]. Sex disaggregated data analysis, which means to analyze data in females and males separately, often detects effects and underlying mechanisms that would not otherwise have been seen in combined analysis. Importantly, a dominant effect or response in one sex might be wrongly attributed to both sexes. Moreover, the exclusion and under-representation of females in clinical trials and preclinical research has raised major concerns for the efficacy and safety of drugs. The Food and Drug Administration (FDA) had to withdraw ten prescription drugs from the market due to their detrimental side effects in women between the years 1997-2000 [44]. In the year 2000, the FDA took steps to remove phenylpropanolamine from all drug products due to the side effect of bleeding into the brain or tissue it causes in women but not in men [36, 39, 44]. Despite these reports, the majority of articles published in 2009 in several journals across major biological disciplines (immunology, neuroscience ,pharmacology, general biology ,endocrinology, behavioral physiology, zoology, reproduction and physiology) failed to report the sex of the animal or the cell line used [44]. Over 50% of articles in immunology and general biology fields failed to report the sex of the animals used [44]. In publications that stated the sex of the animal, a male dominance was evident. Interestingly, studies that employed both sexes failed to analyze results separately by sex [41, 44]. In 2016, two major North American health funding agencies, the US National Institutes of Health (NIH) and the Canadian Institutes of Health Research (CIHR), made a decision to implement a policy where scientists are expected to justify the use of single sex in their research, in efforts to encourage integrating sex as a biological variable (SABV) in animal and human studies [39, 45, 46]. Integration of sex and gender in biomedical research has had direct translational application. In 2019, The FDA approved a new pre-exposure prophylaxis (PrEP) drug for the prevention of infection with HIV only for males and transgender women due to the exclusion of females and transgender men from clinical trials [47].

This section summarizes sex-specific differences documented in innate and adaptive immunity, possible driving factors for sex differences in immune response, and sex bias in the context of allergic asthma.

1.2.2 Sex Differences in Immunity

There is a striking difference in the magnitude of immune responses to allergens, pathogens or self-antigens, between males and females [40, 48]. Generally, females tend to mount a stronger immune response through exhibiting a better ability to detect foreign antigens and recruiting more immune cells which allow them to clear infections more efficiently compared to males [36, 37]. This disparity is reflected in a male bias in susceptibility of various infectious diseases such as tuberculosis (TB) and cutaneous leishmaniasis [37, 40]. However, a heightened immune response in females also contributes to enhanced susceptibility to inflammatory diseases and autoimmune disorders (e.g. arthritis, systemic lupus erythematosus (SLE) and multiple sclerosis) compared with males [37]. Notably, sex dimorphism in immune responses impact disease manifestation and disparities observed between the sexes in terms of susceptibility to infectious diseases, autoimmune diseases, incidence of malignancies and responses to vaccines [37, 40]. Moreover, sex-based immunological differences and disease prevalence are maintained throughout life, where sex-related differences can be influenced by age. For example, in some instances sex-related differences in immune responses is evident following puberty indicating the involvement of hormonal factors and genetic factors associated with sex chromosomes [49]. Thus, inclusion of SABV in research design needs to take age into consideration when interpreting results.

1.2.2.1 Sex Differences in Innate & Adaptive Immunity

Across diverse species, sex dimorphism in immune responses have evolved from insects to lizards, birds and mammals [37, 38, 48]. Sex dimorphism displayed in both innate and adaptive immune responses suggests that certain sex-related differences in immune function may be germline encoded and associated with the composition of sex chromosomes [50, 51].

The X chromosome contains a number of immune-related genes such as those that encode for TLR7, TLR8, IL-2 receptor subunit gamma (IL2RG), IL-13 receptor subunit alpha 2 (IL13RA2), Forkhead-box-P3 (FOXP3), and C-X-C chemokine receptor 3 (CXCR3) [52]. The innate detection of pathogen-associated molecular patterns by PRRs also differs between the sexes [53]. PRRs such as TLR7 and TLR8, as well as IRAK1 a key regulator of the TLR-dependent signalling pathway, are located on the X chromosome [54, 55].

Interestingly, TLR7 expression is higher in neutrophils and macrophages from females compared to males, whereas TLR4 expression is higher in males [37, 56]. The current hypothesis is that TLR7 encoded on the X chromosome may escape the process of gene silencing on the second X chromosomes (known as X inactivation) in females leading to the higher expression of TLR7 in females thus resulting in more robust pathogen recognition and clearance [56]. Peripheral blood mononuclear cells (PBMCs) isolated from females that are exposed to TLR7 ligands in vitro, have higher secretion of IFN-α compared to males [57, 58]. In addition, plasmacytoid dendritic cells (pDCs) isolated from both mice and human females secrete higher levels of IFN-α upon TLR7 ligand stimulation also, females have elevated basal levels of IFN regulatory factor 5 (IRF5) compared to males [58, 59]. These sex-based differences are reflected in disparities in innate immune responses of pDCs from HIV-1 infected patients. For example, pDCs from HIV-1 infected females exhibit greater expression of interferon stimulated genes (ISGs) and have significantly higher production of IFNα compared to pDCs from HIV-1 infected men following *ex vivo* stimulation [60, 61]. Transcriptional analysis of IFN-responsive genes in macrophages (MF-ISGs) revealed enhanced expression of MF-ISGs in unstimulated macrophages from female C56BL/6J mice compared with males [51]. In contrast, males have higher levels of NK and cell activity than females in circulation [58, 62]. Sex differences in group 2 innate lymphoid cells (ILC2) are also reported in various organs and tissues [50, 58]. For example, adult female asthma patients have higher levels of ILC2s and macrophages compared to male patients [63, 64]. Studies in both mouse models and humans revealed that activity of innate immune cells such as macrophages, ILC2s, neutrophils, monocytes and DC are generally higher in adult females than males [58, 60, 65]. APCs from males are less efficient at presenting peptides compared to females [58, 66, 67].

Sex-related differences are also reported in the production of cytokines and chemokines by innate immune cells [36]. PBMCs stimulated with lipopolysaccharide (LPS) from healthy male donors exhibit higher production of TNF-α and IL-6 compared to females, while females elicit higher levels of IL-8 production compared to males [68, 69]. LPS-challenged male-derived macrophages have higher expression of TLR4 and produce significantly higher levels of IL-1β and inflammatory chemokine Interferon gamma-induced protein 10 (IP-10) compared to LPS-challenged female-derived macrophages [70].

PBMCs from healthy male donors produce higher levels of IL-10 following stimulation with viruses (influenza and Herpes-simplex-1) or TLR8 and TLR9 ligands [71]. During SARS-CoV-2 infection, male patients were reported to have higher levels of pro-inflammatory cytokines, including IL-6 and IL-8, compared to females [60, 72]. Thus, it is evident that biological sex strongly influences the innate immune system which in turn can shape adaptive immune responses.

In general, humoral and cell-mediated adaptive immune responses are more vigorous in females compared to males, demonstrated in response to antigenic stimulation, vaccinations and infections [38, 58, 60]. Sex-related differences are reported in lymphocyte subsets including CD4+ T cells, CD8+ T cells and B cells [73, 74]. Clinical studies demonstrated that females have higher levels of CD3+ and CD4+ T cell counts compared to males, as well as females have an elevated CD4+/CD8+ ratio compared to males [60, 74, 75]. In contrast, males have higher levels of CD8+ T cells compared to females [60, 75, 76]. However, females display an enhanced activity of both CD4+ and CD8+ T cells compared to males [76]. For example, transcriptional analyses of T cells isolated from females and stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin have higher expression of inflammatory and cytotoxic genes including IFN-γ, IL-12 receptor β2 (IL-12Rβ2), lymphotoxin β (LTβ), granzyme A (GZMA) and granulysin (GNLY), compared to those from males [77]. Additionally, adult female mice exhibit higher Th1 (e.g., IFN-γ) and Th2 (e.g., IL-4) cytokine responses following parasitic infections (*Leishmania major* and *Plasmodium chabaudi*) compared to male mice, suggesting that females may have better protection and responses to parasitic infections [78, 79].

It is important to note that sex differences in distribution and activity of CD4+ T cell subsets depend on the stage of infection and the type of antigen encountered. Largely, adult females exhibit greater antibody responses than males, where females tend to have higher basal immunoglobulin levels and higher B cell frequencies compared to males [58, 74, 76]. Moreover, following immunization, females have higher vaccine-specific IgG titers which contribute to potentially better protection and longer-term durability in vaccinated females compared to males [80]. However, the enhanced immune activation and higher antibody titre in adult females following immunization can also result in more adverse effects compared to males [80].

Overall, there are clear sex-related differences in both innate and adaptive immune responses which contribute to sex-dependent variation in foreign antigen recognition, levels of immune cells recruitment, antibody-mediated functions, as well as in pathogen control and clearance. Thus, these sex-specific immunological differences can influence the prevalence and susceptibility to autoimmune diseases, infectious diseases and malignancies between females and males.

1.2.2.2 Factors Contributing to Sex Difference in Immunity

As detailed above, biological sex strongly influences the innate and adaptive immune responses. Multiple factors can contribute to sex-based differences in immunological responses including environmental factors (e.g. diet, smoking and gut microbiota), genetic factors and hormonal mediators [36, 58, 81] (Figure 1). For instance, the gut microbiota plays a critical role in influencing intestinal and systemic immune responses, and recent evidence demonstrate that sex-dependent differences in the microbiome composition impacts sex disparities in immune responses [49, 73, 82]. Additionally, genetic differences between males and females particularly in relation to sex chromosomes can contribute to sex disparity in immune responses, primarily because several immune-related genes are located on the X chromosome [52] as discussed above. Moreover, sex hormones (e.g., estrogen, progesterone and testosterone) can directly influence the effector function of immune cells [49, 83]. This section will focus on genetic and hormonal mediators that drive sex-based differences in immune responses.

Figure 1

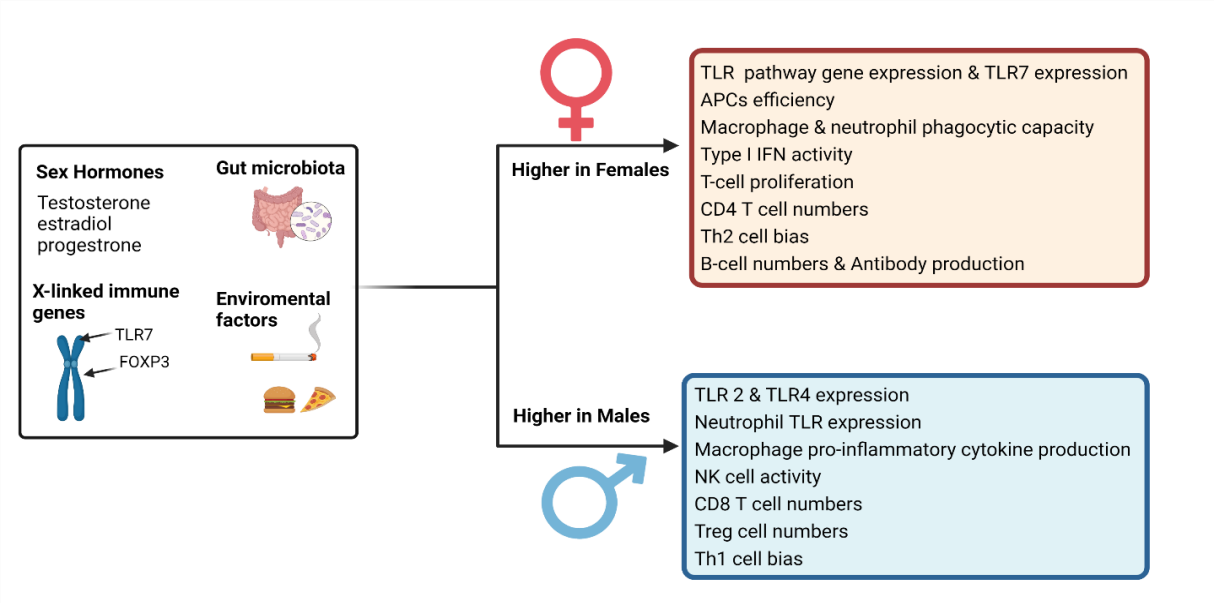


Figure 1: Sex-specific differences in innate & adaptive immunity. Multiple environmental and physiological factors contribute to sex-specific differences in immune responses. Sex chromosomes, sex hormone levels, sex-specific differences in microbiota composition and environmental factors (e.g., smoking and diet) are among some of the factors that influence differential immune responses between male and female (box on the left). Females display greater phagocytic activity by innate immune cells, increased T-cell proliferation and higher levels of B cells and antibodies which can provide a greater protection from infectious diseases. However, these heightened responses increase susceptibility to chronic inflammation and autoimmune diseases in females (Red box). Males have higher levels of CD8+ T cells and NK cell numbers, and certain enhanced Th1 responses (blue box). TLR, Toll like receptor; APCs, Antigen-presenting cells; NK, Natural Killer. This figure was modified from Seminars in Immunopathology, 2019. 41(2): p. 239–249. This figure was created using BioRender.com.

1.2.2.2.1 Impact of X & Y Chromosomes in Immune Response

The X chromosome encodes approximately 1,100 genes, while the Y chromosome encode around 55 genes [41]. Males carry one Y chromosome inherited from the father and one X chromosome inherited from the mother, while females carry two X chromosomes one inherited from each parent [41]. Typically, X-linked genes in females undergo a process of X inactivation that silences genes on one of the X chromosomes in females as they have two X chromosomes in order to balance the dosage of gene expressions between males and females [84]. The random process of X inactivation is controlled by the X-linked non-coding Xist RNA, which coats one of the X chromosome making it inactive whereas the other X remains active [85]. However, studies show that approximately 15% of X-linked genes escape the process of X inactivation in humans, and approximately 3% in mice [86–88]. The X chromosome contains several immune-related genes including pattern recognition receptors (e.g. TLR7and TLR8), co-stimulatory molecules (e.g. CD40 ligand (CD40L)), cytokine receptors (e.g. IL2RG and IL13RA2) and transcriptional factors (e.g. Foxp3) [52, 89]. Genes uniquely encoded on the X chromosome that escape the process of X inactivation can become highly expressed in females which can lead to a heightened immune response in inflammatory conditions and result in a sex-biased immune phenotype [52, 59]. Females display higher expression of TLR7 in response to viral antigens compared to males, with evidence of TLR7 escape X inactivation resulting in more robust immune response to viral infections [56, 90, 91]. Studies in both mice and humans demonstrate higher IFNα production in pDCs derived from females in response to HIV-1-encoded TLR7 ligands compared to males, resulting in stronger secondary activation of CD8+ T cells in females [60, 61]. Similarly, female mice exhibit higher expression of TLR7 in B cells which is correlated with stronger antiviral antibody responses following influenza vaccination compared to male mice [92]. Additionally, the dosage of TLR7 is a vital pathogenic factor in autoimmune disease SLE, which has a strong female bias [56, 60]. Notably, X-inactivation provides females with protection from X-linked gene mutations associated with conditions such as X-linked severe combined immunodeficiency (XSCID) and IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which are more prevalent in males compared to females [93, 94].

In males, all immune-cell lineages harbour the X-linked gene mutations. However, in females the X-inactivation offers restriction in the expression of these mutations and thus reduces the extent of manifestation in immune deficiencies. The X chromosome encodes a predominant number of microRNAs (miRNAs) that are involved in immunity. Approximately 10% of all microRNAs detected in the human genome are encoded on the X chromosome [95]. While, the Y chromosome only encodes for two known miRNAs [95]. miRNAs are non-coding RNAs that regulate gene expression and plays a key role in immune homeostasis, and there is evidence that X-linked miRNAs can contribute to sex-differences in immunity [52, 89, 95]. Dysregulation of miRNA expression, especially those located on the X chromosome, are associated with immune-related disorders in the development of certain cancers and autoimmune diseases, thus contributing to sex-specific bias in many immune-mediated diseases [52, 89, 96]. The critical challenge in interpreting sex-related differences in immune responses is to distinguish between what is due to the expression of X-linked genes as opposed to influence of sex hormones.

The "four core genotypes" (FCG) mouse model was established to distinguish between sex chromosome effects and hormonal effects in animal model studies [97–99]. In this model, the mice sex chromosome complement (XX vs. XY) is not linked to the mice's gonadal sex (testes or ovaries) [88, 99]. Studies that performed gonadectomy of FCG mice to deplete gonadal steroids showed that sex chromosome complement impacts susceptibility to viral infection and autoimmune disorders [100, 101]. In experimental autoimmune encephalitis (EAE) and lupus mouse models, XX mice have worse disease progression and severity with increased IL-13Rα2 expression, and decreased Th2 cytokines (IL-4, IL-5, IL-13) that are associated with protection from disease in EAE, compared to XY mice [100]. In gonadectomized FCG mice infected with coxsackievirus B3 (CVB3), XY mice show decreased susceptibility to infection that correlated with increased FoxP3+ Treg cell activation compared to XX mice [101]. However, the question remains how much of the results obtained from the FCG model can be translated to humans.

Some studies have investigated inherited disorders in humans that have more or fewer than two sex chromosomes such as Klinefelter and Turner syndromes that illustrate the effects of the X chromosome. Klinefelter syndrome occurs in patients that are phenotypically males but have an extra X chromosome (XXY) which results in modestly lower testosterone levels compared to males (XY), but significantly higher than females (XX).

These patients have increased oestrogen levels and elevated gonadotrophins [58, 102]. Patients with Klinefelter syndrome display a blood cytokine profile similar to that of females in response to LPS and other TLR ligands [69, 102]. These results suggest that the X chromosome contributes to sex-related differences in cytokine secretion independent of sex hormone levels. Interestingly, females (XX) exhibit higher levels of pro-inflammatory cytokines IL-1β and TNF-α expression in response to TLR4 stimulation compared to Klinefelter patients [69]. Additionally, transcriptional analysis of neutrophil maturation genes and ISGs in Klinefelter syndrome patients demonstrated that differences in neutrophil phenotype between adult female and male neutrophils are hormonally driven independent of X chromosome gene dosage effects [103]. In contrast, patients with Turner's syndrome who are phenotypically female but with only one X chromosome (X0) have reduced lymphocyte counts and lower antibody production compared to females with XX chromosomes [90]. These studies have clearly demonstrated that sex-related differences in immune response in part can be driven by chromosomal make up and independent of hormones. However, hormones or sex steroids have a profound impact on immune functions.

1.2.2.2.2 Impact of sex steroids on immune response

Sex dimorphism on immune responses is also attributed to sex steroids or hormone actions and associated receptors expressed on immune cells [83]. Equally, the shift in sex steroid levels upon puberty, aging, menopause and pregnancy, are associated with changes in immune responses and susceptibility to inflammatory disorders [49]. Sex steroids (hormones) are produced in the gonads, adrenal cortex and in peripheral tissues such as kidney, liver and fat [104, 105]. Sex steroids are present *in utero* as both female and male fetuses are exposed to maternal estrogens and the testes in developing male fetuses synthesize testosterone [105–107]. However, there are limited studies that have examined sex steroid synthesis and levels in different tissues and organs, including the lungs [105, 108].

Estrogen derivatives that regulate the estrous, menstrual and reproductive cycles include estrogen (E1), estradiol (E2 or 17β-estradiol) and estriol (E3) [83, 104]. Estradiol is the most potent form of sex hormone present in adult females and males, but in females it varies during the menstrual cycle, whereas E3 is produced at high levels during pregnancy [83, 104].

Progesterone (P4) is present in both sexes, in females the level of P4 varies with fluctuations in the menstrual cycle and it is also produced at high levels during pregnancy [109]. Testosterone is produced in both males and females, but is synthesized at high levels in males and is converted to the physiologically active metabolite dihydrotestosterone (DHT) by 5α-reductase or to estradiol by aromatase [49, 105].

Sex steroids can modulate and influence cellular processes through both genomic and non-genomic regulation [110]. The sex steroid effects are mediated through binding to respective nuclear receptors including androgen receptors (AR), estrogen receptor alpha (ERα), estrogen receptor beta (ERβ) and progesterone receptor (PR-A and PR-B) [105, 111, 112]. The binding of sex steroids to respective receptors and subsequent dimerization results in sex steroid receptors to translocate into the nucleus and bind to the estrogen responsive elements (ERE) to directly regulate specific target genes [111, 113]. EREs and androgen response elements are located in the promoters of multiple innate immunity genes, indicating that sex steroids can directly influence immune responses [77, 114]. Rapid "non-genomic" sex steroid signaling can be mediated through G protein-coupled receptor 30 (GPR30) and through ER or AR localized in the inner plasma membrane [105, 115]. Sex steroid receptors are expressed on immune cells with varying degrees as well as on non-immune cells such as epithelial cells, endothelial cells and neuronal cells [104, 111, 116–119]. ERα has been identified to be expressed on macrophages, monocytes, DCs, NK cells, mast cells, T cells, and B cells [120, 121]. CD4+ T cells have higher expression of ERα compared to ERβ, whereas monocytes and CD8+ T cells have low expression of both ERα and ERβ [122]. In contrast, B cells express more Erβ compared to Erα [123].

AR is expressed on multiple immune cells including macrophages, neutrophils, T cells and B cells [117]. Generally, androgens such as testosterone have been reported to induce a suppressive effect on immune functions including cytokine production [113]. Testosterone treatment of primary cultured macrophages and macrophage cell-lines show significant reduction in TLR4 expression [114]. Consistent with this, macrophages isolated from orchiectomized mice showed significantly higher TLR4 expression compared to macrophages isolated from sham gonadectomized mice [114]. Treatment of mouse macrophage cell lines (IC-21 and RAW 264.7) with testosterone results in downregulation of LPS-induced activation of p38 MAPK and nitric oxide synthase production [116].

These studies suggest that testosterone down regulates classical pro-inflammatory responses or immune activation via TLR signaling. In contrast, *in vitro* treatment of CD4+ T lymphocytes with DHT results in enhanced IL-10 production, indicating that testosterone and DHT increase the synthesis of anti-inflammatory cytokines [124]. Consistent with this, studies have demonstrated that splenocytes isolated from DHT-treated female mice have higher production of IL-10 compared to splenocytes isolated from placebo-treated female mice [124]. Male patients that suffer from androgen deficiencies including hypogonadal disorders and Klinefelter's syndrome, exhibit increased levels of inflammatory cytokines (including TNF-α, IL-1β, IL-4 and IL-2), CD4+/CD8+ T cell ratios and serum antibody levels (IgG, IgA and IgM), compared to healthy male subjects [102, 125]. Moreover, androgen replacement treatment (ART) in these patients decreases these aforementioned parameters [102]. These studies demonstrated that reduced testosterone levels lead to enhanced cellular and humoral immunity.

P4 is generally reported to promote an anti-inflammatory state in various tissues and cell culture systems [49, 83]. PR is expressed on multiple immune cells (e.g. DC, macrophages, NK cells, and T cells) as well as non-immune cells (e.g. epithelial cells, endothelial cells and neuronal cells) [49, 83, 109, 126]. Interestingly, P4 does not only act via binding to PR but it can also bind to glucocorticoid receptor (GR), which presents an alternative mechanism for P4-mediated influence on immune responses [127]. P4 can suppress innate immune responses including macrophages, DCs and NK cell activity [127–133]. In rodent bone-marrow derived DCs (BMDCs), *in vitro* treatment with P4 results in the downregulation of TLR3 and TLR3 via GR as well as reduced production of TNF-α, IL-1β, IL-6 and IL-12p40 [127, 129, 130]. Thus, P4 can suppress TLR-induced cytokine production through PR and GR on DCs. Additionally, PR expression is higher in DCs isolated from female rats compared to males, which may explain the reduced cytokine production (e.g. TNF-α, IL-1β, IL-6, IL-10 and IL-12p40) in DCs from female rats compared to males [127, 129, 130]. Moreover, the binding of P4 to PR can directly influence the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) resulting in suppression of gene transcription downstream of the NF-κB pathway to reduce inflammatory responses [128, 133]. LPS-stimulated macrophages in the presence of P4 results in increased expression of SOCS1 (suppressor of cytokine signaling), along with reduced inducible nitric oxide synthase (iNOS) and TLR4 expression, and decreased NF-κB activation [132].

NK cells isolated from PBMCs are susceptible to P4-induced cell death, that can be prevented with P4 antagonist treatment [131]. Murine or human T cells treated with P4 induces a Th2 environment and skew naive T cells away from Th1 type response, with increased secretion of IL-4, IL-5, and IL-10 [134–136]. In T-cell lines, supplementing culture media with P4 promotes a Th2 environment with enhanced levels of IL-4 and IL-5 secretion [137]. Similarly, treatment of human DCs with P4 induces Th2 responses and increased secretions of IL-27, IL-10 and IL-13 [134]. Murine T cells cultured with addition of P4 also display bias towards Th2 environment with increased secretion of IL-4 and reduced IFN-γ production [136]. P4 can reduce susceptibility to infections at diverse mucosal sites [109, 138]. For example, administration of P4 to progesterone-depleted adult female mice has been shown to protect in an influenza A virus (IAV) infection model with improved lung function [138]. Additionally, P4 induces the proliferation of pulmonary cells, including epithelial cells, and promotes lung tissue repair through upregulation of epidermal growth factor amphiregulin (AREG) in the lungs [138].

Estradiol can influence the function of APCs, including DC and macrophages [139–141]. For example, CD11c+ DCs isolated from mice treated with 17β-Estradiol show higher production of IFN-γ in response to IL-12 and IL-18 [140, 141]. Additionally, *in vitro* studies demonstrate that estrogen preferentially promotes the differentiation of bone marrow (BM) progenitor cells into functional CD11c+ DCs [140]. Consistent with these findings, pDCs isolated from estrogen receptor alpha knockout (ERα−/−) lupus prone female mice show a decreased production of IL-6 following stimulation with TLR9 ligands [142]. Also, LPS-activated macrophages isolated from ovariectomized female mice treated with 17β-Estradiol exhibit significant increase in the production of both iNOS and pro-inflammatory cytokines including IL-1 β, IL-6, and TNF-α [118, 143, 144]. Estrogen can modulate immune responses by regulating inflammatory mediators including cytokines and chemokines [83, 112]. Estrogen treatment of orchiectomized male mice show significant enhancement of LPS-induced IFNγ, nitric oxide and iNOS production in splenic lymphocytes from estrogen-treated male mice compared to placebo- treated male mice [145]. Additionally, estrogen treatment enhanced the production of chemokines such as MCP-1, MCP-5 and eotaxin [145, 146]. Further, transcriptional analysis demonstrated that estrogen could induce a selective up-regulation/down-regulation of miRNA expression in splenic lymphocytes which mediate LPS-induced IFNγ and iNOS production [145].

Moreover, 17β-Estradiol enhances the cytotoxicity of NK cells and NKT cells, as well as increased production of IFN-γ by NK cells [141, 147]. In contrast, there are several reports that describe a suppressive action of estrogen to pro-inflammatory responses, including reduced production of IL-6 by macrophages [111, 148–150]. ERα-mediated effect of estrogen on monocyte/macrophage cell line (RAW 264.7 cells) show a reduction in LPS-induced pro-inflammatory response and concurrent resolution of inflammation through SOCS and STAT3 signaling pathways [150]. Additionally, treatment of naive mice with17-β-estradiol induces the upregulation of FoxP3 expression in CD4+ CD25− T cells and expansion of Tregs [151]. These recent studies suggest that estrogen functions as an immunomodulatory agent [119, 149], as low dose of estrogen (<0.1 nM) can enhance Th1 responses and production of proinflammatory (such as IL-1, IL-6, and TNF-α), and high (>10 nM) or sustained concentrations of estrogen promotes Th2 responses with reduced pro-inflammatory cytokines and suppressed NF-κB activity [119, 152]. High concentrations of estrogen also attenuate chemokine production and intervenes in the recruitment of leukocytes and monocytes into several tissues [153, 154]. During pregnancy estrogen levels are high and studies demonstrates that this skews immune environment from Th1 to Th2 [155]. However, high concentrations of estrogen can stimulate B cells to produce antibodies [156]. Therefore, findings from *in vivo* and *in vitro* experiments indicate that the effects of estrogens on both cell-mediated and humoral immune responses is dependent on the dose of estrogen administrated, the timing of estrogen treatment, and the cell population examined.

Overall, it is evident that the inclusion of sex and hormonal status in preclinical and clinical studies is vital for better understanding of immunity related functions, its influence on health and disease, and consequently for the development of novel therapeutics specifically for the progression towards personalized medicine.

1.3 Asthma and Allergic Airway Inflammation

1.3.1 General Overview

Asthma is a chronic inflammatory respiratory condition characterized by airway hyperresponsiveness (AHR), bronchoconstriction and airway inflammation, which results in respiratory symptoms such as shortness of breath, coughing and wheezing [157, 158].

In recent decades, asthma prevalence has risen considerably affecting ~339 million people globally [159–161]. In Canada, asthma impacts 3.8 million (10%) people with an estimated economic burden of more than $2 billion annually [162, 163].

Asthma prevalence varies based on sex across the lifespan [164, 165]. During childhood, boys have higher asthma prevalence compared to girls as well as boys are at higher risk of being hospitalized due to asthma exacerbation [165, 166]. However, during puberty there is a shift in asthma prevalence and morbidity from males to females. In adults, there is a striking increase in asthma prevalence and severity in women compared to men that is maintained until around the time of menopause, when a decline in asthma prevalence is noted in post-menopausal women [164, 166, 167]. The sex shift in asthma prevalence can be correlated with changes in sex steroids [167], which suggests an influence of sex steroids on asthma pathogenesis. Notably, studies have reported variations in symptoms of asthma through the menstrual cycle where 30–40% of women reported pre- or peri-menstrual deterioration in symptoms of asthma [168, 169]. On the contrary, other studies have reported no difference in women visiting the emergency department for worsening of asthma symptoms during their menstrual cycle [170, 171]. Therefore, it remains unclear how sex steroids through the menstrual cycle affect asthma pathogenesis in women. Moreover, the cellular and molecular mechanisms associated with the sex shift in asthma prevalence and severity at puberty remains undefined. The sex disparity in asthma underlines the need for further studies to be conducted on investigating sex steroid-gene interactions (particularly X-chromosome genes) at different stages in life.

Asthma is considered a multifactorial disorder but major factors in the etiology of asthma are attributed to complex interactions between genetic susceptibility, environmental factors (aeroallergens, air pollution and climate) and host factors (allergic sensitization, infections, obesity and nutrition) [165].

These multiple factors play a role in driving the heterogeneity in asthma. Thus, it is now widely recognized that asthma is not a single disease but a broad diagnosis describing variable clinical presentations and symptoms (phenotypes), each of which may develop through distinct molecular and cellular mechanistic pathways and pathophysiological mechanisms (endotypes) [172]. It is important to note that so far there is no overall consensus on asthma endotypes and phenotypes [173]. Asthma phenotyping allows for robust clinical evaluation and assessment of comorbid factors directing therapeutic approaches to improve asthma control [127]. While majority of asthmatics are responsive to inhaled beta agonists and corticosteroids, approximately 10-15% of patients are not responsive to corticosteroid therapies and develop uncontrolled severe asthma [174, 175]. These patients are associated with most of the asthma-related economic burden in Canada. Females are more likely to require urgent care for asthma despite more frequent use of inhaled corticosteroids and develop severe asthma compared to males [174, 176]. With the emergence of technologies such as multi-omics approaches (transcriptomics, epigenomics, microbiomics, metabolomics, and proteomics) understanding the variability in asthma is improving which has led to the development of new therapeutic strategies to control asthma in patients who respond to medications with varying efficacy [177]. Defining various inflammatory endotypes is essential to improve therapeutic precision and development of new therapies, especially for those with steroid-unresponsive severe asthma. A broad characterization of asthma inflammatory endotypes are either type 2 (T2) high or T2-low [173]. Further endotyping studies have characterized the inflammatory responses based on cellular responses such as eosinophilic, neutrophilic, mixed-granulocytic (eosinophils and neutrophils both equally elevated) [178]. Allergens such as house dust mite (HDM) contain many immunogens such as bacterial endotoxin, fungal spores, proteases etc that are recognized by different PRRs, activating different innate immune pathways and skewing adaptive immune responses. It is thus likely that the different asthma endotypes are based on the composition of the allergen and subsequent differences in host response.

The T2-high endotype features eosinophilic airway inflammation which involve high levels of serum IgE with increased Th2 cells and cytokines such as IL-4, IL-5, IL-9 and IL-13 in the lungs [172] (Figure 2). T2-high endotypes are associated with phenotypes that are classified into three groups; allergic asthma, late-onset eosinophilic asthma and aspirin-exacerbated respiratory disease (AERD) [172].

Conversely, T2-low endotype features neutrophilic or mixed-granulocytic airway inflammation which involves a mixture of Th1 or Th17 skewed signature which can be associated with poor responsiveness to corticosteroid treatment [172, 179] (Figure 2). T2-low endotypes are associated with phenotypes that are classified into three groups; obesity-associated asthma, neutrophilic asthma and paucigranulocytic asthma [179]. The classification of the various endotypes with inflammatory phenotypes provides a granular approach to delineate asthma immunopathogenesis. However, the immunological processes that drive asthma pathogenesis and heterogeneity are complex and often interconnected. In this thesis, I will be focusing on allergic asthma.

Figure 2

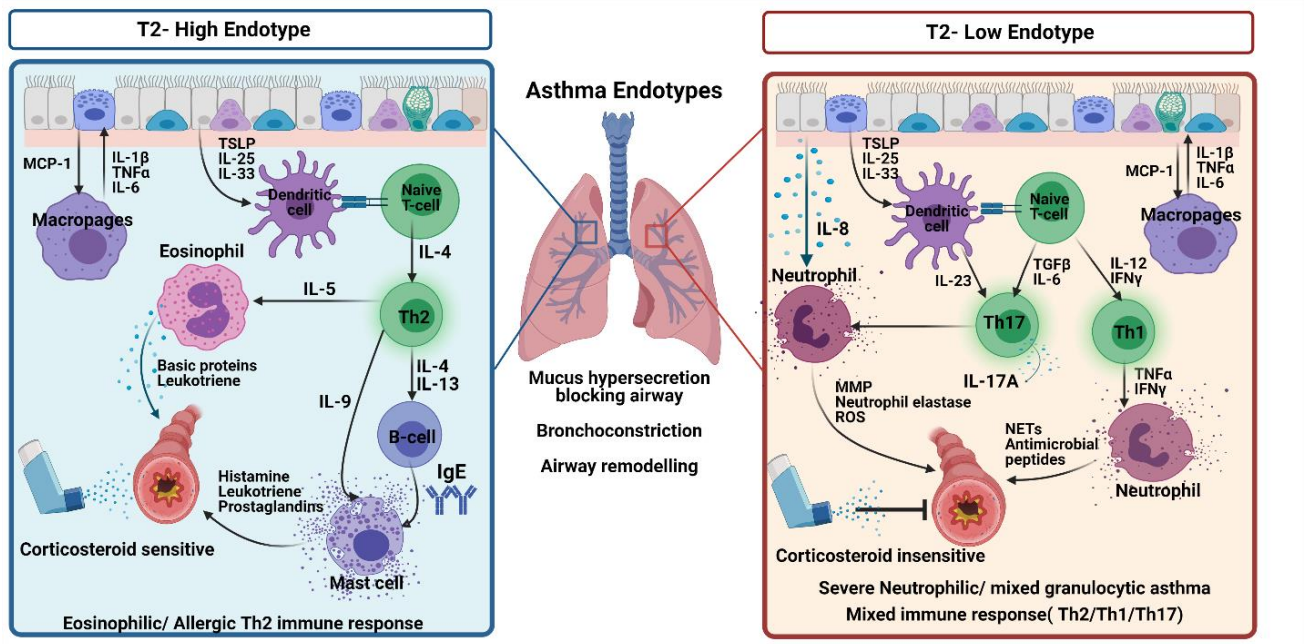


Figure 2: T2-low and high asthma endotype. Left panel demonstrates Th2-high endotype which involves a dominant eosinophilic airway inflammation with characteristic features such as elevated levels of Th2 cells and cytokines including IL-4, IL-5, IL-9 and IL-13. Right panel demonstrate T2-low endotype that involves neutrophilic or mixed-granulocytic airway inflammation that contribute to poor response to corticosteroid treatment. Figure created with BioRender.com.

1.3.2 Allergic Asthma

Allergic asthma is a prominent asthma phenotype with a complex type 2 (T2) inflammatory network that induces a range of mild to severe asthma symptoms [180]. Allergic asthma is induced through exposure and sensitization to environmental allergens such as animal dander, house dust mite (HDM) and pollen [181]. After the sensitization phase, subsequent future re-encounter of the individual to the same allergens drives the clinical symptoms of allergic asthma [181]. Allergic asthma is associated with T2 high eosinophilic airway inflammation which is present in around 50–60% of adults and children with asthma [160]. Several immune cell types are involved in orchestrating the initiation and propagation of the allergic response including bronchial epithelial cells, DCs, eosinophils, Th2 cells, Th9 cells, mast cells and B cells [180]. Details of the immunological responses elicited in allergic asthma are discussed below.

1.3.2.1 Immunobiology of Allergic Asthma

The lung is a central organ that functions for continuous gaseous exchange which constantly makes the lung a target for insults from numerous airborne allergens, pathogens, and a variety of toxicants [182, 183]. The airway epithelium is the first line of defence providing protection from these constant environmental insults or foreign particles in the air we breathe, and thus plays an active role in preserving immune homeostasis in the lung [184]. Consequently, the airway epithelium is considered an immunologically active barrier in the lung that encompasses a wide range of highly specialized cells. These cells respond to stimulation through production of pro-inflammatory cytokines which leads to the recruitment and activation of mucosal innate immune cells. Allergens such as HDM which contain proteases that can disrupt barrier integrity through cleaving epithelial tight junctions (TJs) [184]. The initiation of allergen sensitization involves PRRs detecting allergens and stimulating airway epithelial cells (AECs) to produce pro-inflammatory cytokines, which includes cytokines that are classified as alarmins, resulting in the recruitment and activation of mucosal innate immune cells such as DCs, which then propagate adaptive type 2 immune pathways [184, 185].

DCs are potent APCs that upon encountering allergens in the airways internalize and process allergens into small peptides and present these antigenic peptides on the cell surface via MHC class II, generating MHC-peptide complexes [186]. Additionally, allergen exposed DCs upregulate the expression of OX40 ligand (OX40L) which promote DC migration towards the lung draining lymph nodes [181, 186]. Overall, activated AECs due to allergen exposure secrete 'alarmins' which are epithelial-derived cytokine mediators, primarily IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) [185]. These alarmins can prime pulmonary DCs, induce the expression of OX40L and promote the migration of DC to lymph nodes to activate naïve CD4+ T cells. IL-33 and IL-25 can also activate ILC2s [185]. Activated ILC2s produce IL-5 and IL-13 and thus amplify eosinophilic airway inflammation [185]. The migration of allergen-loaded DCs to the lymph nodes allow them to interact with naïve CD4+ T cells and subsequently drive T-cell activation and differentiation into Th2 cells [187]. Depending on the microenvironment and nature of the antigen, DCs can induce tolerance through Treg cells or drive T cells into distinct T cell subsets (Th1, Th2, Th17) [187]. Allergen-specific activated Th2 cells secrete IL-4, IL-5, IL-9 and IL-13 to mediate inflammatory and remodelling changes in the airway mucosa [160, 188]. IL-5 and IL-9 are major cytokine that promote eosinophilia, especially IL-5 which mediates survival and maturation of eosinophils [189, 190]. IL-13 enhances mucus production by goblet cells and increases AHR [190]. IL-4 is vital for the initial development of Th2-mediated inflammation during primary allergen sensitization, as well as following secondary allergen exposure [188, 191, 192]. IL-4 also drives B-cell class switching and IgE production [193]. Thus activated Th2 cells also regulate B cell activation and stimulate production of antigen-specific IgE antibodies [194].

IgE antibodies can bind to high-affinity receptor, FcεRI, on immune cells particularly basophils and mast cells [186, 193]. The IgE-primed mast cell release inflammatory mediators, such as histamine, prostaglandins, and leukotrienes [193]. IL-5 and IL-9 are major cytokines that promote eosinophilia, especially IL-5 which mediates survival and maturation of eosinophils [189, 190]. Eosinophil accumulation at the bronchial level can lead to epithelial cell damage through degranulation of eosinophils and release of highly charged basic proteins which mediate toxic effects on epithelial cells, thus inducing AHR and bronchial wall remodelling [195, 196].

Additionally, the constant ongoing inflammation, tissue damage and repair can lead to airway remodelling [197]. Moreover, eosinophils are the source of multiple pro-inflammatory cytokines including IL-5, granulocyte–macrophage colony-stimulating factor (GM-CSF) and eotaxin, which further recruit and activate more eosinophils augmenting airway inflammation [195]. IL-13 enhances mucus production by goblet cells and increases AHR [190].

Overall, the inflammatory cascade triggered by allergens in allergic asthma leads to increased mucus production, eosinophilia, bronchoconstriction, smooth-muscle contraction, and eventually the resulting chronic inflammation with persistent lung tissue damage and repair mechanisms which drive lung remodeling or fibrosis, overall making it difficult to breathe [181, 191].

Figure 3

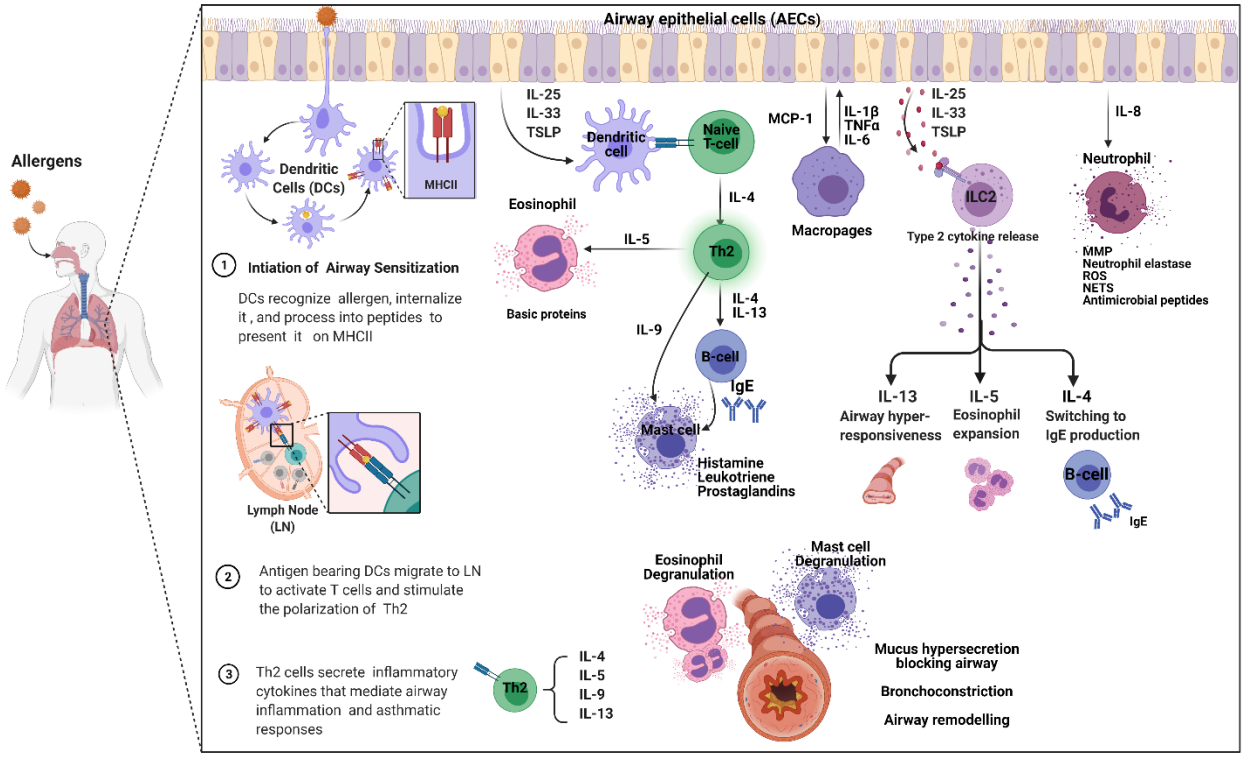


Figure 3: Immunobiology of allergic asthma. Left panel illustrates allergen sensitization process initiated by various mechanisms. (1) Allergen internalization by DCs and antigen processing to display antigenic peptides with MHC II on cell surface. (2) Antigen loaded DCs migrate to lymph nodes and present antigenic peptides to naïve T cells, to induce T-cell activation and polarization into a distinct Th2 cell phenotype. (3) Allergen-activated Th2 cells engage with B cells inducing the activation and proliferation of allergen-specific B cells. Th2 cells secrete IL-4 and IL-13, promoting B cells to undergo class-switching to IgE. Allergen specific IgE binds to high-affinity receptors on mast cell surface resulting in degranulation of mast cells and release of inflammatory mediators such as histamine. Moreover, airway epithelial cells (AEC) produce alarmin cytokines (IL-33, IL-25 and TSLP) that activate and promote various downstream mechanisms associated with inflammation and airway hyperresponsiveness. Adapted from "Allergic Airway Sensitization", and created by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

1.3.2.2 Pulmonary Leukocytes

The lung harbors innate and adaptive immune cells to generate a potent immune response which is vital for the resolution of invading pathogens and to limit inflammation-induced damage, processes that are critical to maintain lung homeostasis [182, 183]. During homeostatic conditions, macrophages are the most abundant immune cells in the lung that regulate the innate immune defence of the airways [198]. Macrophages are effector cells of the innate immune system that populate tissues of various organs with the capacity to function as APCs, phagocytosis and secretion of both pro-inflammatory and antimicrobial mediators [182, 199]. Thus, macrophages play a key role in eliminating pathogens and maintaining homeostasis [200, 201]. The lung is populated with two distinct types of macrophages, the interstitial macrophages which reside in the parenchyma and alveolar macrophages which are present near epithelial cells of the alveoli [201]. The phenotype of alveolar macrophages is shaped by the unique lung microenvironment of high oxygen tension and exposure to surfactant-rich fluid [200, 202]. Both these two tissue-resident macrophage populations are long lived and generally function to maintain tissue homeostasis and contribute to host defense mechanisms such as pathogen recognition, and initiation and resolution of lung inflammation as required [201]. Following inflammatory insults, alveolar macrophages initiate innate immune responses in the lung, and bone marrow-derived monocytes are recruited to the lung that further differentiate into alveolar macrophages to enhance the numbers [202, 203].

Depending on the inflammatory microenvironment, alveolar macrophages can alter their phenotypes and have been classified into classically activated macrophages (M1 macrophage) and alternatively activated macrophages (M2 macrophage) [203, 204]. M1 macrophages can be stimulated through microbial factors which results in the release of various pro-inflammatory cytokines including IFNγ, TNFα, IL-1, IL-6, IL-8, and Leukotriene B4 (LTB4) [203, 204]. This leads to exacerbation of inflammation to allow for pathogen clearance, as well as the production of nitric oxide (NO) which enhances killing of the pathogens [203, 204]. In comparison, M2 macrophages are associated with resolution of inflammation through release of anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β), efficient clearance of apoptotic cells by phagocytosis and repair of damaged tissues [203, 204].

Therefore, macrophages can contribute to airway inflammation through production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα [205, 206]. Macrophages are implicated in many endotypes of asthma including allergic asthma, neutrophilic and glucocorticoid-resistant asthma [205, 206].

Eosinophils are a major cell type recruited to the lungs in allergic asthma, promoting eosinophilic airway inflammation. Eosinophils are granulocytes that develop in the bone marrow and account for more than 5% of the circulating leukocytes [207]. Eosinophils have a lifespan of approximately 8-18 hours in the bloodstream and mostly are located in tissues and persist for at least a week [208]. Cytokines that are important for development of eosinophils include granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-3 and IL-5 [207]. The IL-5 and eotaxin chemokine family are major chemoattractants of eosinophils [207].

Eosinophils develop in the bone marrow and have granules that comprise of different components such as eosinophil peroxidase (EPX), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and major basic protein (MBP) as well as numerous cytokines, chemokines, and growth factors [208, 209]. A major function of eosinophils is to undergo degranulation of cytotoxic granule proteins that disrupt the cell membrane and are highly cytotoxic to parasitic, bacteria and infected mammalian cells [208, 209]. Eosinophils can impact the adaptive immune response by binding antibodies and inducing antibody-dependent cellular cytotoxicity (ADCC) to bacteria and parasites and infected cell targets [208, 209]. Even though the strong cytotoxic properties of eosinophils are crucial for fighting infections particularly parasitic helminths infections, it can lead to tissue damage and destruction which is associated with multiple pathologies [208, 209]. The infiltration of eosinophils into the bronchial mucosa that occurs in allergic asthma induces epithelial cell damage, airway wall remodelling and AHR, via the release of multiple eosinophilic granules [196, 197, 210]. For example, *in vitro* studies demonstrate that eosinophil-derived granules such as MBP and EPX exert toxic effects on pulmonary epithelial cells and cause lung tissue damage [195, 211]. The constant repair processes as a consequence of repeated lung tissue damage can lead to increased airway smooth muscle (ASM), deposition of extracellular matrix (ECM) proteins and goblet-cell hyperplasia, thereby causing structural changes in the lung [197, 212].

Eosinophils are implicated in airway remodelling through promoting increased ASM proliferation, goblet cell differentiation and mucus production [197, 212, 213]. Moreover, eosinophil degranulation promotes AHR worsening through MBP action that reduces neuronal M2 muscarinic receptor function on parasympathetic nerves in the lungs triggering constriction of the airways [195, 210, 211]. Also, MBP can stimulate basophils and mast cells to secrete histamine, which is a key mediator of AHR [195]. Additionally, eosinophils produce a number of pro-inflammatory cytokines including IL-13, which increases AHR and contributes to mucus hypersecretion by enhancing goblet cell differentiation [195, 196, 212]. Eosinophils also produce TGF-β which stimulates proliferation of fibroblasts and enhanced collagen production, thereby inducing airway remodelling [197, 212, 213].

Neutrophils are another key immune cell that are trafficked to the lung in response to an inflammatory insult or infection [182, 183]. Neutrophils are multifunctional granulocytes that are highly abundant in the circulation and represent 40% to 70% of leukocytes [214]. Neutrophils tend to have a short life span of approximately 7–10 hours [215]. However, activated neutrophils due to inflammatory stimulus can survive up to 48 hours and are programmed to undergo apoptosis [215]. Neutrophils destined for apoptosis are recognized and phagocytosed by alveolar macrophages which helps to resolve inflammation in the lung [204]. Upon antigenic stimulation, neutrophils can migrate rapidly to the site of inflammation or infection and have the capacity to phagocytose pathogens, undergo degranulation and release neutrophil extracellular traps (NETs) to trap and kill invading pathogens [216]. Additionally, activation of neutrophils can induce severe tissue damage attributed to secretion of toxic factors including cationic antimicrobial peptides, proteases and reactive oxygen species (ROS) [216]. Therefore, it is critical for acute inflammation to be resolved to not lead to chronic inflammation as the influx of neutrophils can results in severe tissue damage.

In allergic asthma neutrophil degranulation leads to the direct release of cytotoxic granule components including neutrophil elastase (NE), matrix metalloproteinase 9 (MMP-9), ROS, myeloperoxidase (MPO) and NETs, which are highly damaging to the airway mucosa and contribute to AHR and lung remodeling which impact ASM contraction [217–220]. For example, NE can induce mucus hypersecretion by promoting mucus cell metaplasia and airway mucus gland hyperplasia [221].

MMP-9 can stimulate lung dendritic cell maturation and recruitment which mediate allergen sensitization and induces Th2 responses, thus facilitating key asthma hallmarks [220, 222]. Additionally, MMP-9 can directly degrade ECM proteins thus causing structural changes in the airway walls [219, 220, 222]. MMP-9 has also been implicated in promoting the infiltration of inflammatory cells into the airway lumen (eosinophils, neutrophils, basophils, mast cells, and macrophages), thus augmenting the inflammatory cascade in the lung and the subsequent induction of AHR [217–220, 222]. As previously discussed in section 1.3.1, asthma patients that predominantly have neutrophilic inflammation tend to display resistance to inhaled corticosteroids therapy to asthma [172, 178, 179], which demonstrate a fundamental role of neutrophils in severe asthma. Overall, pulmonary leukocytes play a crucial role in maintaining lung homeostasis and fighting invading pathogens. However, in allergic asthma they amplify the inflammatory response and secrete toxic inflammatory mediators that contribute to asthma pathogenesis.

1.3.3 Factors Contributing to Sex Differences in Allergic Asthma

1.3.3.1 General Overview

Sex-based differences are evident in pathophysiology, incidence, morbidity, and mortality of various respiratory diseases, including asthma, over the course of a human life span [223, 224]. In children and adults, some respiratory disorders can occur disproportionately in males and females with distinct degrees of diseases severity and symptoms in each sex. In general, clinical and epidemiological studies showed a female predominance in most chronic respiratory disorders with a higher degree of exacerbation rate, severity hospitalizations and mortality in females compared to males [224–226]. In adulthood, males are at a higher risk of developing idiopathic pulmonary fibrosis (IPF) and emphysema, whereas adult females are more likely to suffer from asthma, chronic obstructive pulmonary disease (COPD), chronic bronchitis, cystic fibrosis, pulmonary arterial hypertension and pulmonary complications associated with influenza infections [223, 224]. The current COVID-19 pandemic has also highlighted sex disparities in morbidity and mortality of patients infected with the SARS-CoV-2 Virus [227]. In general, global data indicate that male patients have a higher case fatality rate for COVID-19 than females [227, 228].

However, in some countries such as India, females have a higher COVID-19 case fatality rate than males, which could be related to access to health services [229]. The pandemic has also shed some light on our limited understanding of individual factors that interact with sex to influence lung susceptibility and resilience, particularly factors such as age, ethnicity, actions of sex steroids, immunological and behavioural risk factors [72, 230].

Sex-based differences in asthma prevalence and severity is well-established globally and changes throughout life [167, 223, 224, 231]. During childhood, boys have increased prevalence and hospitalization rates for asthma exacerbation compared to girls [231]. However, at the onset of puberty there is a decline in asthma prevalence and morbidity in males. By adulthood, females predominantly have increased asthma prevalence and severity compared to males [167]. Additionally, females are at a higher risk of developing unresponsiveness to available corticosteroid therapies [5, 232]. This rise in asthma prevalence in adult females compared to males continues until around the time of menopause, when there is a noted decline in asthma prevalence [167, 233]. It is noteworthy, that the switch in asthma prevalence based on sex at the onset puberty is concurrent with fluctuations in sex steroid levels and implies a role of sex steroids in influencing asthma pathogenesis. Other factors that have been proposed to drive sex bias in asthma include environmental exposure and lung physiology. This section will outline sex dimorphism in lung physiology and the role of sex steroids and its association with driving sex bias in asthma.

1.3.3.2 Sex Dimorphism in Lung Physiology

There are prominent intrinsic differences between males and females in the anatomy and physiology of the respiratory system which can influence sex disparity in various lung disorders such as respiratory distress syndrome [224, 234, 235]. The anatomical differences between males and females in the lung are observed at the 16th week of gestation wherein the female fetuses' lungs mature and develop more rapidly than those of male fetuses [234]. From the 26th to 36th weeks of gestation, female fetuses exhibit lower specific airway resistance than males. In addition, female fetuses display a more mature phospholipid profile that reflects earlier production of surfactant components compared to males [234, 236].

Pulmonary surfactant is composed primarily of lipids (~80-90%) and proteins (~20-10%) that are vital in reducing alveolar surface tension at the air/liquid interface within the alveoli of the lung , thus preventing alveolar and airway collapse and allowing for cyclic ventilation of the lungs [237, 238]. Earlier production of pulmonary surfactant components in female fetuses improves the patency of small airways and consequently enhances airflow rates [234]. It remains unclear how environmental exposures impacts sex differences in prenatal lung development. Oestradiol is mainly secreted by the placenta, whereas testosterone is also produced by the fetal testes [239, 240]. Notably, sex steroids are implicated in exerting modulatory effects on lung development before and during the neonatal period [239, 240]. This is supported by the observation that sex steroid receptors (AR, Erα, Erβ and PR) are expressed in epithelial cells and mesenchymal cells of the lung [239]. During lung maturation, estrogens generally have been shown to have a stimulatory effect whereas androgens have an inhibitory effect [239–241].

Estrogens have stimulatory effects on both alveologenesis and the production of pulmonary surfactant during neonatal and pubertal periods [239–241]. While androgens delay pulmonary surfactant production in male fetuses through altering epidermal growth factor and TGF-β signaling events [239]. Consequently, premature male neonates are at a higher risk of developing respiratory distress syndrome than females [240]. While lung maturation and development are more rapid in female fetuses than in males, females have smaller lungs and fewer respiratory bronchioles at birth compared to males [234]. Across the human life span female lung volume is on average 10–12% smaller than that of males with similar height and age [240, 242]. Additionally, the length of the diaphragm at different lung volumes (functional residual capacity and residual volume) is significantly shorter by approximately 9% in females compared to that in males. [242]. The total number of alveoli and the alveolar surface area are also larger for males compared to females [240].

These intrinsic sex dimorphisms in the lung and airway development both *in utero* and during maturation into adulthood can account for sex differences in asthma prevalence and pathophysiology [224, 234, 235]. In early childhood, boys with asthma have increased incidence of wheezing compared to girls, which may be due to the decreased airway size (smaller airway diameters relative to lung volumes) contributing to the increased incidence of wheezing in young males [243, 244]. Moreover, boys have increased atopy particularly between the age of 1 to 8 years

old compared to girls, which suggests a greater allergen sensitization in males compared to females pre-puberty [243, 244]. The sex-related differences in atopy at early childhood are also accompanied by differences between the immune profiles in females and males pre-puberty [244]. As such, boys with asthma have higher level of peripheral eosinophil counts, total IgE levels, IL-5, and IL-13 responses compared to girls [244]. Airway hyperresponsiveness to methacholine is a fundamental feature of asthma with sex-disparity in sensitivity to inhaled methacholine stimulation being reported [166, 245]. Generally, there is a greater sensitivity to inhaled methacholine in adult females compared to males [245, 246]. This sex difference has been attributed to disparity in lung and airway sizes [247]. Thus, the differences in pulmonary anatomy between males and females can influence lung function and contribute to sex-differences observed in asthma. Importantly as explained above, these differences are mediated by sex hormones.

1.3.3.3 Sex Steroids

The reported male predominance of asthma before puberty and the shift following puberty to female predominance suggests a role of sex steroids in influencing asthma pathogenesis [167]. As described above, sex steroids play a role in lung development before and during the neonatal period [239, 240]. Additionally, sex steroid receptors (AR, Erα, Erβ and PR) are expressed in epithelial cells and mesenchymal cells of the lung, further emphasizing that sex steroids are important physiological modulators of lung development and pathophysiology as detailed above [239, 240]. Consequently, there are several studies demonstrating a role for sex steroids in modulating lung function and AHR in asthma [239]. Sex-specific differences exist in airway responsiveness to methacholine stimulation, wherein male C57BL/6 mice exhibit higher responsiveness to methacholine compared to female mice [245, 246, 248]. This sex disparity in sensitivity to methacholine has been attributed to the actions of testosterone on vagus nerve-mediated reflex pathways [248]. This indicates that androgens can influence airway smooth muscle contraction thus influencing lung function in health and disease. ERα gene polymorphisms are associated with lung function decline in female asthma patients [249]. ERα knockout C57BL/6J mice exhibit significantly increased airway responsiveness to inhaled methacholine compared to wild type mice in the absence of immunologic stimulation [239].

Previous studies have demonstrated that ERα and ERβ are expressed on ASM in both males and females, and that they play a role in regulating intracellular [Ca2+] and ASM contractility [250, 251]. ER decreases intracellular calcium [Ca2+], thereby inducing relaxation of ASM [251]. In asthmatic ASM, ERβ expression is significantly enhanced and the activation of ERβ using pharmacological agonist results in decreased ASM proliferation *in vitro* [250]. Mast cell degranulation have a profound role in asthma pathogenesis through impacting ASM function [252]. Mast cell line (RBL-2H3) exposed to E2 and allergen induce mast cell activation and degranulation [253], suggesting that E2 increases allergen-induced mast cell degranulation. ERβ knockout C57BL/6J mice intranasally challenged with mixed allergens showed increased recruitment of inflammatory cells and collagen deposition compared to ERα knockout and wildtype mice [254]. These studies suggest an involvement of ERs and oestrogens in lung remodeling and function.

Periodic worsening of asthma severity in adult females through the menstrual cycle is also reported [167, 255–258]. Approximately 30–40% of female asthma patients report worsening of asthma symptoms, increased oral corticosteroid usage and emergency department visits during the premenstrual or menstrual phases of the menstrual cycle [167, 255–258]. The aggravation in asthma symptoms can be attributed to hormonal fluctuations during the menstrual cycle which in turn changes the immune environment and play a significant role in the pathophysiology of asthma [167]. Premenstrual asthma occurs in the late luteal phase of the menstrual cycle and it is associated with poorly controlled asthma [257]. During the beginning of the luteal phase there is a peak in progesterone which diminishes at the end of the phase, progesterone plays a role in smooth muscle relaxation thus it is suggested that worsening of asthma symptoms may be caused by the sudden decline in plasma progesterone levels [256, 259]. While another study demonstrated that in the luteal phase of the menstrual cycle the levels of testosterone in both sputum and serum are significantly increased which enhances bronchial reactivity [258]. However, the luteal phase is characterized by a Th2-biased immune environment which also may account for exacerbations of asthma symptoms [260]. In contrast, other studies have found no differences in the phase of the menstrual cycle of asthma symptoms worsening or requiring emergency department visits in multiple phenotypes of asthma [170, 171]. Thus, it is unclear whether the menstrual cycle of females alters asthma symptoms, and whether these are due to the differences in sex steroid levels.

There are some studies that have tracked asthma symptoms in females taking hormonal oral contraceptives to examine the influence of sex steroids on airway inflammation [167, 261, 262]. Females taking hormonal oral contraceptives show increased asthma risk compared to females not on oral contraceptives [261–263]. Some studies have reported that female asthma patients taking oral contraceptives experience increased wheezing [262, 263]. Conversely, other studies report a decrease in asthma symptoms and wheezing in females taking oral contraceptives suggesting that oral contraceptives had a protective effect [264, 265]. Whereas, another longitudinal study reported no differences in asthma symptoms in females taking oral contraceptives compared to females not taking oral contraceptives [266]. These contradictory findings from various studies can be attributed to different forms of birth control medications used and small sample sizes employed in some. Overall, it remains unclear whether the observed increase in asthma symptoms is a hormonal effect mediated by sex steroids or due to other confounding factors such as differential body mass index, smoking and respiratory infection.

Chapter 2: Thesis Overview

2.1 Study Rationale

Biological sex is becoming increasingly recognized as a critical variable in influencing prevalence, severity and response to therapy in respiratory diseases such as asthma [224, 239, 267, 268]. Asthma is a chronic inflammatory disease that causes narrowing of the airways, leading to critical symptoms such as coughing, wheezing and shortness of breath [181, 188, 191, 269, 270]. Approximately 3 million Canadians suffer from asthma with disease prevalence and severity being more prominent in boys during childhood, but this trend shifts around puberty, when asthma prevalence and severity rises in females in adulthood [162, 163, 181, 191, 269, 270]. Often, females are more likely to have uncontrolled asthma and corticosteroid-unresponsiveness disease with primarily Th17-driven neutrophilic inflammation, making it considerably more difficult to treat [163, 166, 271, 272]. This sex bias has been shown to impact the inflammatory processes in the lung, which may drive sex-related disparities in the prevalence and severity of airway inflammation [167, 273, 274]. Even though sex-related differences are well established in asthma, sex as a biological variable is largely ignored in most preclinical murine model studies. Thus, there is an increasing recognition of the need to include both males and females in preclinical studies to understand molecular factors that contribute to sex disparities in disease processes and treatments. This emphasizes the need to disaggregate and analyze data by sex in preclinical research, particularly in the context of a disease such as asthma where a sex disparity is prominent. Moreover, the inflammatory profile in the lung in response to allergen exposure differs considerably based on the genetic background of the mouse strain used [275]. Thus, one factor that could influence sex-differences in response to allergen is the genetic background of mice. Therefore, in this thesis, I examine sex-based differences in immune response in an allergen-challenged murine model of airway inflammation, in two different strains, BALB/c and C57BL/6NJ mice.

2.2 Overall Hypothesis

Allergen-driven inflammatory responses such as accumulation of leukocytes in the lungs and increase in the abundance of pro-inflammatory cytokines and proteins, will be higher in females compared to males.

2.3 Specific Aims

To examine the hypothesis there were two specific aims;

(1) To characterize sex-related differences in an acute house dust mite (HDM)-challenge murine model of airway inflammation, using both BALB/c and C57BL/6NJ strains of mice.

(2) to define sex-related differences in selected bronchial proteins that are enhanced by allergen exposure, in murine and human bronchoalveolar lavage fluid (BALF).

Chapter 3: Materials & Methods

3.1 Mice

Female and male (7-8 weeks old) BALB/c and C57BL/6NJ mice were obtained from Charles River Laboratories. Note that the widely used C57BL/6J mice strain has a mutation in NLRP12 (Nod-like receptor pyrin domain containing 12) which leads to a defect in neutrophil migration and impaired CXCL1 production by macrophages [276]. The sub-strain C57BL/6NJ does not have this mutation, and therefore we used C57BL/6NJ for this project [276]. Mice were housed in the central animal care facility at the University of Manitoba. Upon arrival, mice were randomly assigned to cages with a maximum of five mice per cage (separated by sex) by the animal care facility staff and acclimatized for one week before the start of the experiment.

3.2 Mouse Model of Allergen-Challenged Airway Inflammation

The mouse model protocol used in this study (summarized in Figure 4). The protocol used and time point for sample collection was previous studies performed in our lab [279]. This study was approved by the University of Manitoba Animal Research Ethics Board (protocol number AC11394 (B2018-038)) and was compliant with the ARRIVE guidelines for experimental design and reporting of data [277]. Female and male mice (N=10 each) were challenged with daily intranasal (i.n.) administrations of 35 μL of 0.7 mg/mL whole house dust mite (HDM) protein extract (Greer Laboratories, Lenoir, NC, USA) in saline, per mouse. The HDM extract used in this study was with low endotoxin level (~800 EU/vial) [278]. HDM instillations were performed once daily for five consecutive days for two weeks. Mice were lightly anesthetized with 5% isoflurane for HDM instillation. All HDM challenge was performed in the morning between 10 am and noon, and the health status of the mice was visually monitored daily for activity levels and grooming. Naïve female and male mice (N=10 each) were used as paired controls. Naïve and HDM-challenged mice were sacrificed 24 hours after the last HDM challenge for sample collection. Sample collection and processing are detailed as follows.

Figure 4

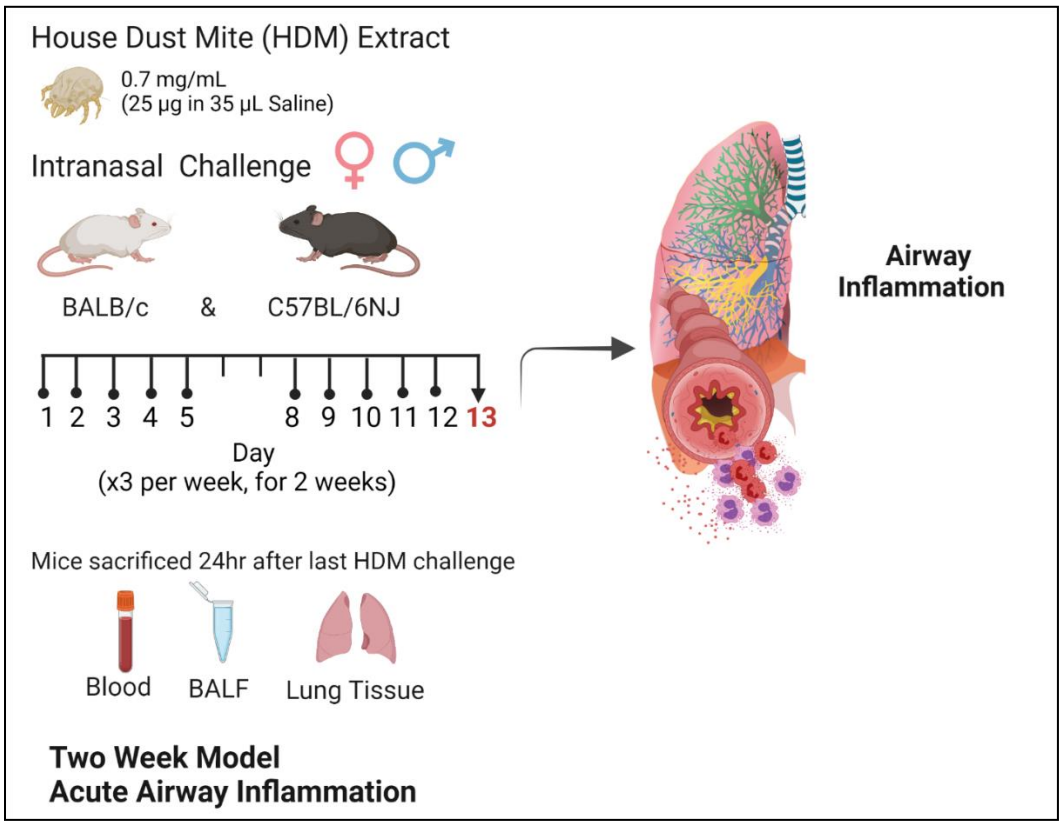


Figure 4: HDM-challenge murine model of airway inflammation. Female and male BALB/c and C57BL/6NJ mice, 7-8 weeks of age were intranasally challenged with 35 µl of whole HDM extract (0.7 mg/mL) in saline for 2 weeks. Outcomes are assessed 24 hr after the last HDM challenge. Figure was created with BioRender.com.

3.3 Bronchoalveolar Lavage Fluid (BALF) Sample Collection

Mice were anesthetized with intraperitoneal (i.p) administration of sodium pentobarbital (90 mg/Kg), the mice trachea was dissected using dissection scissors and a 20-gauge polyethylene catheter was inserted in the mice trachea. A 1 mL syringe filled with saline was connected to the catheter, saline was gently administered into the lung and aspirated to collect the lavage fluid. This procedure was performed two times and the BALF sample was kept on ice. BALF collected was centrifuged at 150 × g for 10 minutes at 4°C and the supernatant was aliquoted and stored at -80°C until used. The cell pellet was used for cell differential assessment.

3.4 Blood Sample Collection

The diaphragm was carefully cut to ensure that the lung is not punctured. Blood was collected through cardiac puncture. . The blood collected was transferred to a 1.7 ml eppendorf tube and left to stand at room temperature (RT) for 30 minutes. The sample was centrifuged at 600× g for 10 minutes at RT. Serum collected, aliquoted and stored at -20°C until used.

3.5 Lung Tissue Sample Collection

Mouse lungs have one large left lobe and four right lobes (Figure 5). The large left lobe was removed using sterile sharp scissors and placed in cryopreservation tube and immediately snap-frozen in liquid nitrogen, and stored at -80°C until used for homogenization to obtain lung tissue homogenates for protein abundance analysis. The bottom right lobe (post-caval lobe) was excised and cut into smaller pieces using a disposable scalpel and transferred into a 1.5 mL eppendorf tube containing 1 mL RNAlater® solution (Invitrogen). The sample was kept at 4°C in RNAlater® for 24 h and stored at -80°C until further analysis.

Figure 5

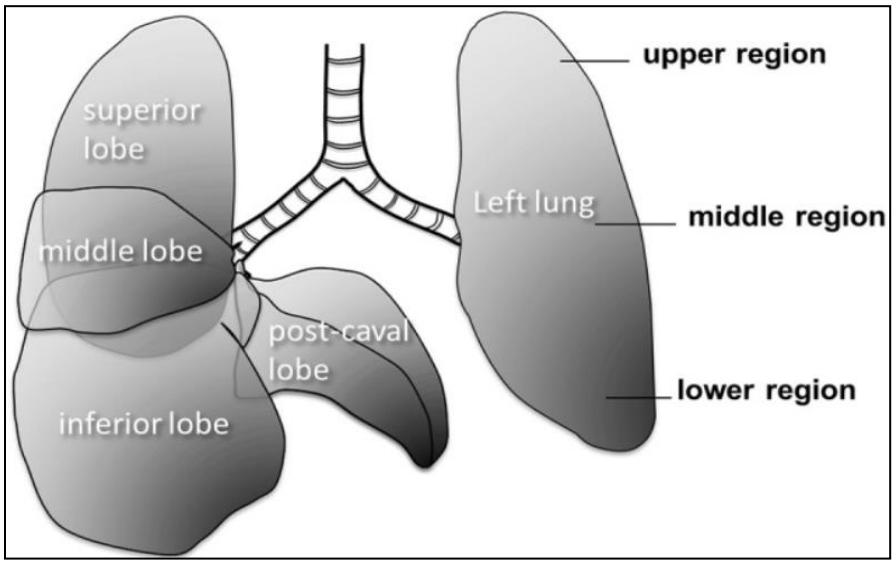


Figure 5: Anatomy of the mouse lung. The left lung has one distinctly large lobe and the right lung consist of four smaller lobes. The right lung comprises of a superior lobe, middle lobe, inferior lobe and post-caval lobes. This figure was obtained from Front Physiol. 2015; 6:146. This Figure was modified and created using BioRender.com.

3.6 BALF Cell Differential Assessment

The cell pellet obtained from BALF was resuspended in 1 mL sterile saline for quantification of total and cell differentials. Cells were stained using a modified Wright-Giemsa staining method (Hema 3® Stat Pack, Fisher Scientific, Hampton, NH, USA) and counted using a Carl Zeiss Axio Lab A1 (Carl Zeiss, Oberkochen, Germany) microscope. Cell differentials (macrophages, eosinophils, neutrophils and lymphocytes) were assessed by two different persons in a blinded fashion in 10 image frames at 20X magnification per slide.

3.7 Preparation of Lung Tissue Homogenates

The snap-frozen left lung lobe was thawed, transferred to an eppendorf tube on ice containing 500 µL of T-Per Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL, USA) and protease inhibitor cocktail (Cell Signalling, Davers, MA, USA). The lung tissues were homogenized on ice using the Cole-Parmer LabGEN 125 homogenizer (Cole-Parmer Canada, Quebec, Canada). Lung homogenates were centrifuged at 10,000 × g for 10 minutes at 4°C. Total protein concentrations in the lung tissue lysates (supernatants) were determined using a Bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). The tissue lysates were aliquoted and stored at -80 °C until further used.

3.8 Assessment of Cytokine Abundance

Concentrations of a panel of cytokines were measured in BALF and lung tissue lysates using a V-PLEX Mouse multiplex Meso Scale Discovery (MSD) assay based on the manufacturer's instructions (Meso Scale Discovery, Rockville, MD, USA). The panel of 29 cytokines evaluated in the multiplex V-PLEX were Interferon-γ (IFN-γ), tumor necrosis factor alpha (TNF-α), Interleukin (IL)- 1β, IL-12p70, IL-10, IL-2, IL-4, IL-5, IL-6, IL-9, IL-15, IL-16, IL-17A, IL-17C, IL-17E/IL-25, IL-17F, IL-21, IL-22,IL-23, IL-17A/F, IL-27p28/IL-30, IL-31, IL-33, keratinocyte chemoattractant (KC) /GRO, Interferon gamma-induced protein 10 (IP-10) , monocyte chemoattractant protein 1(MCP-1), macrophage inflammatory protein 1(MIP-1 α), MIP-2 and

MIP-3α. Lung tissue lysates and BALF were diluted 1:2 for MSD analysis. Data were analyzed using the Discovery Workbench 4.0 software (Meso Scale Discovery). Any values that were below the lower limit of detection were assigned half the value of the lowest detectable standard. The levels of IL-16 detected by MSD in lung tissue lysates were above the highest detectable standard. Therefore, IL-16 concentrations in lung tissue lysates were evaluated by an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (R&D Systems, Minnesota, USA). The lung tissue lysates were diluted 1:100 for detection within the dynamic range of the standards used in the ELISA kit.

3.9 Detection of Total and HDM-specific IgE Antibodies

Total IgE levels in serum were determined by a Mouse Uncoated ELISA Kit (Thermo Fisher, Catalog # 88-50460-22) according to the manufacturer's instructions. Serum samples were diluted 1:50 for BALB/c mice and 1:25 for C57BL/6NJ mice. An indirect IgE-ELISA method was used to detect HDM-specific IgE levels in serum. Costar™ 96-well flat-well high-binding plates (Thermo Fisher Scientific) were coated with 100 µL per well of HDM extract (10 µg/mL) in PBS and incubated overnight. Following washing, the plates were blocked with 3% BSA (w/v) in PBS (200 µL/well) and incubated at 4°C overnight. To detect HDM-specific IgE antibodies, serum samples from BALB/c and C57BL/6NJ mice were precleared by overnight incubation with Protein G Sepharose beads in a 2:1 ratio (40 µL of serum with 20 µL of beads per sample) at 4°C. Subsequent to washing the plates, pre-cleared serum samples was added to the plate and incubated for 2 h at RT. A biotin-anti-mouse IgE (1:5000 dilution in PBS containing 1% BSA) was used as the secondary antibody (2 h at RT) followed by streptavidin-HRP (100 µl per well, 1:5000 dilution) incubated for 20 min at RT, and 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) per well for detection. The optical densities were read at 450 nm with background absorbance at 570 nm using a BioTek Synergy 4 Microplate reader.

3.10 Western Blots with Mouse BALF

BALF samples were concentrated by acetone precipitation (150 µL BALF in 1ml cold acetone) incubated at -20°C overnight. The samples were centrifuged at 10,000 x g at 4°C for 10 min, supernatant discarded and the protein pellet was re-dissolved in 13 µL Milli-Q water and 5 µL NuPAGE LDS Sample Buffer (Invitrogen cat# NP0007). The samples were spiked with recombinant human granulysin (20 ng per sample: R&D systems) as loading control for western blots. BALF samples (20 µL each) were used for western blots. Proteins were separated on a 4%–12% Bis-Tris Protein gels (Invitrogen) and transferred onto nitrocellulose membranes (EMD Millipore, Thermo Fisher Scientific) for western blotting. The membranes were blocked with 5% (w/v) milk powder in TBST (20 mm Tris-HCl pH 7·5, 150 mm NaCl, 0·1% Tween-20) at 4°C overnight. The membranes were probed with antibodies against eosinophil peroxidase (EPX; Fitzgerald, 70R-5444), S100A8 (Abcam, ab 220174), S100A9 (Abcam, ab105472), coronin1A/TACO (Abcam, ab228635), properdin (Abcam, ab254275), lipopolysaccharide-binding protein (LBP; Abcam, ab233524), CD5 antigen-like protein (CD5L; Abcam, ab45406), heterogeneous nuclear ribonucleoprotein U (hnRNPU; Abcam, ab20666), glycosyl-phosphatidylinositol-specific phospholipase D (GPI-PLD; Abcam, ab192543) and calponin (Abcam, ab46794). HRP-linked secondary antibodies (Cell Signalling) and Amersham ECL detection system (GE Healthcare) were utilised for the development of the membranes and detection of protein bands. The band intensity was analyzed by Alpha Innotech Imager with AlphaView software.

3.11 Western Blots with Mouse Lung Lysate

A murine lung lobe was collected and homogenized as described above. The total protein concentration was also quantified using a BCA assay (Thermo Scientific) from murine lung tissue lysates as detailed above. Equal amounts of protein (50 µg of total protein) were resolved on Nu-PAGE 4-12 % protein gels (Invitrogen) and transferred onto nitrocellulose membranes (EMD Millipore, Thermo Fisher Scientific) for western blotting. The membranes were blocked with 5% (w/v) milk powder in TBST (20 mm Tris-HCl pH 7·5, 150 mm NaCl, 0·1% Tween-20) at 4°C overnight.

The membranes were probed with antibodies against estrogen receptor alpha (Erα; Abcam, ab241557), estrogen receptor beta (Erβ; Abcam, ab288) and G protein-coupled receptor 30 (GPR30; Abcam, ab275397) and incubated at 4°C overnight. HRP-linked secondary antibodies (Cell Signalling) and Amersham ECL detection system (GE Healthcare) were utilised for the development of the membranes and detection of protein bands. The band intensity was analyzed using Alpha Innotech Imager with AlphaView software.

3.12 Human BALF Sample Processing and Western Blots

Female and male participants (N=6 each) were exposed to nebulized inhalation (2 min) with allergens (birch, grass or HDM, based on individual sensitivity). The study participants were enrolled with informed consent at University of British Columbia (UBC) by Dr. Christopher Carlsten (collaborator). The participants were all non-smokers and atopic asthma patients. The human BALF samples were obtained by bronchoscopy performed 24 h post allergen exposure by collaborator Dr. Christopher Carlsten (UBC). BALF samples obtained (1 mL) were stored at -80 until used. Human BALF samples (200 μL) were concentrated to ∼30 μL using Amicon Ultra-3 kDa filters (MilliporeSigma) and spiked with recombinant mouse MCP5 as loading control (10 ng per sample: Invitrogen). The BALF samples (30 μL) were resolved on 4%–12% Bis-Tris Protein gels (Invitrogen) and then transferred to nitrocellulose membranes (EMD Millipore, Thermo Fisher Scientific) for western blotting. The membranes were blocked with TBST (20 mm Tris-HCl pH 7·5, 150 mm NaCl, 0·1% Tween-20) containing 5% milk powder (w/v) at 4°C overnight. The membranes were probed with antibodies against human S100A9 (Abcam, ab63818), S100A8 (Abcam, ab 92331), eosinophil peroxidase (EPX; Fitzgerald, 70R-5444), coronin1A/TACO (Abcam, ab228635), properdin (Abcam, ab254275), lipopolysaccharide-binding protein (LBP; Abcam, ab233524), CD5 antigen-like protein (CD5L; Abcam, ab45406), heterogeneous nuclear ribonucleoprotein U (hnRNPU; Abcam, ab20666), glycosyl-phosphatidylinositol-specific phospholipase D (GPI-PLD; Abcam, ab192543) and calponin (Abcam, ab46794).

After washing with TBST (6 × 10 min), the membranes were incubated with secondary antibody (anti-rabbit HRP in 1% (w/v) milk powder in TBST, 1:5000; Cell Signalling) for 45 min at RT. ECL (GE Healthcare) was used for detection and bands were analyzed by densitometry analysis using an Alpha Innotech Imager with AlphaView software.

3.13 Statistical Analysis

In this thesis, all data reported were disaggregated by sex (reporting data in female and male separately) and analysed based on SAGER guidelines for sex- and gender-based analyses (SGBA) [43, 45]. To evaluate the effect of biological sex and HDM treatment as independent variables, two-way repeated measures analysis of variance (ANOVA) with Tukey's multiple comparisons test was used for cell differentials in BALF and serum levels of total and HDM-specific IgE.

Paired comparison of total cells and cell differential ratio (HDM/naïve) between males and females was determined using a Mann-Whitney U test. In naïve mice, for cell types that had a cell count of zero a denominator of 1 was assigned to calculate the ratio of HDM/naïve. Mann-Whitney U test was used to compare cytokine abundance in BALF and lung tissue lysates between males and females in naïve, HDM-challenged mice and for the ratio of HDM/naïve. Comparative assessment of protein abundance in BALF between males and females in western blot densitometry was performed using a Mann-Whitney U test. A value of $p<0.05$ was considered to be statistically significant. All Statistical analyses were performed using GraphPad Prism (version 9.1; GraphPad Software).

Chapter 4: Results

Characterization of Sex-Related Differences in Allergen House Dust Mite-Challenged Airway Inflammation in Two Different Mice Strains

Chapter 4: Characterization of sex-related differences in house dust mite-challenged airway inflammation, in two different strains of mice

This section contains some text and figures from a first author manuscript submitted to *Scientific Reports*

Manuscript Title: Characterization of sex-related differences in allergen house dust mite-challenged airway inflammation, in two different strains of mice.

Manuscript ID: Submission ID 0f925481-c8d3-4c93-98b3-29863785949f (Manuscript under revision)

Authors: **Dina Mostafa**, Hadeesha Piyadasa, Mahadevappa Hemshekhar, Anthony Altieri, Andrew J. Halayko, Christopher D. Pascoe and Neeloffer Mookherjee.

Author contributions in the submitted manuscript:

DM, HP and CP performed mouse model experiments, samples collection and processing. DM, HP, HM and AA performed the various outcome assessments and data analyses. DM, HP, HM and CP contributed to the development of the scientific concepts. DM wrote the first draft of the manuscript. CP and AJH provided significant intellectual input in the study design and extensively edited the manuscript. NM conceived, obtained funding and directly supervised the study, and extensively edited the manuscript. All authors reviewed and edited the manuscript.

4.1 Abstract

Background: Asthma prevalence and disease severity is higher in adult females compared to males. Even though sex-related differences in asthma are well established, sex as a biological variable is neglected in preclinical research using murine models, leading to a limited understanding of sex-related variability in allergen-driven response in murine models. Additionally, there are no quantifiable molecular readouts or biomarkers that define sex bias.

Objective: To characterise sex-related differences in immune responses and define sex-specific molecular markers in an allergen HDM-challenged murine model of allergic airway inflammation, in two different strains of mice.

Methods: Female and male mice (n=10 in each group) of two commonly used genetic backgrounds, BALB/c and C57BL/6NJ were challenged (intranasally) with HDM (0.7 µg/ml saline) for 2 weeks (once per day, X5 instillations per week). Immune cell accumulation in BALF was assessed using modified Wright-Giemsa stain. A panel of 29 cytokines were quantified using the multiplex Meso-scale discovery platform in BALF and lung tissue homogenates. ELISA was performed to detect total and HDM-specific IgE antibodies in serum. Statistical significance was determined using two-way repeated measures ANOVA with Tukey's multiple comparisons test. Mann-Whitney U test, a nonparametric test, was used to compare cytokine levels in females and males.

Results: There were distinct sex-related differences in immune responses to the HDM challenge, and these differences were strain-dependent. Serum HDM-specific IgE titres were approximately 2-fold higher in female BALB/c mice compared to males, suggesting a more robust systemic immune response in females compared to males. While there was no difference in serum IgE levels (total or HDM-specific) between the sexes in C57BL/6NJ mice. Following HDM challenge, leukocytes accumulation in BALF, particularly eosinophils, neutrophils and macrophages, was approximately two times higher in female BALB/c mice compared to males. In contrast, eosinophils accumulation was higher in male C57BL/6NJ mice compared to females.

Examining the abundance of a panel of 29 cytokines in BALF and lung tissue lysates demonstrated that HMD-induced specific cytokines such as IL-17 IL-30, IL-9 and MIP-3 were significantly higher in female BALB/c mice compared to male. Whereas HDM-mediated increase in IL-25, IP-10, MCP-1, MIP-1, TNF and IL-21 were higher males compared to female BALB/c mice. These results indicated that the cytokine profile in females showed a predominantly Th17-skewed response to inhaled HDM challenge compared to males, in BALB/c mice. In contrast, the cytokine profile in male BALB/c mice showed a pro-inflammatory Th1-biased response compared to females, following inhaled HDM challenge in this model. Conversely, C57BL/6NJ female mice showed higher levels of IL-1β, IL-33, IP-10, MIP-2, IL-21 and GROα/KC compared to male mice. Only one cytokine, IL-9, was modestly higher in male C57BL/6NJ mice compared to females, suggesting a more skewed inflammatory cytokine response in female C57BL/6NJ mice in response to HDM challenge. Overall, these results clearly indicated that the inflammatory cytokine response following HDM challenge is sex-dependent and influenced by mice strain.

Conclusion: This chapter defines objective biomarkers of sex dimorphism in HDM-mediated airway inflammation, in two commonly used murine strains, which will be a valuable resource for preclinical asthma research.

4.2 Rationale & Introduction

Asthma is a chronic respiratory disease with a high prevalence rate impacting more than 300 million people worldwide and presenting as a major health care burden [280]. The incidence and severity of asthma as well as several other chronic inflammatory conditions, are characterized by sexual dimorphism in immune responses thus, biological sex is being considered as an effect modifier in chronic disease [267]. In general, females tend to mount a more vigorous immune response required for resolution of infections, where males tend to suffer more from frequent and severe infectious diseases [58, 74]. However, this heightened immune response in females also manifests into higher susceptibility to autoimmune, inflammatory and allergic diseases such as asthma [58, 81]. Prior to puberty, asthma is predominantly more prevalent in boys compared to girls, but in adulthood asthma disproportionately affects women compared to men in disease prevalence and severity [162, 181, 191, 269, 270].

Similarly, clinical studies have shown that women are more likely to have uncontrolled asthma as well as a steroid-unresponsive phenotype compared to males [163, 166, 271, 272]. Despite a clear sex-bias demonstrated in clinical studies for asthma pathophysiology and response to therapy, the effect of biological sex is poorly explored in preclinical studies with animal models in asthma research.

Various allergen challenge models are used in mice to induce an allergic response for the investigation of the pathogenesis of allergic lung diseases [281, 282]. The two most widely used models are the ovalbumin (OVA) and HDM model challenge models, where predominantly female mice are challenged with either OVA or HDM to induce allergic airway inflammation [283]. In mouse models of OVA-induced airway inflammation, female mice have been shown to exhibit enhanced eosinophilic inflammation, higher OVA-specific and total serum IgE and IgA levels, increased eosinophilic responses and increased levels of Th2 cytokines (IL-4, IL-5, IL-10, IL-13 and TGF-β compared to male mice following allergen challenge [284–286]. The majority of studies exploring sex differences in these murine models have been with sensitization of mice with OVA in the presence of alum as a Th2-skewing adjuvant. However, the use of OVA as an allergen to mimic human asthma is not optimal due to development of allergen tolerance leading to desensitization [287]. Thus, recent studies have favoured HDM as an allergen due to its clinical relevance to human allergic asthma. HDM is a prevalent natural allergen with approximately 85% of asthmatics worldwide being sensitized to HDM [287, 288]. However, sex-related differences in HDM-mediated immune responses remain poorly characterized in murine models. The genetic background of mice can also influence the nature of the airway inflammatory responses such as distribution of immune cell infiltration and concentration levels of cytokines, which impacts the severity of airway inflammation [275, 289]. This indicates that mice strain variability may also impact sex-specific differences in murine models of airway inflammation. Given the paucity of data on sex differences in murine models of HDM-induced airway inflammation, there is a need to identify sex-specific inflammatory responses and markers. Furthermore, there is limited understanding of the contribution of mice strain variability to sex differences and interplay between genetic variability and sex differences in airway inflammation.

In this study, we used a 2-week HDM-challenge murine model to characterize sex-related differences in airway inflammation in both BALB/c and C57BL/6NJ mice. The HDM-challenge model described in this thesis results in robust airway inflammation, preceding lung remodeling and fibrosis [279]. Various outcomes that I assessed to examine the influence of sex and strain difference included leukocyte accumulation in BALF which is indicative of immune cell infiltration in the lungs, serum IgE levels (total and HDM-specific) and profiled the abundance of a panel of 29 different cytokines in BALF and lung tissues. Various inflammatory mediators described in this study will provide sex specific biomarkers that are differentially elevated in female and male mice in response to HDM, in two different mice strains. These results will be a valuable resource that can be used in experimental murine models of allergen-mediated airway inflammation, such as for preclinical studies in asthma.

4.3 Results

4.3.1 Serum Level of HDM-Specific IgE is Significantly Higher in Female BALB/c mince compared to Males

IgE antibodies play a pivotal role in propagating airway inflammation following allergen exposure [290]. We have previously shown that HDM-specific IgE antibodies are enhanced in adult female BALB/c mice following repeated instillation of HDM for two weeks [279]. Thus, we assessed serum levels of total and HDM-specific IgE. There were no significant differences in the serum levels of total IgE and HDM-specific IgE between naïve female and male mice, in both strains (Figure 6). HDM-challenge resulted in an increase in total IgE levels in BALB/c mice serum, which was significant in female mice ($p<0.01$) and showed a trend of increase ($p=0.08$) in males (Figure 6A). HDM-specific IgE levels increased in serum obtained from female but not male BALB/c mice, following HDM challenge (Figure 6A). There was a trend in the increase of total IgE ($p=0.07$) and HDM-specific IgE ($p=0.08$) in female C57BL/6NJ mice, in response to HDM (Figure 6B). Whereas the circulating levels of total or HDM-specific IgE did not increase in male C57BL/6NJ mice (Figure 6B). These results showed that HDM challenge resulted in an increase in allergen HDM-specific serum IgE selectively in female mice.

These results demonstrate a sex- and strain-specific difference in systemic antibody response in response to HDM instillations. To further determine sex-related differences in local mucosal responses, we examined molecular endpoints of airway inflammation, leukocyte differentials and cytokine profile in the lungs.

Figure 6

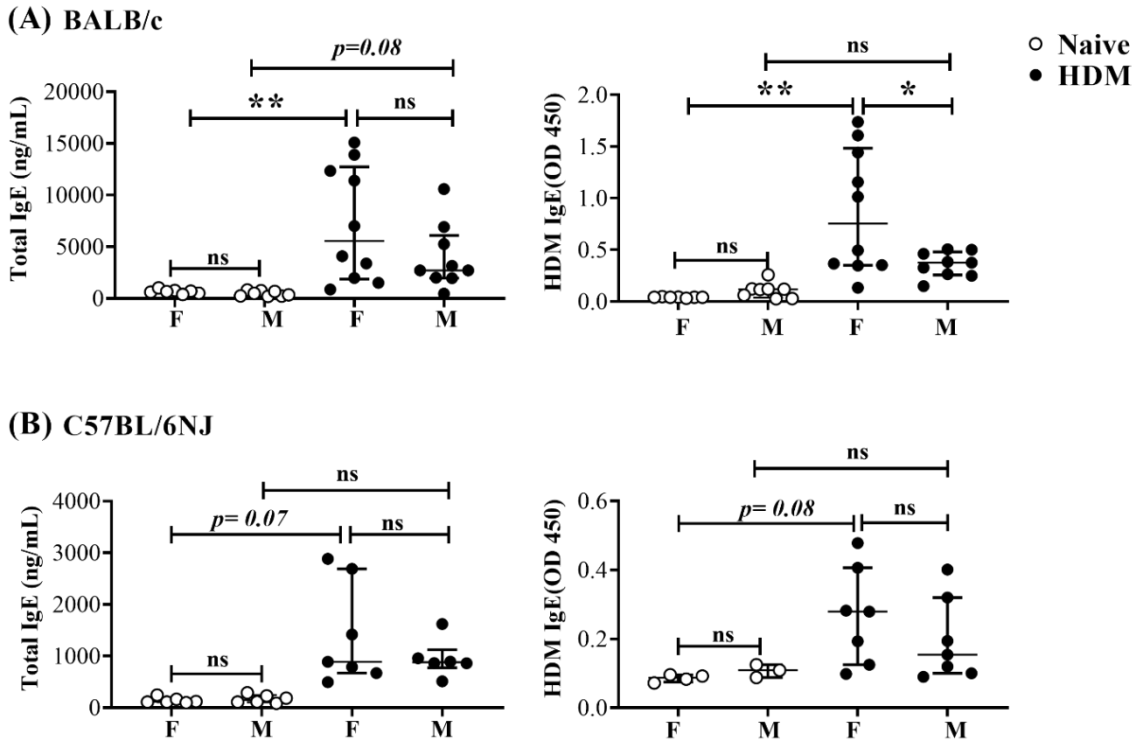


Figure 6: Total and HDM-specific IgE antibodies in serum. Mice were challenged with 35 µL of 0.7 mg/mL whole HDM protein extract in saline (i.n) per mouse, for 2 weeks (one per day X5 per week). Blood was collected from **(A)** BALB/c and **(B)** C57BL/6NJ mice, 24 h after the last HDM challenge and serum used for assessment of total IgE and HDM-specific IgE antibodies by ELISA. Each dot represents an individual mouse. Statistical significance was determined using two-way repeated measures ANOVA with Tukey's multiple comparisons test. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

4.3.2 Sex-Related Differences in Leukocyte Accumulation in the Lungs in Response to Inhaled HDM

We have previously shown that repeated instillation of HDM for two weeks results in a significant increase in specific leukocytes such as eosinophils and neutrophils, known to promote airway inflammation, in the BALF of female BALB/c mice [279, 291]. Based on our previous studies, we examined total cell counts and leukocyte differentials in BALF samples obtained from female and male mice, 24 h after the last HDM challenge. In naïve mice of both strains (BALB/c and C57BL/6NJ) the primary cell type in BALF at baseline was macrophages (~90% of total cells), with no significant sex-related differences (Supplementary Figure 1). There were no statistically significant differences in total cell accumulation, and in any of the leukocytes examined, between female and male naïve mice of both strains (Supplementary Figure 2). Following repeated HDM instillation for two weeks, eosinophils were the major cell type in the BALF (~55% of total cells) of both strains of mice (Supplementary Figure 1). To account for the variability in cell counts at basal level in naïve mice (Supplementary Figure 2), we assessed the ratio of cell counts in each HDM-challenged mouse compared to the average count from the naïve group (HDM/naïve), for total cells and each cell type examined in BALF.

In female BALB/c mice, total cell accumulation was ~3-fold higher ($p<0.0001$), eosinophils by ~8-fold ($p<0.0001$) and macrophages by ~3-fold ($p<0.0001$) compared to males, in response to HDM (Figure 7 and Supplementary Table I). Neutrophil accumulation in the BALF of female BALB/c mice were >250-fold higher ($p<0.003$) compared to males, in response to HDM (Figure 7C and Supplementary Table I). These effects were not observed in C57BL/6NJ mice. In contrast to BALB/c, HDM-driven eosinophil accumulation in the BALF of male C57BL/6NJ mice was ~2-fold higher compared to females (Figure 7B and Supplementary Table I). These results demonstrated that overall composition of leukocytes in the lungs, as well as specific sex-related differences in response to HDM, are dependent on the strain of the mice.

Figure 7

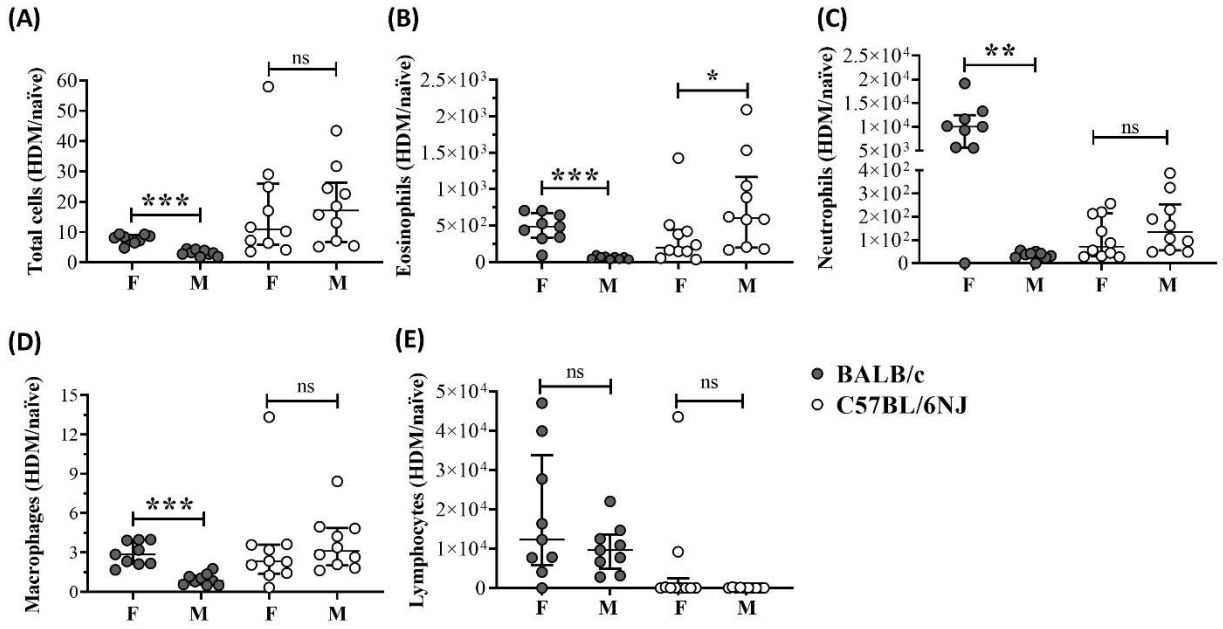


Figure 7: Leukocyte differentials in brochoalveolar lavage fluid (BALF). BALB/c mice (n=9 each, female and male, per group) and C57BL/6NJ (n=10 each, female and male, per group) were challenged (i.n) with 35 µL of 0.7 mg/mL whole HDM protein extract in saline (per mouse) for 2 weeks (one per day, X5 per week). BALF was collected 24 h after the last HDM challenge. **(A)** Total cells, **(B)** eosinophils, **(C)** neutrophils, **(D)** macrophages and **(E)** lymphocytes were assessed in BALF with modified Wright-Giemsa stain**.** Graphs represents ratio of cell counts in BALF obtained from each HDM-challenged mouse compared to the mean value from naïve group (HDM/naïve), for each strain. Mann-Whitney U test was used to compare the ratio (HDM/naïve) of cell counts between females and males.

4.3.3 Sex-Related Differences in Lung Cytokine Profile in Naïve Mice

To define sex-related differences in cytokine profiles in the lung, we measured the abundance of 29 cytokines and chemokines in BALF and lung tissue lysates using the multiplex MSD platform. Out of the 29 cytokines measured, 18 were detected in the BALF and all 29 were detected in the lung tissue lysates. There were distinct sex-related differences in the abundance of specific cytokines at baseline levels in the lungs of naïve mice of both strains. BALF of naïve female BALB/c mice had significantly higher levels (~2-fold) of IL-5, IP-10 and MIP-1α, and 4-fold higher IL-33 abundance, compared to males (Figure 8A). The lung tissue lysates of naïve female BALB/c mice had significantly higher levels (<2-fold) of IFNγ, MIP-1α, IL-5, IL-25, and IL-21 was >2-fold higher, compared to males (Figure 8B). Naïve male BALB/c mice had significantly higher levels (<2-fold) of IL-1β and KC/GROα in the BALF (Figure 8A), and IL-17A/F (<2-fold) in the lung tissue lysates (Figure 8B), compared to females.

Naïve female C57BL/6NJ mice showed significantly higher level of IL-5 (~2-fold) in the BALF and lung tissue lysates (<2-fold)) compared to males (Figure 8C and 8D respectively). IFNγ was also significantly higher in the lung tissue lysates of naïve female C57BL/6NJ mice (Figure 3D). Naïve male C57BL/6NJ mice had significantly higher levels (< 2-fold) of MIP-2 and KC in the BALF, compared to females (Figure 8C). The lung tissue lysate of naïve C57BL/6NJ male mice demonstrated a robust sex bias, with the abundance of KC, MIP-2, IL-21 and IL-25 higher (>2-fold), and IL-17A and MIP-3α (<2-fold higher), compared to females (Figure 8D). Comparative analysis of these cytokine profiles (Figure 8) revealed that IL-5 was significantly higher at baseline levels in the lungs (BALF and lung tissue lysates) of females compared to males, in both BALB/c and C57BL/6NJ mice (Figure 8). With the exception of lung tissue lysates from BALB/c mice, KC/GROα was higher in naïve males compared to females, in both strains of mice (Figure 8). Similarly, IL-17 was significantly higher in lung tissue lysates of naïve males compared to female, in both strains of mice (Figure 8B and 8D), albeit IL-17A/F noted in BALB/c and IL-17A in C57BL/6NJ. These results clearly demonstrated sex- and strain-related differences in the cytokine profile of the lungs at baseline levels in naïve mice. Based on these results we corrected for baseline values to assess HDM-driven change in cytokine abundance as follows.

Figure 8

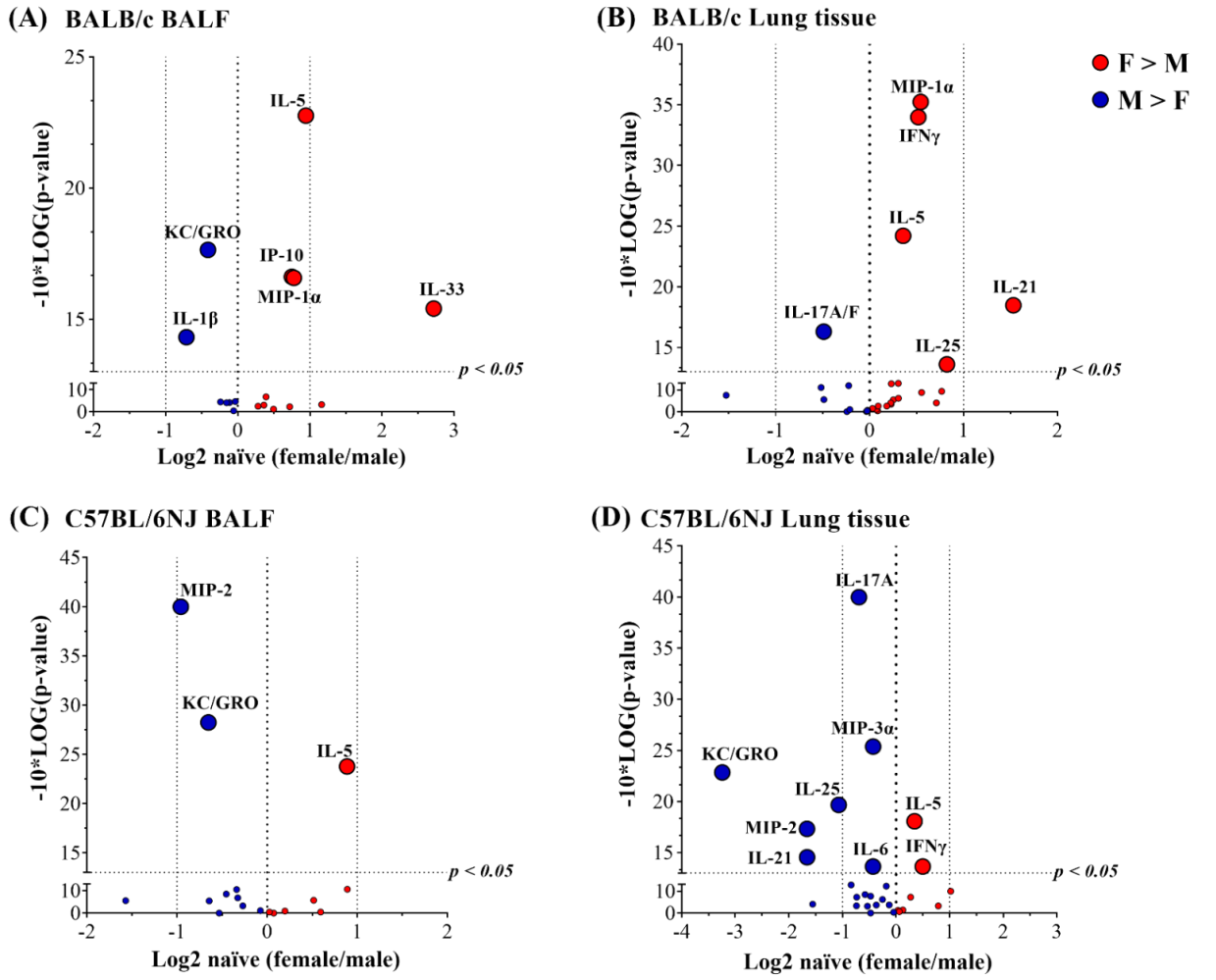


Figure 8: Sex-related differences in cytokine abundance in the lung of naïve BALB/c and C57BL/6NJ mice. A panel of 29 cytokines were measured by multiplex Meso Scale Discovery (MSD) platform in **(A)** BALF and **(B)** lung tissue lysates obtained from naïve BALB/c mice (n=9 each, female and male), and **(C)** BALF and **(D)** lung tissue lysates obtained from naïve C57BL/6NJ mice (n=10 each, female and male). Log2 values of average concentrations (pg/mL) of each cytokine was used for the volcano plots to examine the differences between female and male mice. Cytokine abundance significantly higher in females compared to males have positive values (shown in red), and those significantly higher in males compared to females have negative values (shown in blue), as represented on the x-axis of the volcano plots. Dotted lines represent a fold change of 2 (x-axis) and $p<0.05$ (y-axis).

4.3.4 Sex-Related Differences in Secreted Cytokine Profile in BALF in Response to HDM

To determine HDM-driven change for each cytokine measured, we assessed the ratio of concentration in the BALF obtained from each HDM-challenged mouse compared to the mean concentration from the group of naïve mice i.e., HDM/naïve (Supplementary Table II). To determine sex-related differences, we compared the mean HDM/naïve values for each cytokine between female and male mice, in both strains of mice independently (Figure 9 and Supplementary Table II). Of the 29 cytokines examined, 16 cytokines were increased by >2-fold in the BALF of female and male mice of both strains, in response to HDM (Supplementary Table II), with specific sex-related differences as follows.

Strikingly, an HDM-driven increase in IL-17A was >150-fold ($p<0.01$) in females and only 8-fold in male BALB/c mice, compared to naïve mice (Supplementary Table II). IL-17A was the only cytokine that was significantly higher (by ~16-fold) in the BALF of female BALB/c mice compared to males, following HDM challenge (Figure 9A). This result indicates that HDM drives a Th17-skewed response in the BALF of female BALB/c mice compared to males. In contrast, male BALB/c mice had significantly higher levels (by ~2-fold) of TNF, IP-10, MCP-1 and MIP-1α in the BALF, compared to females (Figure 9A and Supplementary Table II), indicating a Th1-biased response in secreted protein profile.

In contrast to BALB/c, HDM-driven secreted cytokine profile in the BALF of C57BL/6NJ mice demonstrated a clear female-bias with significantly higher levels of IL-1β, KC/GRO, IL-33, IP-10 and MIP-2 (between 2 and 4-fold) compared to males (Figure 9B and Supplementary Table II). None of the cytokines examined were significantly higher in the BALF of male C57BL/6NJ mice compared to females (Figure 9B). These results indicated that HDM results in significantly higher levels of specific Th1-bias cytokines in the BALF of female C57BL/6NJ mice compared to males.

Figure 9

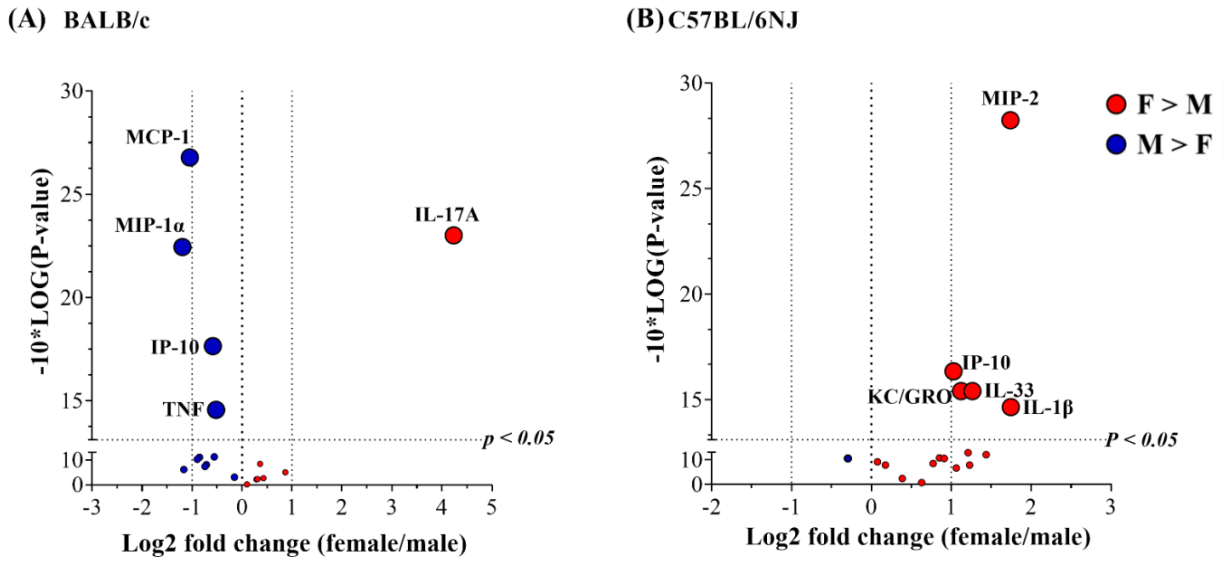


Figure 9: Sex-related differences in HDM-mediated secreted cytokines in BALF. (A) BALB/c mice (n=9 each, female and male, per group) and **(B)** C57BL/6NJ (n=10 each, female and male, per group) were challenged (i.n) with 35 µL of 0.7 mg/mL whole HDM protein extract in saline per mouse, for 2 weeks. A panel of 29 chemokines and cytokines were measured in BALF collected 24 h after the last HDM challenge, by multiplex Meso Scale Discovery (MSD) platform. Ratio of concentration of each cytokine in the BALF of each HDM-challenged mouse compared to the mean concertation obtained from the group of naïve mice represents HDM-driven fold change (HDM/naïve). Log2 values of average HDM/naïve fold change obtained from female and male BALF were used for the volcano plots. Positive values on the x-axis of the volcano plots (shown in red) are cytokines that are significantly higher in females compared to males, and negative values (shown in blue) are those that are significantly higher in males. Dotted lines represent a fold change of 2 (x-axis) and $p<0.05$ (y-axis).

4.3.5 Sex-Related Differences in HDM-Mediated Increase in Cytokine Abundance in Lung Tissue Lysates

Similar to that described above for BALF, we assessed the ratio of abundance in the lung tissue lysates obtained from each HDM-challenged mouse compared to the mean abundance in lung tissue lysates from the group of naïve mice i.e., HDM/naïve for each cytokine (Supplementary Table III). To determine sex-related differences, we compared the mean HDM/naïve values for each cytokine between female and male mice, in both strains of mice independently (Figure 10 and Supplementary Table III). The abundance of 19 cytokines in BALB/c and 23 cytokines in C57BL/6NJ were significantly increased in the lung tissue lysates, in response to HDM challenge compared to naïve mice (Supplementary Table III).The HDM-driven increase in the abundance of IL-17A, MIP-3α, IL-30 and IL-9 was significantly higher (between 1.5 and 2-fold) in the lung tissue lysates of female BALB/c mice compared to males (Figure 10A). Male BALB/c mice had significantly higher abundance of IL-21 (>2-fold) and IL-25 (<2-fold) in lung tissue lysates compared to females (Figure 10A).

In contrast to BALB/c mice, female C57BL/6NJ mice did not demonstrate an IL-17-skewed response to HDM. Instead, female C57BL/6NJ mice showed significantly higher (>2-fold) abundance of KC/GRO, MIP-2 and IL-21 in the lung tissue lysates compared to males, in response to HDM (Figure 10B). IL-9 was the only cytokine that was significantly higher (~4-fold) in the lung tissue lysates of male C57BL/6NJ mice compared to females, in response to HDM (Figure 10B).

Figure 10

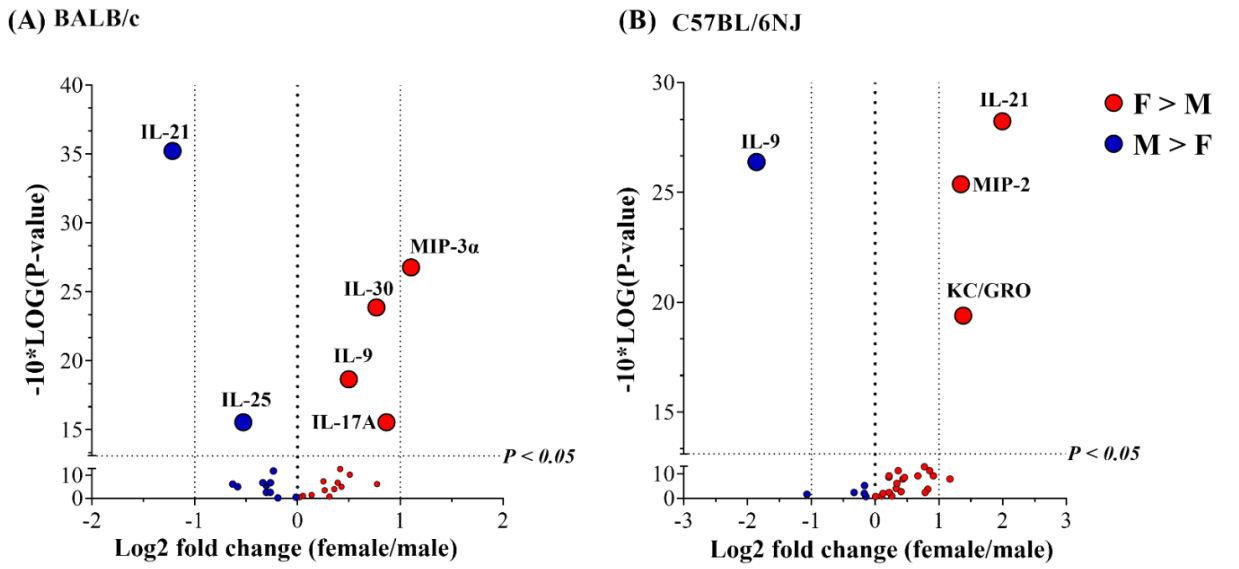


Figure 10: Sex-related differences in HDM-mediated increase in the abundance of cytokines in lung tissue lysates. (A) BALB/c mice (n=9 each, female and male, per group) and **(B)** C57BL/6NJ (n=10 each, female and male, per group) were challenged (i.n) with 35 µL of 0.7 mg/mL whole HDM protein extract in saline per mouse, for 2 weeks. A panel of 29 chemokines and cytokines were measured in the lung tissue lysates collected 24 h after the last HDM challenge, by multiplex Meso Scale Discovery (MSD) platform. Ratio of the abundance of each cytokine in the lung tissue lysates from each HDM-challenged mouse compared to the mean abundance obtained from the group of naïve mice represents HDM-driven fold change (HDM/naïve). Log2 values of average HDM/naïve fold change obtained from female and male BALF were used for the volcano plots. Positive values on the x-axis of the volcano plots (shown in red) are cytokines that are significantly higher in females compared to males, and negative values (shown in blue) are those that are significantly higher in males. Dotted lines represent a fold change of 2 (x-axis) and $p<0.05$ (y-axis).

4.4 Discussion & Conclusion

The predominance of females' susceptibility to inflammatory respiratory diseases such as asthma may be related to differential immune responses to an allergen challenge [272]. The differences in immune response remain insufficiently characterized in murine models of airway inflammation. In this study, we examined sex-related differences in immune response to allergens in the lung of two different mouse strains (BALB/c and C57BL/6NJ). we focused on assessing leukocyte accumulation in BALF, the levels of circulating total and HDM-IgE antibodies and the abundance of 29 different inflammatory cytokines and chemokines in BALF and lung tissue. we used a two-week HDM-challenged murine model of airway inflammation, which results in robust airway inflammation preceding lung remodelling and fibrosis [279]. Thus, this model is ideal to delineate sex-specific differences in airway inflammation. As this study was limited to a 2-week intranasal challenge period, outcomes or endpoints in the context of airway remodeling were not assessed. In this study, we demonstrated specific strain-dependent sex disparity in BALF leukocyte accumulation and in circulating IgE antibodies, in naïve mice and in response to HDM. Notably, we identified a sex-dependent cytokine signature in the lung of both naïve and HDM-challenged mice. Remarkably, specific sex-biases in molecular endpoints identified in this study were different between the two different mouse strains used in this study (summarized in Figure 11). Overall, the results in this study defined sex- and strain-dependent differences in immune response in a mouse model of allergen-challenged airway inflammation.

Cellular and mediator profiles in BALF are a determinant of inflammatory status and asthma severity [292]. we demonstrated that female BALB/c mice showed higher levels of total leukocyte accumulation in the lungs, and specifically higher accumulation of eosinophils, neutrophils and macrophages in BALF following HDM-challenge. These results are in line with previous studies demonstrating that female mice elicit a stronger cell accumulation in the lungs in an OVA-induced airway inflammation model [284–286]. Similar to results in this study, Fuseini and colleagues previously reported that HDM-induced BALF eosinophils and neutrophils were more elevated in female BALB/c mice compared to male mice [293]. These studies emphasize that sex-bias in cell infiltration in the lung is observed regardless of the allergen used.

It is possible that endogenous sex steroids play a role in mediating the sex differences in HDM-induced leukocyte accumulation, in particular eosinophils and neutrophil accumulation in the

lungs [293]. Further, testosterone has been shown to reduce the expression of TLR4 on macrophages in response to endotoxin [114]. However, the mechanism on how sex steroids modulate these responses is not addressed directly in this study. Nevertheless, the results in this study clearly demonstrate differences in sex bias in leukocyte accumulation in the lungs in response to allergen challenge, and these were also different in BALB/c compared to C57BL/6NJ mice. These results indicate that the genetic background of mice is a contributing factor to sex difference in airway inflammation. This becomes an important factor to consider in preclinical studies while translating results from murine studies to relevance in human. It is important to note that kinetics of differential leukocyte population trafficking to the lung could possibly influence the disparity in inflammatory profile observed between males and females as well as in the different mouse strains. As all outcomes measured in this study were performed at one time point, a comparative analysis in future studies is needed to determine whether the sex-related differences are related to kinetics of response.

The cellular composition in BALF reflects the overall inflammatory cell profile in the lung, therefore we examined the differential cell percentage in BALF. Among the mouse strains, we observed different patterns of cellular composition of immune cells in the lung. Previous studies have illustrated strain-related differences in cytokines and chemokines profiles, as well as in levels of antibody and the type of immunity associated with allergic lung disease in mouse models [289, 294, 295]. For example, OVA-challenged female BALB/cJ mice showed higher levels of serum IgE, IL5 and IL13 compared to C57BL/6J mice [295]. Similarly, Gueders *et al* demonstrated that eosinophil and neutrophil counts were higher in BALF of C57BL/6 male mice compared to BALB/c following OVA-challenge [289]. Likewise, in this study we illustrated that sex disparities in airway inflammation are different between different mice strains.

The results in this study highlight that the mouse strain used in a preclinical study is a critical factor to consider, particularly in studies using transgenic or knockout mice to define molecular mechanisms that drive sex related disparity in airway inflammation. The recent insights of asthma heterogeneity have established a strong link between several cytokines and asthma disease pathogenesis [190, 296–300]. This is the first study to comprehensively define cytokine signatures related to sex dimorphism in HDM-mediated airway inflammation and provide sex differences in two different strains of mice. Limited previous studies that investigated sex differences in cytokine

response in airway inflammation primarily focused on Th2-skewed cytokine responses [64, 284–286, 301–303]. In this study, we examined sex-related differences in the abundance of 29 cytokines and chemokines in the BALF and lung tissue lysates obtained from allergen-naïve and HDM-challenged mice, in BALB/c and C57BL/6NJ strains.

Interestingly, at baseline we were able to illustrate sex difference in inflammatory cytokines in allergen-naïve mice in both mice strains. Markedly, IL-5 was significantly higher in females compared to males in the BALF and lung tissue lysates from both mice strains. IL-5 exerts a key function in inducing and propagating the pathogenesis of eosinophilic asthma, as it plays a key role in eosinophil activation, survival and recruitment to the airways [189, 304]. The main cells that produce IL-5 include eosinophils, Th2 lymphocytes and ILC2s [305]. ILC2s are implicated in the development of airway inflammation in response to allergen [306, 307]. Interestingly, sex-related differences in basal ILC2s levels have been shown in naive adult mice, female mice show a higher number of lung ILC2s than males [63, 308–310]. The elevated basal levels of IL-5 observed could possibly imply that females are more prone to readily respond to allergen compared to males. This could be due to intrinsic differences in gene expression of lung ILC2s as well as other inflammatory cells. In contrast, KC/GRO (homologous to human IL-8), known to be a potent neutrophil chemoattractant [311], was higher in BALF of naïve male mice compared to females, in both mice strains. While IL-17A in C57BL/6NJ mice and IL-17A/F in BALB/c mice, were higher in the lung tissue of naïve male mice compared to females, IL-17 is associated with the induction and amplification of Th17-mediated neutrophilic inflammation [312, 313]. The results from this study indicate that sex differences in basal cytokine levels in adult naïve mice need to be taken into consideration when evaluating responses in animal models of airway inflammation. The sex bias in basal cytokine levels could be driven by sex disparity in microbial communities either in the lung and gut. Recent studies have provided evidence that biological sex play a role in shaping the gut microbiota and influence lung microbiome [314–317]. Interestingly, assessment of changes in lung microbiome associated with inflammation in two different mouse strains BALB/cj and C57BL/6 revealed that the lung microbiome is sex-dependent [317]. Yet, the role of the lung microbiome from a biological sex point of view is still poorly characterized.

The sex dimorphism in the cytokine profile in allergen-naïve mice could impact on homeostatic immune status and dictate the overall cytokine response following allergen exposure. we showed that HDM-mediates a mixed eosinophil-neutrophil cellular profile along with a significantly higher IL-17A abundance in the lungs of female BALB/c mice compared to males. Comparing HDM-driven cytokine responses, IL-17A was the only cytokine that showed a robust female-bias in the BALF of BALB/c mice in response to HDM challenge. Female BALB/c mice also had significantly higher levels of IL-17A, IL-30, IL-9 and MIP-3α in the lung tissue lysates, compared to males in response to HDM. IL-17A is a key determinant of severe forms of asthma and is particularly involved in promoting neutrophil recruitment and enhancing the severity of allergen-induced airway responses [312, 313]. Further, IL-17A is implicated in inducing glucocorticoid insensitivity in human bronchial epithelial cells [318]. The prevalence of steroid-unresponsive asthma is more frequent in adult female asthmatics compared to males which aligns with the immunophenotype displayed in females [319–321]. These clinical observations are supported by the results in this study which demonstrated a higher IL-17A and thus a Th17-skewed response in HDM-challenged female BALB/c mice. Similarly, the results in this study are in concurrence with previous reports showing higher HDM-induced lung IL-17A expression in female BALB/c mice compared to males [293, 303]. As sex-related differences of IL-17A was not similar in both mice strains in this study, it is not likely that IL-17A solely is responsible for sex disparities in allergic asthma. This study also showed that female BALB/c mice had higher levels of MIP-3α in lung tissue lysates compared to males. MIP-3α (CCL20) is a chemotactic factor for neutrophils and Th17 cells, and is elevated in the sputum of asthma patients following the use of inhaled glucocorticoids [318, 322, 323]. MIP-3α has also been implicated in Th17-mediated unresponsiveness to glucocorticoid in asthma [318, 322]. The role of IL-17 and MIP-3α in asthma severity in females remains undefined. However, the results of this study suggest that the elevated levels of MIP-3α and IL-17 in females, but not in males, could potentially explain the prevalence of severe asthma and poor response to steroid therapy in post-pubertal females.

In contrast to BALB/c mice, C57BL/6NJ female mice showed a Th1-skewed cytokine profile in response to HDM-challenge with higher levels of MIP-2, IP-10, IL-1β and KC/GROα in the lungs, compared to male mice. However cytokines such as IL-33 (in BALF) and IL-21 (in lung tissue lysates), which play a role in promoting and augmenting allergic Th2 airway inflammation, were also higher in female C57BL/6NJ mice in response to HDM [324–327].

This indicates that female C57BL/6NJ mice elicit a mixed Th1/Th2-skewed response following HDM challenge compared to males, and not Th17-skewed which is distinctly different from that noted in BALB/c mice.

Interestingly, the HDM-driven cytokine profile in lung tissue lysates showed significantly higher levels of cytokines associated with Th2-mediated airway inflammation in males compared to females, in both strains of mice. For example, IL-21 and IL-25 were higher in the lung tissue lysates of male BALB/c mice compared to females. IL-21 enhances Th2 cell function as well as airway eosinophilia [328, 329]. IL-25 also augments Th2 responses [330]. Similarly, IL-9 which induces airway eosinophilia and promotes a Th2-skewed immune response by inducing the production of IL-13 [331–333], was higher in the lung tissue lysates of male C57BL/6NJ mice compared to females, in response to HDM. Note that IL-9 was the only cytokine to display a male-bias in lung tissue lysates of C57BL/6NJ mice compared to females, in response to HDM. This was consistent with higher accumulation of eosinophils in the lungs of male C57BL/6NJ mice in response to HDM, compared to females. Taken together, these results suggest that HDM-drives a higher Th2-skewed inflammatory response in the lungs of male mice compared to females, in both BALB/c and C57BL/6NJ mice, albeit there are differences in the specific cytokines elevated in the lungs between the two strains. Surprisingly, in the present study we did not observe sex differences in key Th2 cytokines such as IL-4, IL-5 and IL-13 following HDM- exposure in both mice strains. Prior reports have indicated differential IL-4 and IL-5 cytokine expression between males and females in BALB/c mice in a chronic OVA and HDM model [286, 303]. The discrepancies in findings could possibly be due to a difference in protocols of allergen challenge, as different allergen and endotoxin content can alter inflammatory responses challenge with HDM with low endotoxin content may have resulted in the immunophenotye characterized in this study. Similarly, the time point of measurement of outcomes may influence the differences in cytokine responses. We have only determined the outcomes at a single time point, perhaps sex-related differences in Th-2 cytokines could have been may have been observed at a later time point, example 48 h after the last HDM challenge. In addition, other factors may have influenced the differences in observations, including the estrous cycle in female mice and the influence of differences in sex steroid abundance which was not evaluated in this study.

Nevertheless, the cytokine profile results in this study primarily indicates that in response to HDM, female BALB/c mice elicit a higher Th17-skewed response, and female C57BL/6NJ mice mount a higher Th1-skewed response, compared to males (Figure 11). In contrast, male mice from both strains showed higher levels of Th2-associated cytokines in the lungs in response to HDM (Figure 11). Asthma is an extremely heterogenous disease associated with airway inflammation which is Th2-driven to a mixed Th1/Th17-driven disease in various patients. Mouse models do not directly capture the heterogeneity of asthma patients [334]. Nevertheless, this study provides two different strain-dependent models which may be used to study mechanisms related to sex dimorphism in disparate inflammatory phenotypes in asthma research.

In summary, the findings reported in this study highlight the critical role played by both sex and genetic background in allergen-mediated airway inflammatory response and further highlight the importance of taking both variables (sex and mice strain) into consideration in experimental design and interpretation of data in preclinical studies. Recognition of immune variations among mouse strains is essential for the accurate interpretation of any sex difference that may arise, particularly when translating these findings to human studies. The identification of HDM-induced sex-specific quantifiable markers in this study from BALB/c and C57BL/6NJ mice, provide a valuable resource that can be used in research aimed to identify novel therapeutics and treatment strategies for a sex-based personalized drug development in asthma.

The genetic background of mice is often neglected in the interpretation of data in preclinical studies. In the context of this study, and for preclinical models of HDM-challenged allergic asthma, BALB/c display several advantages as it exhibits a robust Th2 immunological response, but can be used to simulate a Th1 and Th17 responses, all of which are related to clinical observations in asthma. However, mice of C57 background are often used as transgenic mice are more likely to in C57 background. We have clearly shown differences in inflammatory outcomes between the two mice strains used in this study. Immunological variation between inbred mouse strains, as demonstrated in this study, emphasizes the importance of carefully assessing the mice strain selected for addressing the specific study question, and being cognizant that the stain of mice can influence other biological variables such as sex dependent outcomes.

Figure 11

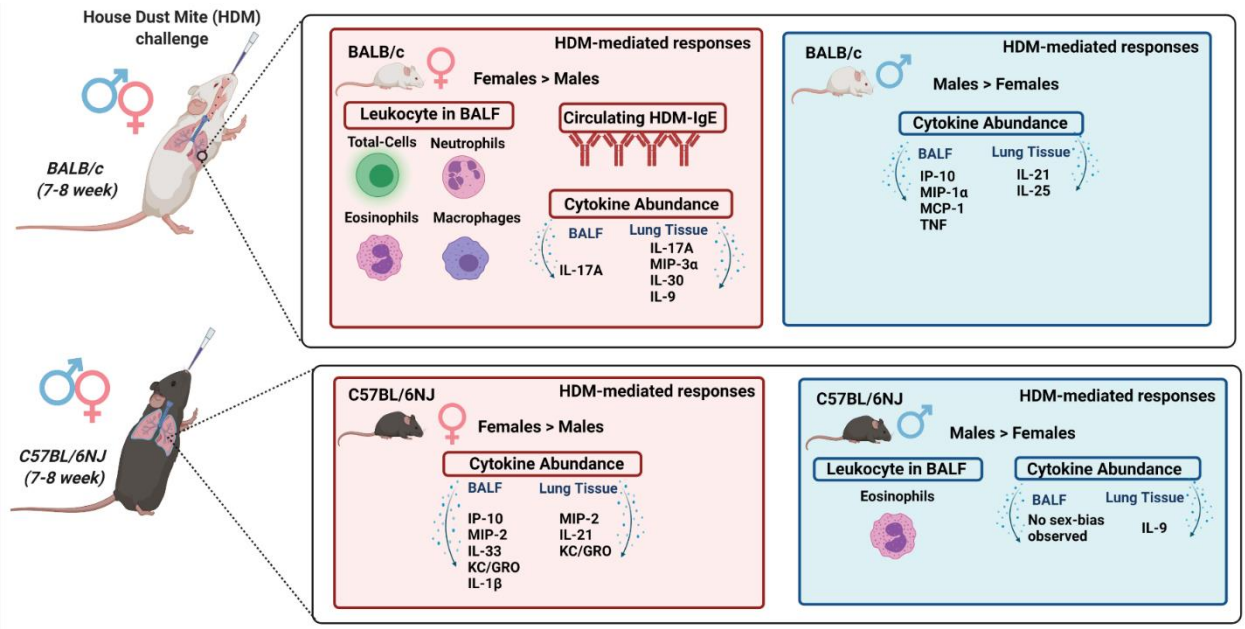


Figure 11: Sex dimorphism in HDM-mediated leukocyte accumulation and cytokine profile in the lungs is strain dependent. This figure summarizes sex-related differences in leukocyte accumulation and the increase in specific cytokines in BALB/c and C57BL/6NJ mice. HDM-mediated increase in the accumulation of total cells, neutrophils, eosinophils and macrophages are higher in female BALB/c mice compared to males. Whereas, male C57BL/6NJ mice have higher accumulation of eosinophils compared to females in response to HDM. Cytokine profile assessments indicate that HDM-driven increase in IL-17 and MIP-3α (a chemoattractant of Th-17 cells) is significantly higher in female BALB/c mice compared to males, suggesting a Th17-skewed response. Whereas HDM-driven cytokine profile in female C57BL/6NJ mice indicate a Th1-skewed response compared to males. In contrast, HDM-mediated cytokine profiles in male mice of both strains show higher levels of Th2-associated cytokines compared to females. Figure was created with BioRender.com.

Chapter 5: Results

Comparative Analyses of Sex-Disparity in Protein Targets in Murine Model & Human BAL Samples

Chapter 5: Comparative analyses of sex-related differences in allergen-mediated increase of specific proteins in murine and human bronchoalveolar lavage fluid.

This section contains some text and figures for a manuscript in final preparation, submitted to *Frontiers in Immunology* as a Brief Research Report.

Manuscript Title: Sex dimorphism of allergen-induced secreted proteins in murine and human lungs.

Manuscript ID: 923986

Authors: Mahadevappa Hemshekhar[#], **Dina Mostafa**[#], Victor Spicer, Hadeesha Piyadasa, Andrew J. Halayko, Christopher Carlsten, Neeloffer Mookherjee

[#]These authors contributed equally

Author contributions for the manuscript in final preparation:

MH and DM performed most of the experiments and data analyses. MH wrote the manuscript and contributed to the development of the scientific concepts. HP performed the animal model experiment with DM. VS performed the bioinformatics analysis. AJH provided significant intellectual input in the development of this study and extensively edited the manuscript. CC was the senior lead for the human exposure study, performed the bronchoscopy and sample collection, provided extensive intellectual input and extensively edited the manuscript. NM conceived and directly supervised the study, is the principal investigator for funding in this study, and extensively edited the manuscript. All authors reviewed and edited the manuscript.

5.1 Abstract

Background: Sex bias in asthma prevalence and pathogenesis has been reported from clinical studies. However, sex-specific changes in allergen-induced proteins in the lung and associated protein biomarkers are not yet defined.

Objective: To define sex-specific allergen-mediated protein changes in BALF of humans and BALB/c mice, with a specific focus on proteins that are commonly enhanced in both mice and human following allergen exposure.

Methods: Previously, our lab utilized an unbiased proteomic approach to characterize allergen-induced changes in secreted proteins (secretome) in the lung of female BALB/c mice (2-week HDM-challenge) and in a controlled allergen exposure human model. Comparative analysis of these two proteomics datasets detected 19 proteins commonly enhanced in both mice and human BALF, in response to allergen challenge. The top 10 protein targets selected from these 19 proteins were examined in BALF of mice and humans (in independent studies) using western blot. Briefly, female and male BALB/c mice (N=10, per group) were intranasally challenged with HDM (0.7 µg/mL in saline) for 2 weeks and BALF was collected 24hr after the last HDM administration. In the human study, female and male participants (N=6 each) were exposed to nebulized inhalation (2 min) with allergens (birch, grass or HDM, based on individual sensitivity). BALF samples from mice and humans were probed in immunoblots and relative abundance was determined for each protein.

Results: Proteins S100A8, S100A9, properdin and eosinophil peroxidase (EPX) were significantly higher in BALF of female mice compared to males in response to HDM-challenge. Similar to the results in mice BALF, female participants showed higher levels of allergen- induced EPX compared to males in human BALF. Contrary to the mice data, the allergen-induced increase of S100A8 and S100A9 in the human BALF samples were higher in males compared to females. Interestingly, there was certain species-specific sex-bias in allergen-induced increase of proteins in BALF. For example, coronin1A/TACO was significantly higher in female participants compared to males, only in human BALF Whereas the HDM-driven increase in properdin was significantly higher in females compared to males, only in murine BALF and not in humans.

Conclusion: Overall, these results demonstrate that among secreted proteins that are enhanced in response to allergen challenge in the lungs of both mice and humans, sex-related differences in response to allergen is largely species-specific and thus cannot be always translated from mouse models to human studies. These results also show that EPX, a marker of eosinophilic inflammation, is significantly higher in the BALF of females compared to males in both human and mice BALF following allergen challenge. Therefore, EPX will be a useful sex-specific biomarker of airway inflammation in translational research.

5.2 Rationale & Introduction

Asthma is a chronic respiratory disorder that displays a predominance in prevalence in males during childhood that shifts to predominance in females with increasing disease severity in adulthood [224, 239, 268, 335]. Yet, there are no allergen-induced protein biomarkers defined in the lung that stratify this sex disparity. Proteomic analysis is a potent tool for identifying biomarkers to improve clinical diagnosis and help to develop a more personalized treatment approach [336–338]. Previously the Mookherjee lab has performed proteomic profiling using liquid chromatography coupled with mass spectrometry (LC–MS) and detected enhancement in secreted proteins in BALF following allergen challenge in BALB/c mice (females) and in a controlled human exposure model [339, 340]. Comparative bioinformatic analysis of the mice and human proteomic datasets identified a commonly upregulated allergen-mediated protein biosignature in both mice and humans following allergen challenge (Figure 12). In this study, we selected the top 10 targets from this protein biosignature and examined sex-specific differences in the abundance of these 10 protein targets, in both murine and human BALF. we independently examined the abundance of the selected 10 protein targets using western blot and examined sex-specific differences therein in BALF obtained from an HDM-challenged mouse model (as detailed in Chapter 4) and from humans exposed to a nebulized allergen. Human exposures were performed by our collaborator Dr. Christopher Carlsten at the University of British Columbia, and we obtained the BALF samples through this collaboration.

Figure 12

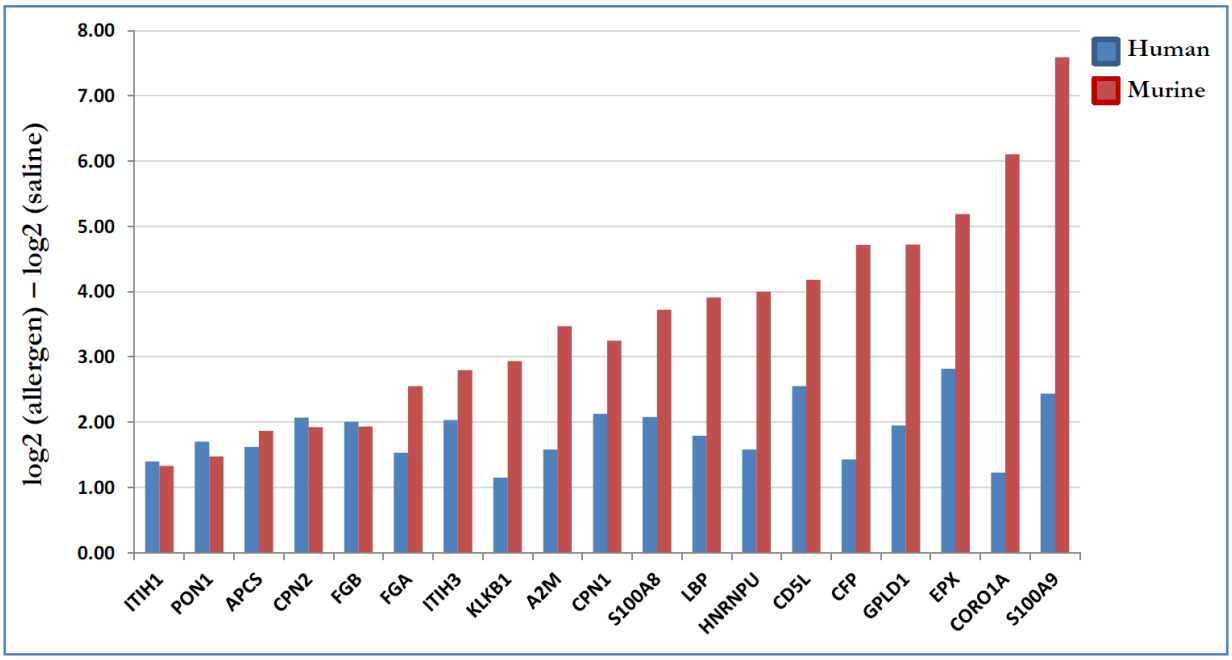


Figure 12: Common allergen-induced lung proteomic signature in murine and human models. BALF samples obtained from female BALB/c challenged with HDM for 2 weeks (Fig. 4) and from human controlled exposure studies were used to characterize the profile of secreted proteins in BALF (secretome). Mice and human BALF samples were analysed by liquid chromatography—tandem mass spectrometry (LC–MS/MS). Comparative analysis between the mice and human proteomic datasets [339, 340] identified 19 common proteins upregulated in both mice and human following allergen challenge. Expression of these 19 proteins was increased ≥2-fold in response to allergen exposure in both mice and human BALF. The values on the Y-axis represent upregulated protein values in BALF in response to allergen challenge normalized to saline obtained from control saline exposure samples.

5.3 Results

5.3.1 Female BALB/c Mice Exhibit Higher Levels of Allergen-Mediated Proteins Abundance in BALF

Proteomic analysis of secreted proteins in BALF allow for the identification of disease biomarkers and provide guidance for novel therapeutic strategies [336–338]. The Mookherjee lab had previously used a proteomic approach using LC-MS to characterize allergen-induced alteration of secreted proteins in BALF (secretome) of female BALB/c mice as well as in the human lung proteome in a controlled human exposure model [339, 340]. A comparative bioinformatic analysis between the human and mouse proteomic datasets revealed 19 common proteins that were upregulated in BALF of both mice and humans in response to an allergen challenge (Figure 12). The abundance of these 19 proteins was increased by ≥2-fold following allergen challenge in both mouse and human BALF. To examine the presence of any sex-specific differences in this common allergen-mediated biosignature, we selected the top 10 protein targets that are upregulated more than 4-fold in the mouse and 2-fold in the human BAL following allergen challenge. These were arbitrary cut-off values to select a set of proteins for further independent confirmation studies. The top 10 proteins that are upregulated more than 4-fold in the mouse and 2-fold in the human BAL following allergen challenge selected for further validation studies were S100A8, S100A9, eosinophil peroxidase (EPX), properdin, coronin1A/TACO, CD5 antigen-like protein (CD5L), glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD), heterogeneous nuclear ribonucleoprotein U (hnRNPU), lipopolysaccharide binding protein (LBP) and calponin. The abundance of these 10 proteins in mouse BALF was further examined using western blots.

BALF from female and male BALB/c mice, naïve and HDM-challenged (N=10 in each group), was obtained 24 h after the last HDM challenge for this study. BALB/c mice were used in this study as the proteomics evaluation from which the protein targets were selected was performed in BALB/c mice. S100A8, S100A9, EPX and properdin levels were significantly enhanced in the BALF of HDM-challenged female mice compared to allergen-naïve mice, but not in male mice (Figure 13). No significant differences were observed in the other proteins examined in response to allergen in either female or male mice (Figure 13 and supplementary Figure 3).

Thus, out of these 10 proteins examined, four proteins in BALF showed significant differences in abundance in female compared to male mice in response to HDM (Figure 13 and supplementary Figure 3). In comparison to male mice, female BALB/c mice displayed a higher fold change in HDM-induced proteins in S100A8 (~2-fold), S100A9 (>3-fold), EPX (>2-fold) and properdin (~5-fold). These results suggest that there is a clear female-bias in specific HDM-induced protein levels in murine BALF.

Figure 13

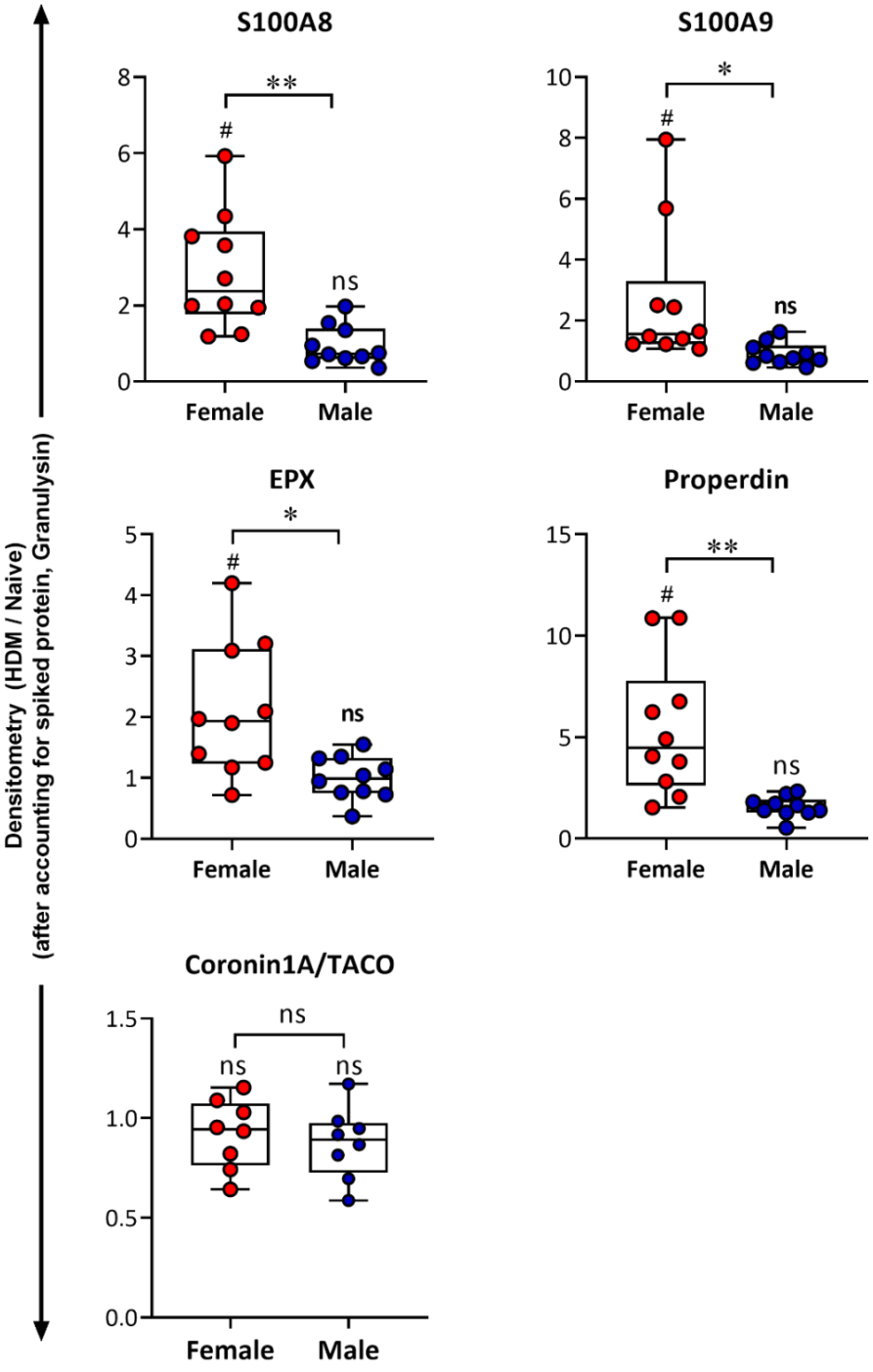

Densitometry (HDM / Naive)
(after accounting for spiked protein, Granulysin)
S100A8
**
#
ns
Female
Male
S100A9
*
#
ns
Female
Male
EPX
*
#
ns
Female
Male
Properdin
**
#
ns
Female
Male
Coronin1A/TACO
ns
ns
ns
Female
Male

Figure 13: Sex-specific protein biomarker signature in BALB/c mice BALF. Female and male BALB/c mice (N=10 each) were challenged with (i.n.) administration of whole HDM extract (0.7 mg/mL) in saline for 2 weeks and BALF was collected 24 h after the last HDM administration. BALF samples were probed in western blots to determine the abundance of selected protein targets. Relative protein expression levels were quantified by densitometry. Protein band intensities were normalized to loading protein (recombinant human granulysin; 20 ng per sample) as a control for protein abundance quantification. Y-axis represents fold change of relative protein abundance of each HDM-challenged mouse compared to mean abundance in naïve mice (HDM/naïve). Each dot represents an individual mouse. Mann–Whitney U-test was used to assess statistical significance (*$p<0.05$, **$p<0.01$, ns=non-significant). A hash (#) denotes a significant difference ($p<0.05$) between HDM-challenged mice versus the naïve group ($p < 0.05$).

5.3.2 Sex-Related Differences in Allergen Mediated Secreted Protein in Human BALF

As detailed above, 10 proteins were selected for assessment by western blot in murine and human BALF in this study. To define sex-specific protein changes in human BALF, we compared the abundance of the selected 10 proteins in BALF of male and females with atopic asthma collected 24 h after inhaled allergen exposure. Female and male adult participants (N=5 each) inhaled nebulized allergen (HDM, birch or Pacific grass, participant-adjusted based on wheal to skin-prick) for 2 minutes. BALF collection was conducted by bronchoscopy 24 h following allergen exposures (by collaborator Dr. Carlsten at UBC).

Similar to mice, four out of the 10 proteins examined displayed sex-related differences in protein abundance in human BALF following allergen exposure (Figure 14 and supplementary Figure 4). Compared to male participants, females showed higher abundance of EPX and coronin1A/TACO (~2-fold) in BALF (Figure 14). Interestingly, EPX was the only protein which showed a clear female bias in both mice and humans (Figure 13 and Figure 14). Contrary to the mice data, allergen-induced increase of S100A8 and S100A9 in the human BALF were higher in males compared to females (Figure 14). These results clearly showed differential increase of specific proteins in the BALF between female and male participants in response to inhaled allergen exposure. These results demonstrate that certain inflammatory responses are similar between mice and human in response to allergen, such as eosinophilic inflammatory mediator EPX. In addition, these results highlight that not all findings in mice can be translated to humans.

Figure 14

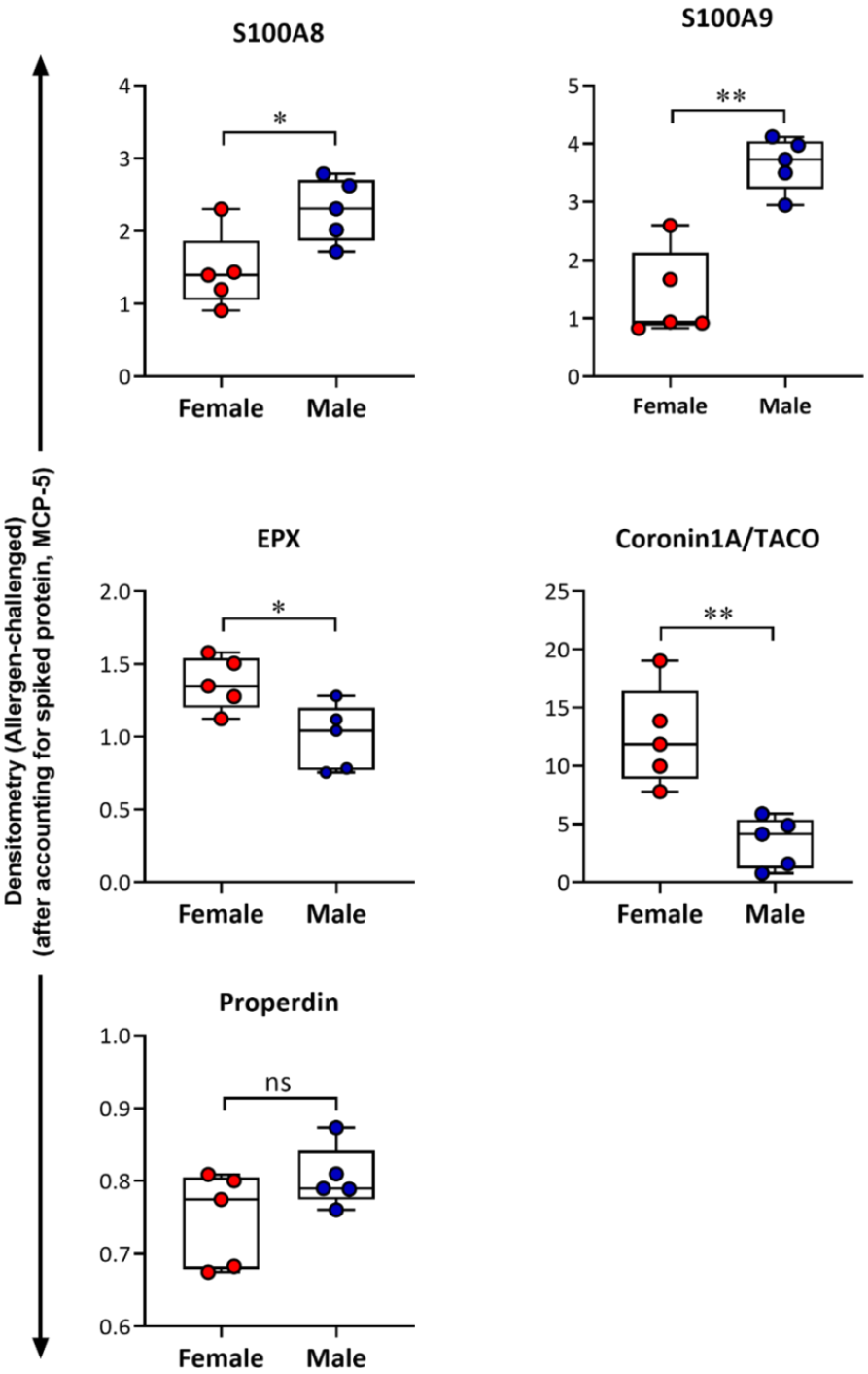

S100A8
S100A9
EPX
Coronin1A/TACO
Properdin
Densitometry (Allergen-challenged)
(after accounting for spiked protein, MCP-5)
Female
Male
*
**
ns

Figure 14: Sex-disparity in allergen-induced secreted proteins in human BALF. Female and male adult participants (N=5 each) inhaled nebulized allergen (HDM, birch or Pacific grass, participant-adjusted based on wheal to skin-prick) for 2 minutes. BALF collection was conducted by bronchoscopy 24 h following allergen exposure. BALF samples were probed in western blots to determine the abundance of selected protein targets. Relative protein expression levels were quantified by densitometry. Y-axis represents protein band intensity normalized to loading protein (recombinant mouse MCP5; 10 ng per sample) as a control for protein abundance quantification. Each dot denotes an individual participant. Mann–Whitney U-test was used to assess statistical significance (*$p<0.05$, **$p<0.01$, ns=non-significant).

5.4 Discussion & Conclusion

Sex disparity in the susceptibility and severity of inflammatory lung diseases have been reported [181, 224, 239, 268, 335]. Yet, there is a lack in reports of any sex-specific protein disease biomarkers from preclinical research using animal models as well as in asthma patients. This is the first study to identify a 19 allergen-induced secreted protein biosignature in the lungs that is enhanced similarly in both mice and humans mined from previous proteomic studies [339, 340]. Further this study identified sex-specific protein changes in response to inhaled allergen in the lungs. In this study, the allergen-induced secreted proteins identified in the BALF of mice that were differentially increased in females compared to males were Properdin, S100A8, S100A9 and EPX (summarized in Figure 15). While, coronin1A/TACO, S100A8, S100A9 and EPX showed sex-related differences in the human BALF following exposure to inhaled allergen (summarized in Figure 15). There may be several contributing factors for differences between mice and humans, such as the amount and type of allergen used. Moreover, the inflammatory environment in inbreb mice are more tightly controlled compared to the variability in humans. The progression and severity of response in humans adds to the variability of protein responses. Finally, the two week HDM-challenge mice model used in this study does not capture all the endotypes related to allergic asthma in humans. These caveats need to be taken into consideration for biomarker identification. Nevertheless, the protein biosignature related to sex dimorphism in response to allergen highlighted in this study provides novel disease biomarkers that will be useful for research using personalized sex-specific approaches.

In this study, we showed that there is an overlap in sex-bias in the increase in EPX in response to allergen in mice and human BALF. The abundance of EPX is significantly higher in females compared to males in the BALF of both mice and humans in response to allergen challenge. EPX is an eosinophil activation and degranulation marker as well as it is a potent marker of airway eosinophils in sputum of asthma patients [341]. Consistent with the results in this study, eosinophilic airway inflammation induced by allergen has been shown to be more severe in females compared to males in both mice and humans [233, 342]. Remarkably, in the current study EPX was the only protein to show a female-bias in allergen-mediated changes in both mice and humans. These results imply that EPX can be considered as a female-biased marker for allergen responses in allergen-challenged murine models and in human studies using inhaled allergen

challenge. These results also suggest that EPX can be investigated as a potential therapeutic target to reduce the severity of eosinophilic inflammation, which may be more effective in females.

We also demonstrated specific disparities between mice and humans in terms of the sex-bias in allergen-enhanced proteins. For example, S100A8 and S100A9 were significantly higher in the BALF of female BALB/c mice compared to males, in response to HDM. However, this sex-bias was reversed in humans, where male participants showed higher levels of allergen-induced S100A8 and S100A9 in BALF compared to females. These results emphasize that results observed in mice might not be exactly replicated in humans. S100A8 and S100A9 are alarmins that belong to the S100 family of calcium (Ca2+) binding proteins, typically present in a heterodimer S100A8 and A9 (known as Calprotectin) [343]. S100A8 and S100A9 are mainly expressed in monocytes, macrophages and neutrophils and are actively released during inflammation [344]. They mediate the severity of inflammatory process by promoting the recruitment of leukocytes and secretion of cytokines [345]. Thus S100A8 and S100A9 proteins have been proposed as biomarkers for diagnosis as well as indicator of inflammatory status and therapeutic responses in multiple chronic inflammatory conditions [343, 345–347]. Within pulmonary diseases, S100A8 and S100A9 are implicated as a mediator in severe asthma pathogenesis through exaggerating neutrophil responses and supressing neutrophil apoptosis [348, 349]. However, there is conflicting evidence regarding the ability of S100A8 and S100A9 proteins to enhance and/or suppress Th2–mediated allergic airway inflammation in murine models [350, 351]. Thus, further research is needed to clarify the precise role of S100A8 and S100A9 in asthma and elucidate the mechanistic pathways involved in asthma pathogenesis. Importantly, the results of this study points towards S100A8 and S100A9 as potential biomarkers and the possibility of therapeutic applications targeting S100A8 and S100A9 proteins specifically for a more personalized sex-specific approach. However, the sex-bias as demonstrated in this study for these proteins are different in mice and humans, which needs to be considered while using these proteins as either biomarkers or intervention targets in preclinical research. Additionally, we demonstrated disparities in the allergen-induced increase in specific proteins between mice and humans. For example, properdin levels were significantly higher in female mice compared to males. However, this sex-specific difference in properdin was not observed in humans. Properdin promotes alternative pathway complement activation and plays a pathogenic role in airway inflammation primarily through regulating Th2 and Th17 immune responses [352].

However, further studies are needed to delineate the properdin mechanism of action in asthma. The results in this study indicate the presence of sex dimorphism of complement-dependent responses in airway inflammation which needs to be taken into consideration for any subsequent studies that target the complement pathway for therapies.

Furthermore, we showed that an allergen-induced increase in coronin-1A/ TACO is significantly higher in human BALF from females compared to males, however this sex disparity in coronin-1A/ TACO abundance was not observed in mice. Coronin-1A regulates cytoskeleton-dependent cellular processes and Ca2+ signaling [353]. It is highly expressed in all hematopoietic lineages and plays a critical role in T-cells in survival, migration and activation [354, 355]. Yet there is limited data on how coronin-1A contributes to asthma pathogenesis. A recent study identified coronin-1A as a potential biomarker in the sputum of patients suffering from frequent exacerbations in various airway diseases (asthma, COPD and chronic bronchitis) [356]. In this study, we demonstrated a female-bias in coronin-1A levels in BALF of asthmatics in response to allergen that was not present in mice.

The main limitation of this study is the small sample size of human BALF samples as well as there were no BALF samples from healthy human participants as controls. This is mainly due to the challenges of obtaining BALF from healthy participants. The novelty of this study is in defining a sex-specific biosignature in BALF of both mice and human which are clinically relevant. In summary, the results from this study highlight the effect of biological sex on allergen-induced proteins in both mice and humans. This emphasises the necessity of a more focused approach in delineating sex differences in airway inflammation to improve clinical outcomes through attention to sex. The discrepancies between mice and humans noted in this study in regards to sex-specific protein changes highlight the challenges in extrapolating biomarkers from inbred mice to humans. Overall, the sex dimorphism of inhaled allergen-medicated secreted protein biosignature in the lungs defined in this study provides novel biomarkers that may be useful in a more personalized sex-specific research approach in preclinical studies in mouse models of allergen-challenge and in studies with human participants.

Figure 15

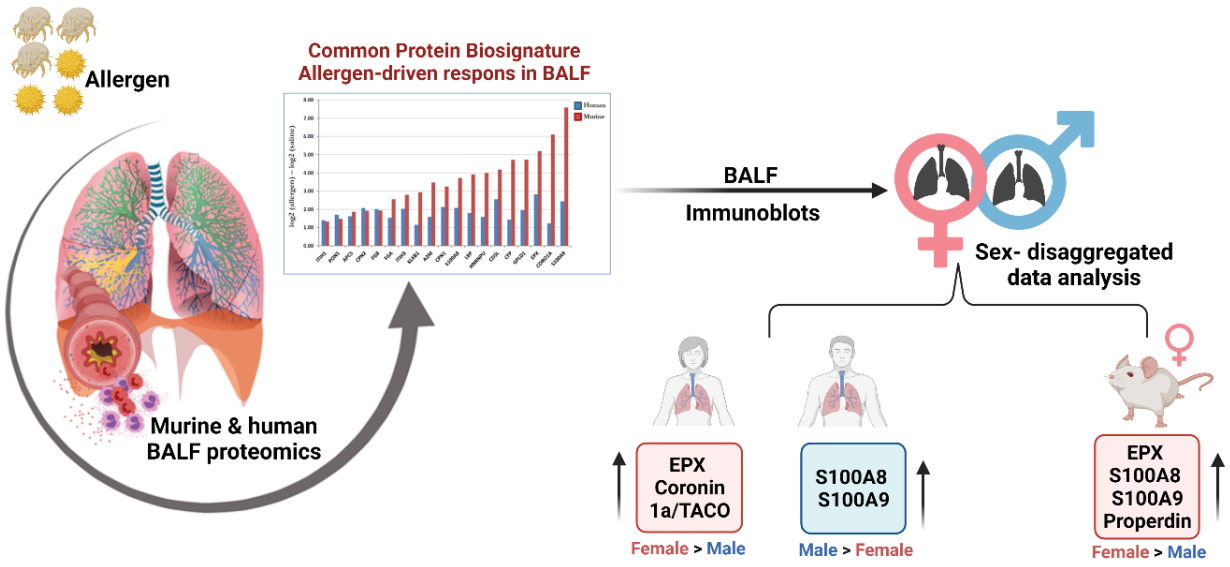


Figure 15: Sex-related differences in specific allergen-induced proteins in BALF of mice and humans. This figure summarizes sex-specific differences in secreted proteins in the lung in both mice and humans. A comparative bioinformatic analysis of proteomics datasets obtained from murine and human BALF secretome studies identified a common panel of proteins that were enhanced in response to allergen challenge in both mouse and human BALF. Independent examination of the abundance of top 10 selected proteins showed sex-bias in response following inhaled allergen challenge. Sex-disaggregated data analysis demonstrated that an inhaled allergen-mediated increase in EPX and coronin1A/TACO is higher in the BALF of adult human females compared to males. Conversely, allergen-mediated increase in S100A8 and S100A9 were higher in BALF of males compared to female human participants. Allergen-driven EPX, S100A8, S100A9 and properdin were higher in the BALF of female mice compared to males.

Chapter 6: Overall Conclusions, Limitations and Significance

6.1 Overall Conclusions

The historical omission of women from clinical trials and exclusion of biological sex as a variable in biomedical research have hindered critical discoveries and the understanding of interventions variation by sex [357]. The immune system displays a marked sex dimorphism in susceptibilities and prevalence to chronic inflammatory conditions between females and males [58, 81, 267]. A clear sex-bias exists in asthma prevalence and severity where in adulthood females have higher prevalence and disease severity compared to males [166, 271, 335]. Further, female asthma patients are more prone to become unresponsive to corticosteroid therapy [168, 224, 239, 240, 358]. Surprisingly little is known about the impact of biological sex on airway inflammation processes, largely due to the disregard of the effects of sex as a biological variable in preclinical studies using murine models in asthma research. Therefore, the overall aim of this thesis was to examine sex-related differences in immune responses and protein markers in a murine model of allergen HDM-mediated airway inflammation and in a controlled human allergen exposure model. In this study, we used the two-week HDM-challenged murine model to characterize sex-related differences in airway inflammation in two different mouse strains (BALB/c and C57BL/6NJ). I demonstrated sex-related differences in circulating HDM-IgE levels as well as leukocyte accumulation in BALF following HDM-challenge, with notable differences between the two mouse strains (summarized in Figure 11). Further, we identified a sex-specific cytokine signature in the lung, both in naïve and HDM-challenged mice that was notably different between BALB/c and C57BL/6NJ (summarized in Figure 11). Additionally, we identified sex dimorphism in allergen-induced protein abundance in BALF of both mice (BALB/c) and humans (summarized in Figure 15).

Allergen-specific IgE antibodies are pivotal in the augmentation of inflammation and asthma pathogenesis and indicate a robust systemic response to allergen sensitization [290, 359, 360]. Findings in this study indicate that females show a stronger IgE antibody response than males in response to inhaled allergen challenge however the intensity of response appears to be influenced by genetic background.

Leukocyte influx to the lung in response to allergen is a key marker of airway inflammation [182, 361, 362].

Both neutrophilic and eosinophilic inflammation contribute to the development of asthma exacerbation and severity particularly neutrophils which are strongly associated with severe asthma and steroid insensitivity corticosteroid resistant asthma [210, 363–365].

Here, we demonstrated that HDM-induced BALF total cells, eosinophils, neutrophils, and macrophages were higher in female BALB/c mice compared to males. This illustrates a clear female bias in cellular infiltration suggesting a stronger inflammatory response in female mice compared to males. Contrary to BALB/c, we demonstrated that male C57BL/6NJ mice have higher eosinophil levels compared to female mice following HDM-challenge. These results not only highlight the presence of sex dimorphism in leukocyte accumulation in response to an inhaled allergen but also variation between mice strains. These findings emphasise the importance for researchers not only to take sex differences into consideration but also genetic variability between the mouse strain. This particularly becomes vital for studies using knockout or transgenic mice to outline mechanisms that examine sex-related differences in airway inflammation. The findings in this study are consistent with the influence of gene-environment interactions in disease development and response.

To date there has been little focus on delineating sex-related differences in cytokine responses, as most of the previous studies have focused on Th2 cytokines in allergen exposure. The novelty of this study is that it outlines a more comprehensive sex-specific cytokine signature in two different mouse strains. Here, we demonstrate sex dimorphism in the profile of 29 cytokines in both naïve and HDM-driven response in BALB/c and C57BL/6NJ mice, in both BALF (secreted proteins) and lung tissue lysates. Interestingly, we showed sex-bias in key inflammatory cytokines in naïve mice, in particular basal IL-5 levels were higher in allergen-naive female BALF and lung tissue lysates compared to males, in both mouse strains. In contrast, naïve male mice showed higher levels of KC/GRO compared to female mice in BALF of both mouse strains. While in lung tissue, naïve male mice showed higher level of IL-17A in C57BL/6NJ mice and IL-17A/F in BALB/c mice, compared to females. These results highlight sex-specific differences in basal cytokine levels in adult allergen-naïve mice which need to be considered when examining changes in cytokine markers in airway inflammation. These findings allude to sex-related differences in the homeostatic immune lung environment which could influence sex-bias in inflammatory responses following allergen exposure.

The results in this study showed that HDM drives a Th17-skewed cytokine response in female BALB/c mice, and a Th1-skewed cytokine response in female C57BL/6NJ mice, compared to males. In contrast, male mice from both strains showed higher levels of Th2-associated cytokines in the lungs in response to HDM. These results highlight a clear strain-dependent sex bias in cytokine responses, further emphasising the importance of considering the influence of mouse strain variability on inflammatory responses and sexual dimorphism. Overall, this study describes two different strain-dependent models with different inflammatory phenotypes which could be used to study mechanisms related to sex dimorphism in asthma research.

Th17-biased inflammatory responses are associated with severe neutrophilic asthma [312, 313, 318, 366, 367], thus female-bias in Th17-skewed responses reported in this study could potentially explain higher prevalence of severe asthma and poor response to steroid therapy reported in clinical studies. Recently, genome-wide analysis of sex-specific gene expression in adult asthmatic patients revealed that IL-17 and related chemokine signaling pathways were highly enriched in female BALB/c mice compared to males [368]. These results are consistent with findings in this study of higher IL-17 levels, and MIP-3α which is a chemoattractant for Th-17 cells, in female BALB/c mice compared to males, which underscores that results in this study are consistent with clinical observations in asthma patients.

Sex differences in lung protein biomarkers are not completely defined from either murine models of airway inflammation or asthma patients. Here, we report sex differences in the abundance of specific proteins in response to an inhaled allergen challenge, secreted in the BALF of BALB/c mice (following a 2-week inhaled HDM-challenge) and human atopic asthma patients. we demonstrated that allergen exposure resulted in higher levels of EPX, a marker for eosinophilic inflammation, in females compared to males in both mice and humans. Therefore, EPX may serve as a valuable sex-specific marker in asthma research and preclinical studies of airway inflammation. Our results also show that there are distinct differences in mice and humans. For example, allergen challenge resulted in enhancement of properdin in mice and not humans, whereas coronin1A/TACO was increased in humans and not mice.

Moreover, S100A8 and S100A9 were significantly higher in females compared to males in mice, whereas human participants showed higher levels of allergen-induced S100A8 and S100A9 in males compared to females.

These findings clearly demonstrate that sex-related protein changes in response to allergen are species specific, and not all results demonstrated in mouse models can be translated in human studies. However, our findings provide specific protein markers that are valuable for preclinical studies in animal models and in human translational studies and highlight the importance of sex stratification in asthma biomarker research.

6.2 Limitations

The main limitation of this study is that the mouse model used here reflects a specific asthma endotype-related immunophenotype (predominantly eosinophilic airway inflammation as determined by leukocyte composition in the lungs post allergen challenge), and does not directly capture the heterogeneity observed in asthma patients. The 2-week acute murine model used in this study allows for delineating predominantly airway inflammation preceding robust lung remodelling thus, any sex-related differences in airway remodeling were not assessed in this study. Also, all outcomes in this study were determined at a single time point after allergen challenge. Therefore, kinetic assessments of leukocyte accumulation and cytokine response might display disparate sex difference profiles at different time points and show similarities between the two strains of mice with different kinetics of response. As this study was limited on examining cell differentials using hematoxylin and eosin stain in BALF, any possible sex differences in different Th subsets were not examined. Therefore, immunophenotyping cells in BALF would be a better approach to be implemented in future studies to examine sex differences in cellular composition in BALF.

6.3 Significance

Overall, the work in this thesis outlines molecular mediators that differentiate sex-related responses following allergen challenge in two different strain-dependent mouse models. Results in this study will provide the foundation for future studies to further understand mechanisms that shape sex-disparity in inflammatory and allergic disease processes. The novelty of this work in delineating sex differences in inflammatory responses in two different mouse strains highlights the impact of genetic variability on immune responses as well as sex-related differences.

Thus, my work provides a valuable resource for researchers and lays the foundation for future work that recognises the importance of taking sex into consideration when developing novel therapies. This work will be vital for ongoing research efforts for developing new treatments for asthma particularly it will benefit translational research and sex-based personalized drug development efforts in asthma.

The inclusion of a sex-disaggregated data analysis in biomedical research will advance the discovery of sex-specific disease biomarkers, and more importantly, promote reproducibility in subsequent validation studies. This approach will facilitate the translation of novel biomarkers from animal models to human studies in a sex-dependent manner. Additionally, the inclusion of biological sex will further promote a deeper understanding of sex dimorphism in disease prevalence and pathophysiology, subsequently leading to the development of more sex-related personalized effective interventions.

Chapter 7: Future Direction

7.1 Future Direction

Asthma prevalence and severity display a clear age and sex bias, affecting boys in childhood and disproportionately affecting women in adulthood [166, 224, 271, 335]. Consequently, the sex–age interaction adds another layer of complexity for understanding the effects of biological sex in asthma, which indicates a role of sex steroids (hormones). Therefore, to evaluate the potential role of sex steroids in mediating sex-related differences reported in this study, it is necessary to 1) examine hormone receptor expression changes following allergen-exposure in lung cells, 2) circulating sex steroids levels in plasma and 3) the effect of estrous cycle on modulating inflammatory response to allergen.

We have generated preliminary data to address some of the aforementioned questions. We assessed the expression of different sex steroid receptors, ERα, ERβ, AR and GPR30 in lung tissue lysates obtained from the animal model used in this study by western blots. Interestingly, I have demonstrated that the expression of ERα and Erβ was significantly increased in female BALB/c lung tissue (Figure 16). In contrast, AR was downregulated in HDM-challenged mice compared to naïve mice in both sexes. GPR30 was downregulated in HDM-challenged male mice compared to allergen naïve BALB/c (Figure 16). These results suggest that sex steroid receptors change in responses to allergen challenge in the lungs.

Previous studies have revealed a differential inflammatory response in each estrous cycle stage (proestrus, estrus, metestrus, and diestrus) in females that are exposed to ozone [369]. This indicates that the estrous cycle stages can influence the inflammatory profile in the lung. Thus, a follow up study is required to examine the effects of fluctuating hormones across the female reproductive cycle on leukocyte accumulation and cytokine responses following allergen exposure. Future studies should include the examination of the abundance of sex steroid panel (cortisol, testosterone, 17β-estradiol and progesterone) in serum in the HDM-challenged mouse model using LC-MS-MS. This is planned in the Mookherjee lab in collaboration with Dr. Kiran Soma at the University of British Columbia (UBC). The Soma Lab utilises multidisciplinary approaches to investigate the role of sex steroid hormones in regulating the nervous system and immune system, thus this collaboration will be extremely beneficial in delineating the role of sex steroid hormones in regulating airway inflammation.

Mass spectrometry is the optimal approach to assess steroid hormones as immunoassays have several limitation including standardisation and antibody sensitivity problems between labs [370, 371]. Additionally, monitoring the mouse estrous cycle in the HDM-challenge mouse model through vaginal cytology will be useful to identify whether the stage of the estrous cycle influences the inflammatory profile observed in mice. The estrous cycle in mice is segregated into four main stages (proestrus, estrus, metestrus, and diestrus) which cycles every 4-5 days [372]. Thus, vaginal cytology at the start of the experiment then day 6 and finally 24 h before sacrificing the mice, will allow monitoring the changes in the estrous cycle in the female mice. Additionally, correlating sex steroid levels with estrous cycle (female mice), and with the differences observed in lung cytokine profiles in both sexes in mice may be able to highlight the impact of sex steroids on airway inflammation following allergen exposure.

Figure 16

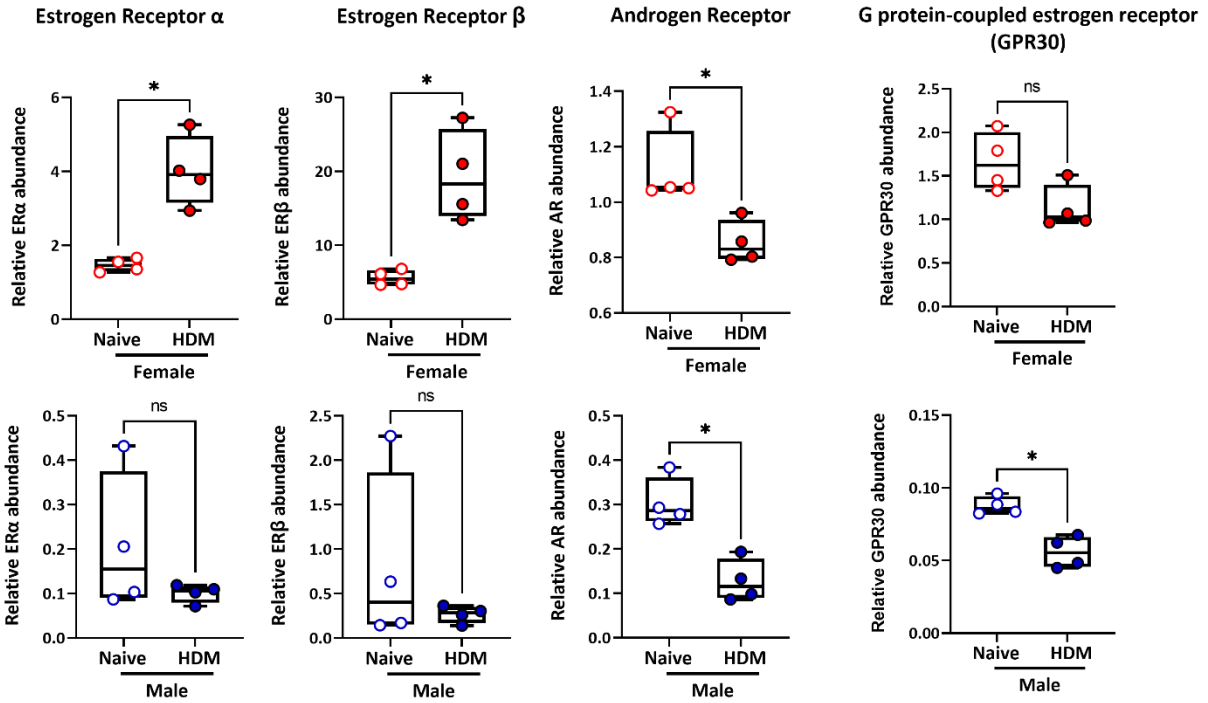


Figure 16: The abundance of ERα, ERβ, AR and GPR30 in BALB/c lung tissue. Female and male BALB/c mice (N=4 each) were challenged intranasally for 2 weeks with 35 µL of 0.7 mg/mL whole HDM protein extract in saline, per mouse. Lung tissue was collected from both allergen-naïve and HDM-challenged mice, 24 h after the last HDM administration. The abundance of each receptor was examined using western blots. Lung tissue lysates (50 µg total protein each) were resolved on Nu-PAGE 4-12 % protein gels (Invitrogen) and primary antibodies (Abcam) were used for probing specific proteins on immunoblots. Horseradish peroxidase (HRP) conjugated to secondary antibodies (Cell Signaling) and Amersham ECL Select (GE Healthcare) were used for detection of the target proteins. Imaging of blots and protein quantification was done by densitometry was performed using AmershamTM Imager 680 and software version 2.0. β-actin abundance was used for normalizing the protein load across lung lysate samples. The Y-axis denotes the abundance of selected proteins (band intensity) following normalization with β-actin. Statistical significance was determined using Mann-Whitney U test (*p<0.05, **p<0.01, ns=non-significant).

7.2 Distinguishing Between the Effects of Sex Chromosomes and Sex Steroids Using the Four Core Genotypes (FCG) Transgenic Mice

Sex-related differences in inflammatory responses are mediated through the effects of sex chromosomes and sex steroids as detailed in the introduction chapter (1.2.3). Sex chromosomes can determine the development of either testis or ovaries and induce indirect hormone-mediated effects. Thus, it is challenging to detangle the effects of sex chromosomes from that of sex steroids in murine models. The development of the FCG mouse model (discussed briefly in chapter 1.2.3.1) allow for differentiating between the effects of sex chromosome and sex steroids [97–99]. In this model, the mice sex chromosome complement (XX vs. XY) is not linked to the mice's gonadal sex (testes or ovaries) [88, 99]. The SRY gene encoded on the Y chromosome is necessary for the testicular gonadal formation, whereas the absence of the SRY gene allow for the development of ovaries [88, 97–99]. The deletion and insertion of SRY gene in mice can result in mice with XX chromosomes that have testes and mice with XY chromosomes that have ovaries (Figure 17). Implementing the 2-week HDM challenge used in this study in the FCG mice will be a powerful tool to further delineate whether the sex-related differences in inflammatory profile is driven by sex steroids or sex chromosome complement. Particularly utilising omics-based approaches such as transcriptomic and proteomics will be able to provide insight into sex-related differences in molecular responses following allergen challenge in the FCG mice.

Figure 17

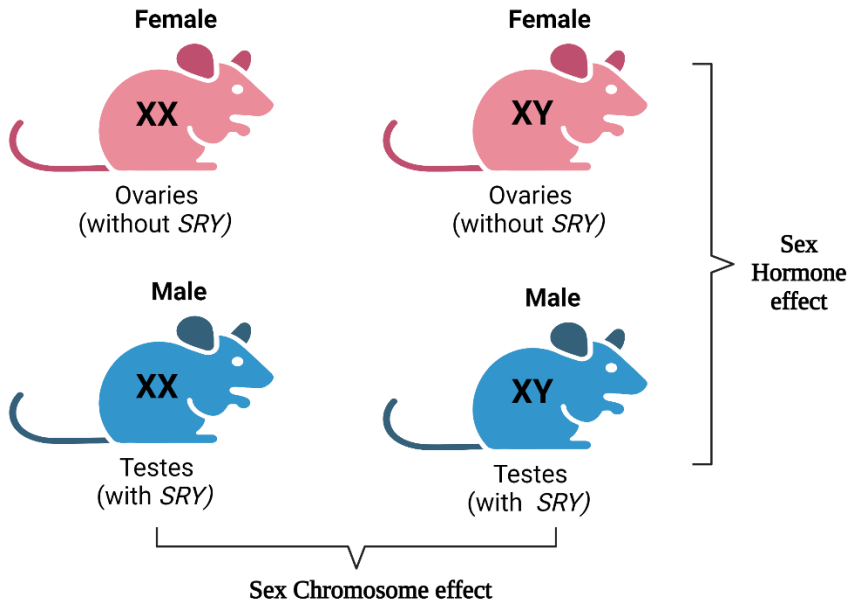


Figure 17: Four core genotypes" (FCG) mouse model. The FCG mouse model distinguishes the effects of sex chromosomes from sex hormones. This model provides a comparison of mice with sex chromosome complement (XX versus XX) but with contrasting gonadal phenotype (ovaries versus testes). The phenotypic results of these mice are dependent on the presence or absence of the Sry gene. This figure was created with BioRender.com.

7.3 Examine Sex-Related Differences in a Chronic Model of Allergic Asthma

The 2-week HDM-challenge model described in this thesis recapitulates airway inflammation preceding lung remodeling. However along with airway inflammation, lung remodeling is a key pathophysiology feature of chronic asthma which involve alteration to composition and structure of the airway walls contributing to clinical manifestation of chronic asthma [373, 374]. Repeated HDM challenge for 5 weeks (chronic model) induces accumulation of collagen, goblet cell hyperplasia and airway thickening and fibrosis along with airway inflammation [291, 375]. Thus, in preliminary studies, we utilised the 5-week chronic model of HDM challenge to examine sex-specific differences in allergen-mediated response. Both female and male BALB/c mice were challenged intranasally with 35 µl of HDM extract (0.7 mg/ml in saline), with five daily intranasal administrations for 5-weeks (Figure 18). we generated preliminary data that demonstrated a significant increase in leukocyte accumulation following repeated HDM challenge for 5 weeks in both female and male BALB/c mice (Figure 18). However, there were no statistically significant differences in leukocyte accumulation between HDM-challenged female and male mice (Figure 18). The chronic allergen exposure model can be used to further assess sex disparity in goblet cell hyperplasia using periodic acid Schiff (PAS) stain in paraffin-embedded lungs sections and collagen deposition in airway tissue using picrosirius red stain. Lung mechanics using a small animal ventilator can be used to examine airway hyperresponsiveness in this model. Transcriptional and proteomic profiling of BALF and lung tissue samples can be used to interrogate sex-specific differences in molecular responses following chronic allergen challenge for defining a sex-specific HDM-induced bronchial biosignature. These approaches will be useful to identify potential avenues for the identification of novel therapeutic targets and disease biomarkers using different allergen challenge protocols that result in distinct immunophenotypes in mice.

Figure 18

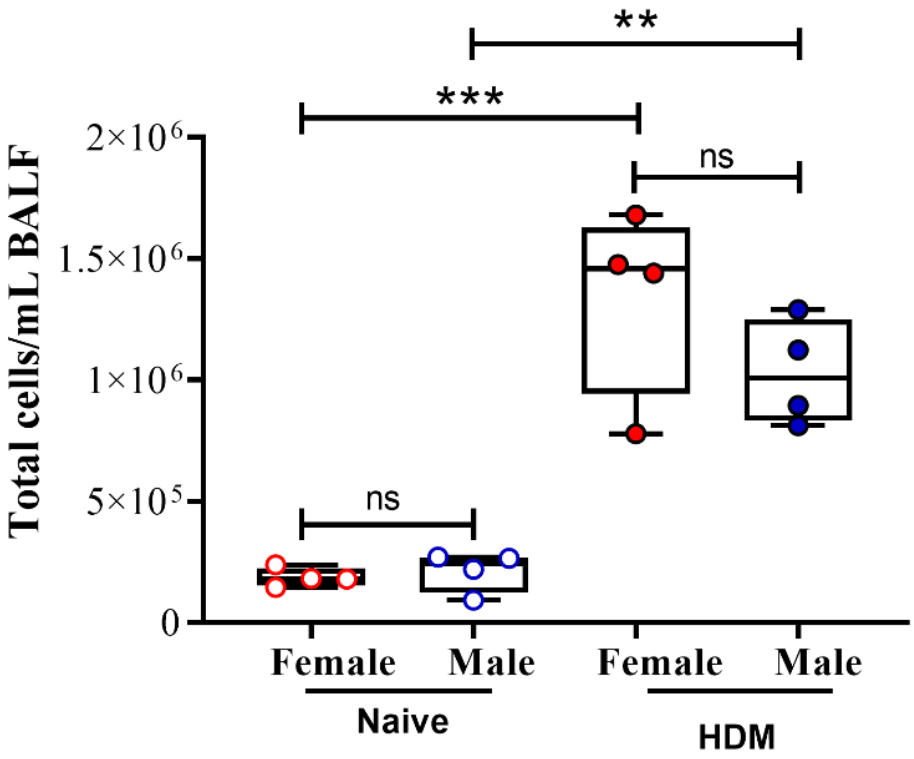


Figure 18: Leukocyte accumulation in BALF of BALB/c mice. Female and male BALB/c mice (n=4 per group) were intranasally challenged by 35 µl of HDM extract (0.7 mg/ml in saline) for 5 weeks. BALF was collected 24 h after the last HDM challenge. Total cell numbers were counted using a hemacytometer. Statistical significance was determined using two-way repeated measures analysis of variance (ANOVA) with Tukey's multiple comparisons test (*p<0.05, **p<0.01, ns=non-significant).

7.4 Examine Sex-Related Differences in Murine Model of Allergic Lung Neutrophilic Inflammation Associated with Severe Asthma

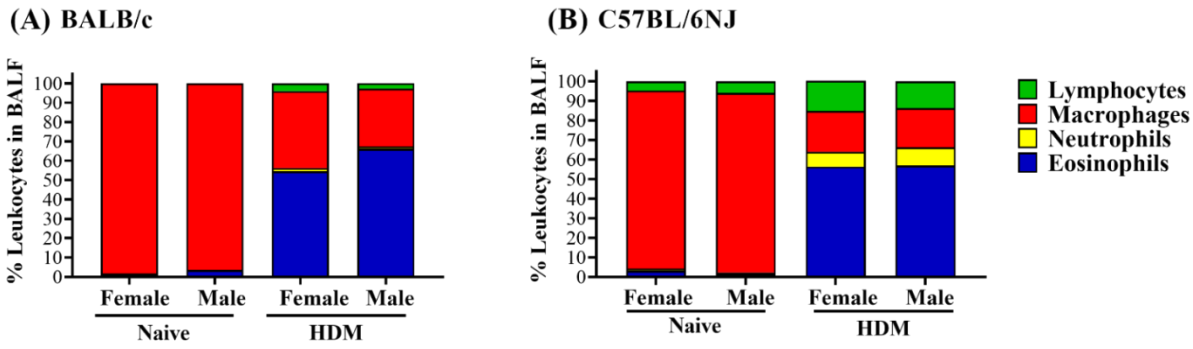
Neutrophilic inflammation is associated with poor responsiveness to corticosteroid treatment as detailed in chapter 1.3.1. Additionally, there is higher prevalence of severe asthma and poor response to steroid therapy in adult females. Moreover, we have demonstrated that female BALB/c mice have higher levels of leukocyte accumulation and IL-17A levels in both BALF and lung tissue in the 2-week HDM model as detailed in chapter 4.1.3. The 2-week HDM murine model used is a predominantly eosinophilic inflammation as demonstrated in leukocyte composition analysis of BALF as detailed in chapter 4.1.3.2. A recent study by Krishnamoorthy and colleagues established a murine model of allergic lung inflammation that has dominant elevation of BALF neutrophils and IL-17 through intranasal administration of LPS and HDM [376]. Thus, utilising this model will be crucial in delineating molecular responses and biomarkers associated with severe asthma. Additionally, utilising transcriptomic and proteomic approaches to outline a sex-specific biosignature in this model can target the unmet need for suitable biomarkers for classifying severe asthma phenotype and predicting sensitivity to inhaled corticosteroids. These studies should also include administration of clinically used inhaled corticosteroids to examine whether there is a sex difference in response to therapy.

In summary, we have briefly outlined possible future approaches to examine the impact of biological sex in allergen-induced airway inflammation. we have presented preliminary data outlined above, that examine contributing factors particularly sex hormones to sex-related differences in airway inflammation. My work in this thesis characterized sex-specific differences in two different mouse strains using a murine model of in allergen HDM-induced airway inflammation. My research emphasises the urgency in the inclusion of sex-disaggregated data analysis in biomedical research and provides key findings that can advance the discovery of sex-specific disease biomarkers.

Appendix

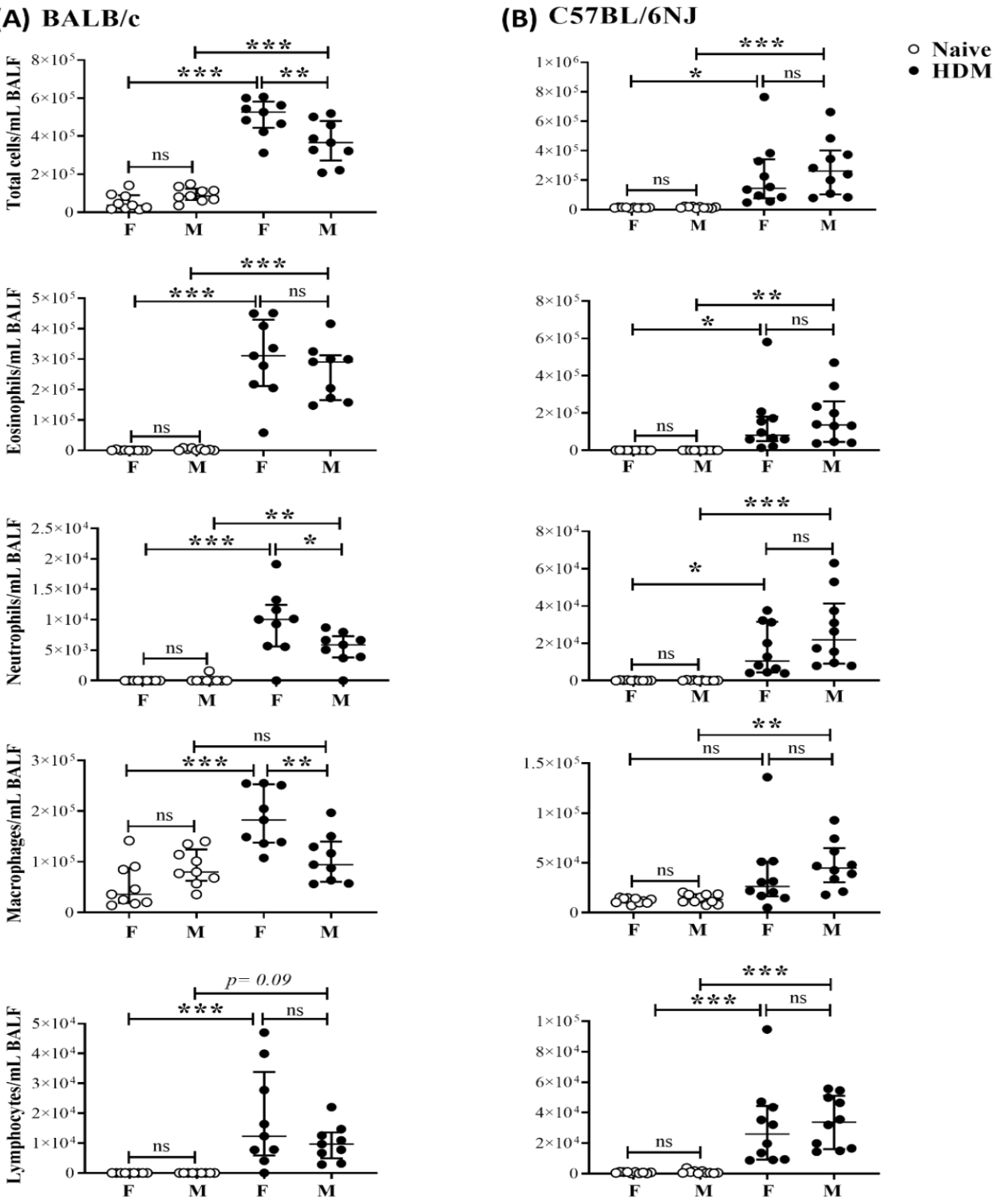
Supplementary Figures

Supplementary Figure 1



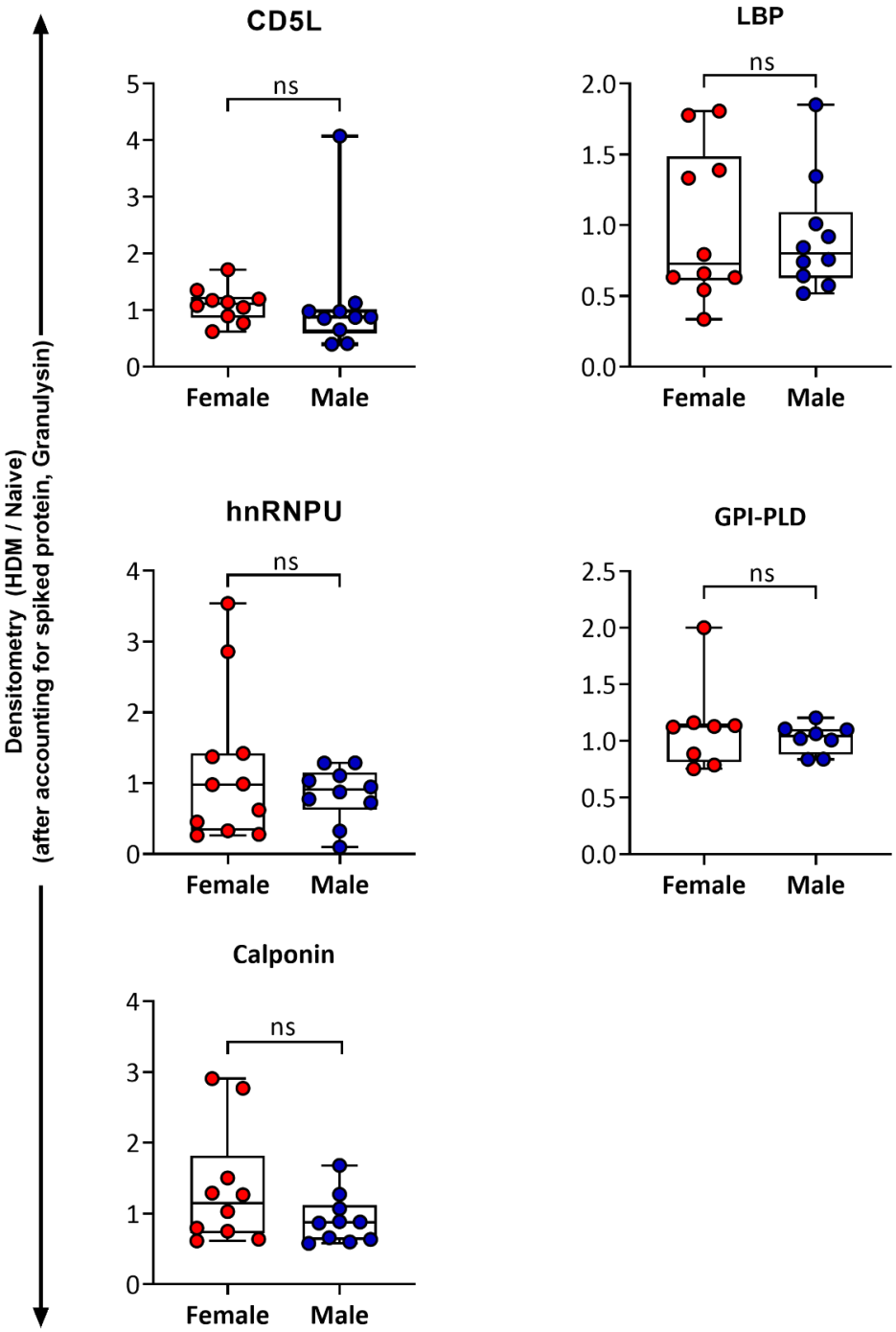
Supplementary Figure 1: Cellular composition of BALF in BALB/c and C57BL/6NJ mice. **(A)** BALB/c mice (n=9 each, female and male, per group) and **(B)** C57BL/6NJ (n=10 each, female and male, per group) were challenged (i.n) with 35 µL of 0.7 mg/mL whole HDM protein extract in saline, per mouse, for 2 weeks. BALF was collected 24 h after the last HDM challenge, and cell differentials assessed with modified Wright-Giemsa stain. Data shown represents mean percentage of each cell type, with total leukocytes set to a 100 percent.

Supplementary Figure 2



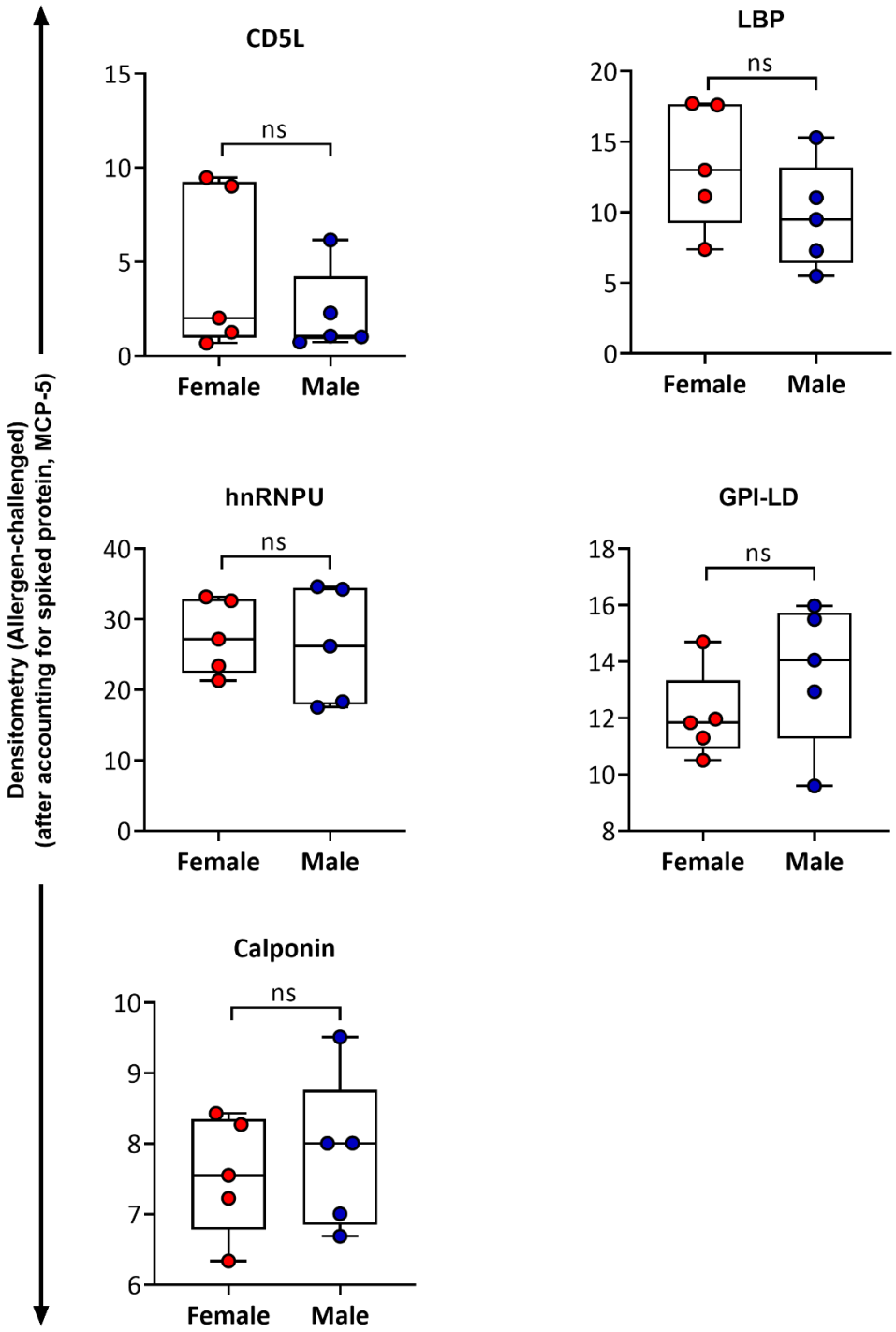
Supplementary Figure 2: Leukocyte accumulation in BALF of BALB/c and C57BL/6NJ mice. (A) BALB/c mice (n=9 each, female and male, per group) and **(B)** C57BL/6NJ (n=10 each, female and male, per group), were challenged (i.n) with 35 µL of 0.7 mg/mL whole HDM protein extract in saline, per mouse, for 2 weeks. BALF was collected 24 h after the last HDM challenge, and total cells and cell differentials were assessed in BALF. Two-way repeated measures analysis of variance (ANOVA) with Tukey's multiple comparisons test was used to determining statistical significance.

Supplementary Figure 3



Supplementary Figure 3: Abundance of selected HDM-induced proteins in BALB/c mice BALF. Female and male BALB/c mice (N=10 each) were challenged with (i.n.) administration of whole HDM extract (0.7 mg/mL) in saline for 2 weeks and BALF was collected 24 h after the last HDM administration. BALF samples were probed in western blots to determine the abundance of selected protein targets. Relative protein expression levels were quantified by densitometry. Protein band intensity were normalized to loading protein (recombinant human granulysin ; 20 ng per sample) as a control for protein abundance quantification. Y-axis represents fold change of relative protein abundance of each HDM-challenged mouse compared to mean abundance in naïve mice (HDM/naïve). Each dot represents an individual mouse. Mann–Whitney U-test was used to assess statistical significance (*$p<0.05$, **$p<0.01$, ns=non-significant). A hash (#) denotes a significant difference ($p<0.05$) between HDM-challenged mice versus the naïve group ($p < 0.05$).

Supplementary Figure 4



Supplementary Figure 4: Expression of selected allergen-induced proteins in human BALF. Female and male adult participants (N=5 each) inhaled nebulized allergen (HDM, birch or Pacific grass, participant-adjusted based on wheal to skin-prick) for 2 minutes. BALF collection was conducted by bronchoscopy 24 h after allergen exposure. BALF samples were probed in western blots to determine the abundance of selected protein targets. Relative protein expression levels were quantified by densitometry. Y-axis represents protein band intensity normalized to loading protein (recombinant mouse MCP5; 10 ng per sample) as a control for protein abundance quantification. Each dot denotes an individual participant. Mann–Whitney U-test was used to assess statistical significance (*$p<0.05$, **$p<0.01$, ns=non-significant).

Supplementary Tables

Supplementary Table I. Cell differentials in BALF of HDM-challenged mice compared to allergen-naïve (HDM/naïve).

	BALB/c			**C56BL/6NJ**		
Cell Differentials	**Female (F)** **HDM/naïve** Fold Change	**Male (M)** **HDM/naïve** Fold Change	***p* value** **(F vs M)**	**Female (F)** **HDM/naïve** Fold Change	**Male (M)** **HDM/naïve** Fold Change	***p* value** **(F vs M)**
Total cells	8.0	3.0	$p<0.0001$	17.0	19.0	NS
Eosinophils	472	60	$p<0.0001$	351	789	$p<0.02$
Neutrophils	9418	34	$p<0.003$	110	165	NS
Macrophages	3.0	1.0	$p<0.0001$	3.0	4.0	NS
Lymphocytes	18121	10028	NS	5331	72	NS

NS = Non Significant

Supplementary Table II: Cytokine abundance in BALF in response to HDM-challenge.

Cytokine	BALB/c			C57BL/6NJ		
	Female (F) HDM/naïve	**Male (M) HDM/naïve**	***p-value*** **(F vs M)**	**Female (F) HDM/naïve**	**Male (M) HDM/naïve**	***p-value*** **(F vs M)**
IFNγ	8.96	13.17	*0.075*	26.78	25.40	*0.123*
IL-10	23.98	39.33	*0.156*	32.41	14.01	*0.052*
IL-12p70	2.33	1.82	*0.145*	0.78	0.96	*0.090*
IL-1β	65.69	36.09	*0.315*	32.49	9.69	***0.034***
IL-2	22.65	25.18	*0.497*	16.12	8.93	*0.085*
IL-4	560.16	936.61	*0.182*	218.16	166.81	*0.579*
IL-5	104.87	189.12	*0.079*	19.71	8.40	*0.166*
IL-6	16.84	31.35	*0.095*	41.30	4.48	*0.051*
KC/GRO	7.90	5.88	*0.549*	9.20	4.23	***0.029***
TNF	2.20	3.16	***0.035***	17.94	10.50	*0.143*
IL-16	38.60	31.60	*0.604*	12.11	6.44	*0.089*
IL-17A	156.01	8.28	***0.005***	406.53	262.95	*0.841*
IL-17C	ND	ND	ND	ND	ND	ND
IL-25	ND	ND	ND	ND	ND	ND
IL-17F	ND	ND	ND	ND	ND	ND
IL-21	ND	ND	ND	ND	ND	ND
IL-22	ND	ND	ND	ND	ND	ND
IL-23	ND	ND	ND	ND	ND	ND
IL-31	ND	ND	ND	ND	ND	ND
MIP3α	1.47	1.19	*0.589*	9.74	8.62	*0.166*
IL-15	ND	ND	ND	ND	ND	ND
IL-17A/F	ND	ND	ND	ND	ND	ND
IL-30	ND	ND	ND	ND	ND	ND
IL-33	3.01	6.76	*0.243*	1.09	0.45	***0.029***
IL-9	ND	ND	ND	ND	ND	ND
IP-10	3.91	5.86	***0.017***	7.59	3.72	***0.023***
MCP1	123.08	254.20	***0.002***	200.05	73.94	*0.063*
MIP1α	3.30	7.56	***0.006***	18.01	8.62	*0.218*
MIP2	3.58	3.35	*0.968*	6.73	2.01	***0.002***

ND=not detected

Supplementary Table III: Cytokine Abundance in lung tissue lysates in response to HDM- challenge.

Cytokine	BALB/c			C57BL/6NJ		
	Female (F) HDM/naïve	**Male (M) HDM/naïve**	***p-value* (F vs M)**	**Female (F) HDM/naïve**	**Male (M) HDM/naïve**	***p-value* (F vs M)**
IFNγ	6.69	6.76	*0.905*	5.78	7.26	*0.579*
IL-10	13.35	15.23	*0.968*	10.85	6.82	*0.123*
IL-12p70	1.00	1.17	*0.065*	1.65	1.42	*0.138*
IL-1β	9.96	9.06	*0.720*	7.17	3.80	*0.123*
IL-2	7.60	11.40	*0.315*	13.44	28.21	*0.684*
IL-4	50.10	61.85	*0.549*	70.09	55.72	*0.393*
IL-5	27.55	34.00	*0.278*	15.89	11.98	*0.529*
IL-6	6.33	5.10	*0.842*	8.38	3.71	*0.166*
KC/GRO	6.71	4.72	*0.095*	3.66	1.41	***0.012***
TNF	2.93	2.46	*0.182*	6.57	3.64	*0.075*
IL-16	2.57	1.93	*0.054*	2.24	1.92	*0.123*
IL-17A	9.09	4.99	***0.028***	10.20	11.26	*0.853*
IL-17C	1.04	1.25	*0.211*	1.50	1.69	*0.631*
IL-25	1.68	2.43	***0.028***	2.95	2.18	*0.166*
IL-17F	0.98	1.51	*0.243*	1.74	1.73	*0.836*
IL-21	3.62	8.41	***0.0001***	24.46	6.14	***0.002***
IL-22	1.45	1.08	*0.314*	2.86	2.39	*0.796*
IL-23	1.26	0.96	*0.211*	1.73	1.94	*0.305*
IL-31	1.64	1.97	*0.549*	3.25	2.53	*0.075*
MIP3α	5.95	2.77	***0.002***	31.76	27.28	*0.579*
IL-15	5.23	1.13	*0.106*	7.45	4.20	*0.424*
IL-17A/F	1.86	1.09	*0.243*	4.42	2.57	*0.578*
IL-30	3.33	1.96	***0.004***	3.18	2.31	*0.143*
IL-33	4.86	6.14	*0.211*	3.39	3.15	*0.796*
IL-9	0.92	0.65	***0.014***	1.29	4.68	***0.002***
IP-10	5.22	4.08	*0.400*	4.94	3.90	*0.248*
MCP1	3.11	2.99	*0.780*	2.57	1.51	*0.052*
MIP1a	4.02	3.97	*0.905*	4.53	4.17	*0.631*
MIP2	5.99	4.98	*0.447*	4.77	1.88	***0.003***

References Cited

1. Jr JC. Principles of innate and adaptive immunity. : 10.
2. Chaplin DD. 1. Overview of the immune response. *Journal of Allergy and Clinical Immunology* 2003; 111: S442–S459.
3. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. *Nat Med* 2005; 11: S45–S53.
4. Sattler S. The Role of the Immune System Beyond the Fight Against Infection. In: Sattler S, Kennedy-Lydon T, editors. *The Immunology of Cardiovascular Homeostasis and Pathology* [Internet] Cham: Springer International Publishing; 2017 [cited 2021 Nov 19]. p. 3–14Available from: http://link.springer.com/10.1007/978-3-319-57613-8_1.
5. Marshall JS, Warrington R, Watson W, Kim HL. An introduction to immunology and immunopathology. *Allergy Asthma Clin Immunol* 2018; 14: 49.
6. Tan LY, Komarasamy TV, RMT Balasubramaniam V. Hyperinflammatory Immune Response and COVID-19: A Double Edged Sword. *Front. Immunol.* 2021; 12: 742941.
7. Saad N, Moussa S. Immune response to COVID-19 infection: a double-edged sword. *Immunological Medicine* 2021; 44: 187–196.
8. Elenkov IJ, Iezzoni DG, Daly A, Harris AG, Chrousos GP. Cytokine dysregulation, inflammation and well-being. *Neuroimmunomodulation* 2005; 12: 255–269.
9. Bennett JM, Reeves G, Billman GE, Sturmberg JP. Inflammation–Nature's Way to Efficiently Respond to All Types of Challenges: Implications for Understanding and Managing "the Epidemic" of Chronic Diseases. *Front. Med.* 2018; 5: 316.
10. Zhong J, Shi G. Editorial: Regulation of Inflammation in Chronic Disease. *Front. Immunol.* 2019; 10: 737.
11. Herold K, Mrowka R. Inflammation—Dysregulated inflammatory response and strategies for treatment. *Acta Physiol* 2019; 226: e13284.
12. Li D, Wu M. Pattern recognition receptors in health and diseases. *Sig Transduct Target Ther* 2021; 6: 291.

13. Koenderman L, Buurman W, Daha MR. The innate immune response. *Immunology Letters* 2014; 162: 95–102.

14. Carrillo JLM, Rodríguez FPC, Coronado OG, García MAM, Cordero JFC. Physiology and Pathology of Innate Immune Response Against Pathogens. In: Rezaei N, editor. *Physiology and Pathology of Immunology* [Internet] InTech; 2017 [cited 2021 Oct 27].Available from: http://www.intechopen.com/books/physiology-and-pathology-of-immunology/physiology-and-pathology-of-innate-immune-response-against-pathogens.

15. Moser M, Leo O. Key concepts in immunology. *Vaccine* 2010; 28: C2–C13.

16. Geginat J, Paroni M, Maglie S, Alfen JS, Kastirr I, Gruarin P, De Simone M, Pagani M, Abrignani S. Plasticity of Human CD4 T Cell Subsets. *Front. Immunol.* [Internet] 2014 [cited 2021 Nov 19]; 5Available from: http://journal.frontiersin.org/article/10.3389/fimmu.2014.00630/abstract.

17. Stenken JA, Poschenrieder AJ. Bioanalytical chemistry of cytokines – A review. *Analytica Chimica Acta* 2015; 853: 95–115.

18. De RK, Tomar N, editors. Immunoinformatics [Internet]. New York, NY: Springer New York; 2014 [cited 2021 Nov 19].Available from: http://link.springer.com/10.1007/978-1-4939-1115-8.

19. Swain SL, McKinstry KK, Strutt TM. Expanding roles for CD4+ T cells in immunity to viruses. *Nat Rev Immunol* 2012; 12: 136–148.

20. Andersen MH, Schrama D, thor Straten P, Becker JC. Cytotoxic T Cells. *Journal of Investigative Dermatology* 2006; 126: 32–41.

21. Charles A Janeway J, Travers P, Walport M, Shlomchik MJ. B-cell activation by armed helper T cells. *Immunobiology: The Immune System in Health and Disease. 5th edition* [Internet] Garland Science; 2001 [cited 2021 Dec 30]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK27142/.

22. Avalos AM, Ploegh HL. Early BCR Events and Antigen Capture, Processing, and Loading on MHC Class II on B Cells. *Front Immunol* 2014; 5: 92.

23. Lin J-X, Leonard WJ. Fine-Tuning Cytokine Signals. *Annu. Rev. Immunol.* 2019; 37: 295–324.

24. Lacy P, Stow JL. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. *Blood* 2011; 118: 9–18.

25. Coondoo A. Cytokines in dermatology - A basic overview. *Indian J Dermatol* 2011; 56: 368.

26. Proudfoot AEI. Chemokine receptors: multifaceted therapeutic targets. *Nat Rev Immunol* 2002; 2: 106–115.

27. Hughes CE, Nibbs RJB. A guide to chemokines and their receptors. *FEBS J* 2018; 285: 2944–2971.

28. Rill W, Iii WR, Iii WR. Regulation of leukocyte migration by activation of the leukocyte. 1991; 349: 4.

29. Karin N, Wildbaum G. The Role of Chemokines in Shaping the Balance Between CD4+ T Cell Subsets and Its Therapeutic Implications in Autoimmune and Cancer Diseases. *Front. Immunol.* [Internet] 2015 [cited 2021 Nov 19]; 6Available from: http://journal.frontiersin.org/Article/10.3389/fimmu.2015.00609/abstract.

30. Charo IF, Ransohoff RM. The Many Roles of Chemokines and Chemokine Receptors in Inflammation. *N Engl J Med* 2006; 354: 610–621.

31. Ono SJ, Nakamura T, Miyazaki D, Ohbayashi M, Dawson M, Toda M. Chemokines: Roles in leukocyte development, trafficking, and effector function. *Journal of Allergy and Clinical Immunology* 2003; 111: 1185–1199.

32. Stow JL, Murray RZ. Intracellular trafficking and secretion of inflammatory cytokines. *Cytokine & Growth Factor Reviews* 2013; 24: 227–239.

33. Del Valle DM, Kim-Schulze S, Huang H-H, Beckmann ND, Nirenberg S, Wang B, Lavin Y, Swartz TH, Madduri D, Stock A, Marron TU, Xie H, Patel M, Tuballes K, Van Oekelen O, Rahman A, Kovatch P, Aberg JA, Schadt E, Jagannath S, Mazumdar M, Charney AW, Firpo-Betancourt A, Mendu DR, Jhang J, Reich D, Sigel K, Cordon-Cardo C, Feldmann M,

Parekh S, et al. An inflammatory cytokine signature predicts COVID-19 severity and survival. *Nat Med* 2020; 26: 1636–1643.

34. Muller WA. Leukocyte-Endothelial Cell Interactions in the Inflammatory Response. *Lab Invest* 2002; 82: 521–534.

35. Spiering MJ. Primer on the Immune System. : 5.

36. Mauvais-Jarvis F, Bairey Merz N, Barnes PJ, Brinton RD, Carrero J-J, DeMeo DL, De Vries GJ, Epperson CN, Govindan R, Klein SL, Lonardo A, Maki PM, McCullough LD, Regitz-Zagrosek V, Regensteiner JG, Rubin JB, Sandberg K, Suzuki A. Sex and gender: modifiers of health, disease, and medicine. *The Lancet* 2020; 396: 565–582.

37. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol* 2016; 16: 626–638.

38. Markle JG, Fish EN. SeXX matters in immunity. *Trends in Immunology* 2014; 35: 97–104.

39. Institute of Medicine (U.S.), Wizemann TM, Pardue ML, editors. Exploring the biological contributions to human health: does sex matter? Washington, D.C: National Academy Press; 2001.

40. vom Steeg LG, Klein SL. SeXX Matters in Infectious Disease Pathogenesis. Heitman J, editor. *PLoS Pathog* 2016; 12: e1005374.

41. Bachtrog D, Mank JE, Peichel CL, Kirkpatrick M, Otto SP, Ashman T-L, Hahn MW, Kitano J, Mayrose I, Ming R, Perrin N, Ross L, Valenzuela N, Vamosi JC, The Tree of Sex Consortium. Sex Determination: Why So Many Ways of Doing It? *PLoS Biol* 2014; 12: e1001899.

42. Deasy BM, Lu A, Tebbets JC, Feduska JM, Schugar RC, Pollett JB, Sun B, Urish KL, Gharaibeh BM, Cao B, Rubin RT, Huard J. A role for cell sex in stem cell–mediated skeletal muscle regeneration: female cells have higher muscle regeneration efficiency. *Journal of Cell Biology* 2007; 177: 73–86.

43. Heidari S, Babor TF, De Castro P, Tort S, Curno M. Sex and Gender Equity in Research: rationale for the SAGER guidelines and recommended use. *Res Integr Peer Rev* 2016; 1: 2.

44. Lee SK. Sex as an important biological variable in biomedical research. *BMB Rep*. 2018; 51: 167–173.

45. Tannenbaum C, Ellis RP, Eyssel F, Zou J, Schiebinger L. Sex and gender analysis improves science and engineering. *Nature* 2019; 575: 137–146.

46. Tannenbaum C, Schwarz JM, Clayton JA, de Vries GJ, Sullivan C. Evaluating sex as a biological variable in preclinical research: the devil in the details. *Biol Sex Differ* 2016; 7: 13, s13293-016-0066–x.

47. Kernel Networks Inc. FDA approves second drug to prevent HIV infection as part of ongoing efforts to end the HIV epidemic. *CMR* [Internet] 2019 [cited 2021 Nov 19]; Available from: https://www.fda.gov/news-events/press-announcements/fda-approves-second-drug-prevent-hiv-infection-part-ongoing-efforts-end-hiv-epidemic.

48. Pennell LM, Galligan CL, Fish EN. Sex affects immunity. *Journal of Autoimmunity* 2012; 38: J282–J291.

49. Bhatia A, Sekhon HK, Kaur G. Sex Hormones and Immune Dimorphism. *The Scientific World Journal* 2014; 2014: 1–8.

50. Jaillon S, Berthenet K, Garlanda C. Sexual Dimorphism in Innate Immunity. *Clinic Rev Allerg Immunol* 2019; 56: 308–321.

51. Gal-Oz ST, Maier B, Yoshida H, Seddu K, Elbaz N, Czysz C, Zuk O, Stranger BE, Ner-Gaon H, Shay T. ImmGen report: sexual dimorphism in the immune system transcriptome. *Nat Commun* 2019; 10: 4295.

52. Libert C, Dejager L, Pinheiro I. The X chromosome in immune functions: when a chromosome makes the difference. *Nat Rev Immunol* 2010; 10: 594–604.

53. Echem C, Akamine EH. Toll-Like Receptors Represent an Important Link for Sex Differences in Cardiovascular Aging and Diseases. *Front. Aging* 2021; 2: 709914.

54. Kawai T. Toll-like receptor signaling pathways. *Frontiers in Immunology* : 8.

55. Schurz H, Salie M, Tromp G, Hoal EG, Kinnear CJ, Möller M. The X chromosome and sex-specific effects in infectious disease susceptibility. *Hum Genomics* 2019; 13: 2.

56. Souyris M, Cenac C, Azar P, Daviaud D, Canivet A, Grunenwald S, Pienkowski C, Chaumeil J, Mejía JE, Guéry J-C. *TLR7* escapes X chromosome inactivation in immune cells. *Sci. Immunol.* 2018; 3: eaap8855.

57. Berghöfer B, Frommer T, Haley G, Fink L, Bein G, Hackstein H. TLR7 Ligands Induce Higher IFN-α Production in Females. *J Immunol* 2006; 177: 2088–2096.

58. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol* 2016; 16: 626–638.

59. Hagen SH, Henseling F, Hennesen J, Savel H, Delahaye S, Richert L, Ziegler SM, Altfeld M. Heterogeneous Escape from X Chromosome Inactivation Results in Sex Differences in Type I IFN Responses at the Single Human pDC Level. *Cell Reports* 2020; 33: 108485.

60. Jacobsen H, Klein SL. Sex Differences in Immunity to Viral Infections. *Front. Immunol.* 2021; 12: 720952.

61. Meier A, Chang JJ, Chan ES, Pollard RB, Sidhu HK, Kulkarni S, Wen TF, Lindsay RJ, Orellana L, Mildvan D, Bazner S, Streeck H, Alter G, Lifson JD, Carrington M, Bosch RJ, Robbins GK, Altfeld M. Sex differences in the Toll-like receptor–mediated response of plasmacytoid dendritic cells to HIV-1. *Nat Med* 2009; 15: 955–959.

62. Yovel G, Shakhar K, Ben-Eliyahu S. The Effects of Sex, Menstrual Cycle, and Oral Contraceptives on the Number and Activity of Natural Killer Cells. *Gynecologic Oncology* 2001; 81: 254–262.

63. Cephus J-Y, Stier MT, Fuseini H, Yung JA, Toki S, Bloodworth MH, Zhou W, Goleniewska K, Zhang J, Garon SL, Hamilton RG, Poloshukin VV, Boyd KL, Peebles RS, Newcomb DC. Testosterone Attenuates Group 2 Innate Lymphoid Cell-Mediated Airway Inflammation. *Cell Reports* 2017; 21: 2487–2499.

64. Melgert BN, Oriss TB, Qi Z, Dixon-McCarthy B, Geerlings M, Hylkema MN, Ray A. Macrophages: Regulators of Sex Differences in Asthma? *Am J Respir Cell Mol Biol* 2010; 42: 595–603.

65. Szaniawski MA, Spivak AM, Bosque A, Planelles V. Sex Influences SAMHD1 Activity and Susceptibility to Human Immunodeficiency Virus-1 in Primary Human Macrophages. *The Journal of Infectious Diseases* 2019; 219: 777–785.

66. Weinstein Y, Ran S, Segal S. Sex-associated differences in the regulation of immune responses controlled by the MHC of the mouse. : 7.

67. Schneider-Hohendorf T, Görlich D, Savola P, Kelkka T, Mustjoki S, Gross CC, Owens GC, Klotz L, Dornmair K, Wiendl H, Schwab N. Sex bias in MHC I-associated shaping of the adaptive immune system. *Proc Natl Acad Sci USA* 2018; 115: 2168–2173.

68. Lefèvre N, Corazza F, Duchateau J, Desir J, Casimir G. Sex Differences in Inflammatory Cytokines and CD99 Expression Following In Vitro Lipopolysaccharide Stimulation. *Shock* 2012; 38: 37–42.

69. Lefèvre N, Corazza F, Valsamis J, Delbaere A, De Maertelaer V, Duchateau J, Casimir G. The Number of X Chromosomes Influences Inflammatory Cytokine Production Following Toll-Like Receptor Stimulation. *Front. Immunol.* 2019; 10: 1052.

70. Marriott I, Bost KL, Huet-Hudson YM. Sexual dimorphism in expression of receptors for bacterial lipopolysaccharides in murine macrophages: A possible mechanism for gender-based differences in endotoxic shock susceptibility. *Journal of Reproductive Immunology* 2006; 71: 12–27.

71. Torcia MG, Nencioni L, Clemente AM, Civitelli L, Celestino I, Limongi D, Fadigati G, Perissi E, Cozzolino F, Garaci E, Palamara AT. Sex Differences in the Response to Viral Infections: TLR8 and TLR9 Ligand Stimulation Induce Higher IL10 Production in Males. Pekosz A, editor. *PLoS ONE* 2012; 7: e39853.

72. Takahashi T, Ellingson MK, Wong P, Israelow B, Lucas C, Klein J, Silva J, Mao T, Oh JE, Tokuyama M, Lu P, Venkataraman A, Park A, Liu F, Meir A, Sun J, Wang EY, Casanovas-Massana A, Wyllie AL, Vogels CBF, Earnest R, Lapidus S, Ott IM, Moore AJ, Yale IMPACT Research Team, Anastasio K, Askenase MH, Batsu M, Beatty H, Bermejo S, et al. Sex differences in immune responses that underlie COVID-19 disease outcomes. *Nature* 2020; 588: 315–320.

73. Taneja V. Sexual dimorphism, aging and immunity. *Vitamins and Hormones* [Internet] Elsevier; 2021 [cited 2021 Nov 20]. p. 367–399Available from: https://linkinghub.elsevier.com/retrieve/pii/S0083672920300637.

74. Klein SL. Immune Cells Have Sex and So Should Journal Articles. *Endocrinology* 2012; 153: 2544–2550.

75. Genetic Control of the CD4-CD8 T-Cell Ratio in Humans.pdf. .

76. Abdullah M, Chai P-S, Chong M-Y, Tohit ERM, Ramasamy R, Pei CP, Vidyadaran S. Gender effect on in vitro lymphocyte subset levels of healthy individuals. *Cellular Immunology* 2012; 272: 214–219.

77. Hewagama A, Patel D, Yarlagadda S, Strickland FM, Richardson BC. Stronger inflammatory/cytotoxic T-cell response in women identified by microarray analysis. *Genes Immun* 2009; 10: 509–516.

78. Barrat F, Lesourd B, Boulouis H-J, Thibault D, Vincent-Naulleau S, Gjata B, Louise A, Neway T, Pilet Ch. Sex and parity modulate cytokine production during murine ageing. *Clinical & Experimental Immunology* 1997; 109: 562–568.

79. Roberts CW, Walker W, Alexander J. Sex-Associated Hormones and Immunity to Protozoan Parasites. *Clin Microbiol Rev* 2001; 14: 476–488.

80. Fischinger S, Boudreau CM, Butler AL, Streeck H, Alter G. Sex differences in vaccine-induced humoral immunity. *Semin Immunopathol* 2019; 41: 239–249.

81. Markle JG, Fish EN. SeXX matters in immunity. *Trends in Immunology* 2014; 35: 97–104.

82. Zheng D, Liwinski T, Elinav E. Interaction between microbiota and immunity in health and disease. *Cell Res* 2020; 30: 492–506.

83. Bouman A, Heineman MJ, Faas MM. Sex hormones and the immune response in humans. *Human Reproduction Update* 2005; 11: 411–423.

84. Disteche CM, Berletch JB. X-chromosome inactivation and escape. *J Genet* 2015; 94: 591–599.

85. Plath K, Mlynarczyk-Evans S, Nusinow DA, Panning B. *Xist* RNA and the Mechanism of X Chromosome Inactivation. *Annu. Rev. Genet.* 2002; 36: 233–278.

86. Carrel L, Willard HF. X-inactivation profile reveals extensive variability in X-linked gene expression in females. *Nature* 2005; 434: 400–404.

87. Berletch JB, Yang F, Xu J, Carrel L, Disteche CM. Genes that escape from X inactivation. *Hum Genet* 2011; 130: 237–245.

88. Cox KH, Bonthuis PJ, Rissman EF. Mouse model systems to study sex chromosome genes and behavior: Relevance to humans. *Frontiers in Neuroendocrinology* 2014; 35: 405–419.

89. Klein SL, Jedlicka A, Pekosz A. The Xs and Y of immune responses to viral vaccines. 2010; 10: 12.

90. Bianchi I, Lleo A, Gershwin ME, Invernizzi P. The X chromosome and immune associated genes. *Journal of Autoimmunity* 2012; 38: J187–J192.

91. Karnam G, Rygiel TP, Raaben M, Grinwis GCM, Coenjaerts FE, Ressing ME, Rottier PJM, de Haan CAM, Meyaard L. CD200 Receptor Controls Sex-Specific TLR7 Responses to Viral Infection. Mossman KL, editor. *PLoS Pathog* 2012; 8: e1002710.

92. Fink AL, Engle K, Ursin RL, Tang W-Y, Klein SL. Biological sex affects vaccine efficacy and protection against influenza in mice. *Proc Natl Acad Sci USA* 2018; 115: 12477–12482.

93. Migeon BR. The Role of X Inactivation and Cellular Mosaicism in Women's Health and Sex-Specific Diseases. *JAMA* 2006; 295: 1428.

94. Brooks EG, Schmalstieg FC, Wirt DP, Rosenblatt HM, Adkins LT, Lookingbill DP, Rudloff HE, Rakusan TA, Goldman AS. A novel X-linked combined immunodeficiency disease. *J. Clin. Invest.* 1990; 86: 1623–1631.

95. Pinheiro I, Dejager L, Libert C. X-chromosome-located microRNAs in immunity: Might they explain male/female differences?: The X chromosome-genomic context may affect X-located miRNAs and downstream signaling, thereby contributing to the enhanced immune response of females. *Bioessays* 2011; 33: 791–802.

96. Peng Y, Croce CM. The role of MicroRNAs in human cancer. *Sig Transduct Target Ther* 2016; 1: 15004.

97. Arnold AP. Mouse Models for Evaluating Sex Chromosome Effects that Cause Sex Differences in Non-Gonadal Tissues. *Journal of Neuroendocrinology* 2009; 21: 377–386.

98. Arnold AP, Chen X. What does the “four core genotypes” mouse model tell us about sex differences in the brain and other tissues? *Frontiers in Neuroendocrinology* 2009; 30: 1–9.

99. Itoh Y, Mackie R, Kampf K, Domadia S, Brown JD, O’Neill R, Arnold AP. Four Core Genotypes mouse model: localization of the Sry transgene and bioassay for testicular hormone levels. *BMC Res Notes* 2015; 8: 69.

100. Smith-Bouvier DL, Divekar AA, Sasidhar M, Du S, Tiwari-Woodruff SK, King JK, Arnold AP, Singh RR, Voskuhl RR. A role for sex chromosome complement in the female bias in autoimmune disease. *Journal of Experimental Medicine* 2008; 205: 1099–1108.

101. Robinson DP, Huber SA, Moussawi M, Roberts B, Teuscher C, Watkins R, Arnold AP, Klein SL. Sex chromosome complement contributes to sex differences in coxsackievirus B3 but not influenza A virus pathogenesis. *Biol sex dif* 2011; 2: 8.

102. Koçar IH, Yesilova Z, Özata M, Turan M, Sengül A, Özdemir IÇ. The effect of testosterone replacement treatment on immunological features of patients with Klinefelter’s syndrome: Immunological features of Klinefelter’s syndrome. *Clinical & Experimental Immunology* 2000; 121: 448–452.

103. Gupta S, Nakabo S, Blanco LP, O’Neil LJ, Wigerblad G, Goel RR, Mistry P, Jiang K, Carmona-Rivera C, Chan DW, Wang X, Pedersen HL, Gadkari M, Howe KN, Naz F, Dell’Orso S, Hasni SA, Dempsey C, Buscetta A, Frischmeyer-Guerrerio PA, Kruszka P, Muenke M, Franco LM, Sun H-W, Kaplan MJ. Sex differences in neutrophil biology modulate response to type I interferons and immunometabolism. *Proc Natl Acad Sci USA* 2020; 117: 16481–16491.

104. Hafner LM, Cunningham K, Beagley KW. Ovarian steroid hormones: effects on immune responses and Chlamydia trachomatis infections of the female genital tract. *Mucosal Immunol* 2013; 6: 859–875.

105. Kadel S, Kovats S. Sex Hormones Regulate Innate Immune Cells and Promote Sex Differences in Respiratory Virus Infection. *Front. Immunol.* 2018; 9: 1653.

106. Pang SF, Tang F. Sex differences in the serum concentrations of testosterone in mice and hamsters during their critical periods of neural sexual differentiation. *Journal of Endocrinology* 1984; 100: 7–11.

107. The postnatal gonadotropin and sex steroid surge-insights from the androgen insensitivity syndrome.pdf. .

108. Sathish V, Martin YN, Prakash YS. Sex steroid signaling: Implications for lung diseases. *Pharmacology & Therapeutics* 2015; 150: 94–108.

109. Hall OJ, Klein SL. Progesterone-based compounds affect immune responses and susceptibility to infections at diverse mucosal sites. *Mucosal Immunol* 2017; 10: 1097–1107.

110. Mielke MM, Miller VM. Improving clinical outcomes through attention to sex and hormones in research. *Nat Rev Endocrinol* 2021; 17: 625–635.

111. Kovats S. Estrogen receptors regulate innate immune cells and signaling pathways. *Cellular Immunology* 2015; 294: 63–69.

112. Klein SL, Roberts C, editors. Sex Hormones and Immunity to Infection [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2010 [cited 2021 Nov 19].Available from: http://link.springer.com/10.1007/978-3-642-02155-8.

113. Trigunaite A, Dimo J, Jørgensen TN. Suppressive effects of androgens on the immune system. *Cellular Immunology* 2015; 294: 87–94.

114. Rettew JA, Huet-Hudson YM, Marriott I. Testosterone Reduces Macrophage Expression in the Mouse of Toll-Like Receptor 4, a Trigger for Inflammation and Innate Immunity. *Biology of Reproduction* 2008; 78: 432–437.

115. Prossnitz ER, Arterburn JB, Sklar LA. GPR30: A G protein-coupled receptor for estrogen. *Molecular and Cellular Endocrinology* 2007; 265–266: 138–142.

116. Benten W. Rapid effects of androgens in macrophages. *Steroids* 2004; 69: 585–590.

117. Lai J-J, Lai K-P, Zeng W, Chuang K-H, Altuwaijri S, Chang C. Androgen Receptor Influences on Body Defense System via Modulation of Innate and Adaptive Immune Systems. *The American Journal of Pathology* 2012; 181: 1504–1512.

118. Miller L, Hunt JS. Sex steroid hormones and macrophage function. *Life Sciences* 1996; 59: 1–14.

119. Straub RH. The Complex Role of Estrogens in Inflammation. *Endocrine Reviews* 2007; 28: 521–574.

120. Phiel KL, Henderson RA, Adelman SJ, Elloso MM. Differential estrogen receptor gene expression in human peripheral blood mononuclear cell populations. *Immunology Letters* 2005; 97: 107–113.

121. Lasrado N, Jia T, Massilamany C, Franco R, Illes Z, Reddy J. Mechanisms of sex hormones in autoimmunity: focus on EAE. *Biol Sex Differ* 2020; 11: 50.

122. Lélu K, Laffont S, Delpy L, Paulet P-E, Périnat T, Tschanz SA, Pelletier L, Engelhardt B, Guéry J-C. Estrogen Receptor α Signaling in T Lymphocytes Is Required for Estradiol-Mediated Inhibition of Th1 and Th17 Cell Differentiation and Protection against Experimental Autoimmune Encephalomyelitis. *J.I.* 2011; 187: 2386–2393.

123. Grimaldi CM, Cleary J, Dagtas AS, Moussai D, Diamond B. Estrogen alters thresholds for B cell apoptosis and activation. *J. Clin. Invest.* 2002; 109: 1625–1633.

124. Liva SM, Voskuhl RR. Testosterone Acts Directly on CD4 $^{+}$ T Lymphocytes to Increase IL-10 Production. *J Immunol* 2001; 167: 2060–2067.

125. Malkin CJ, Pugh PJ, Jones RD, Kapoor D, Channer KS, Jones TH. The Effect of Testosterone Replacement on Endogenous Inflammatory Cytokines and Lipid Profiles in Hypogonadal Men. *The Journal of Clinical Endocrinology & Metabolism* 2004; 89: 3313–3318.

126. Teilmann SC, Clement CA, Thorup J, Byskov AG, Christensen ST. Expression and localization of the progesterone receptor in mouse and human reproductive organs. *Journal of Endocrinology* 2006; 191: 525–535.

127. Jones LA, Kreem S, Shweash M, Paul A, Alexander J, Roberts CW. Differential Modulation of TLR3- and TLR4-Mediated Dendritic Cell Maturation and Function by Progesterone. *J.I.* 2010; 185: 4525–4534.

128. Hardy DB, Janowski BA, Corey DR, Mendelson CR. Progesterone Receptor Plays a Major Antiinflammatory Role in Human Myometrial Cells by Antagonism of Nuclear Factor-κB Activation of Cyclooxygenase 2 Expression. *Molecular Endocrinology* 2006; 20: 2724–2733.

129. Butts CL, Shukair SA, Duncan KM, Bowers E, Horn C, Belyavskaya E, Tonelli L, Sternberg EM. Progesterone inhibits mature rat dendritic cells in a receptor-mediated fashion. *International Immunology* 2007; 19: 287–296.

130. Butts CL, Bowers E, Horn JC, Shukair SA, Belyavskaya E, Tonelli L, Sternberg EM. Inhibitory effects of progesterone differ in dendritic cells from female and male rodents. *Gender Medicine* 2008; 5: 434–447.

131. Arruvito L, Giulianelli S, Flores AC, Paladino N, Barboza M, Lanari C, Fainboim L. NK Cells Expressing a Progesterone Receptor Are Susceptible to Progesterone-Induced Apoptosis. *J Immunol* 2008; 180: 5746–5753.

132. Su L, Sun Y, Ma F, Lü P, Huang H, Zhou J. Progesterone inhibits Toll-like receptor 4-mediated innate immune response in macrophages by suppressing NF-κB activation and enhancing SOCS1 expression. *Immunology Letters* 2009; 125: 151–155.

133. Lei K, Chen L, Georgiou EX, Sooranna SR, Khanjani S, Brosens JJ, Bennett PR, Johnson MR. Progesterone Acts via the Nuclear Glucocorticoid Receptor to Suppress IL-1β-Induced COX-2 Expression in Human Term Myometrial Cells. Sun K, editor. *PLoS ONE* 2012; 7: e50167.

134. Kyurkchiev D, Ivanova-Todorova E, Hayrabedyan S, Altankova I, Kyurkchiev S. Female Sex Steroid Hormones Modify Some Regulatory Properties of Monocyte-Derived Dendritic Cells. *Am J Reprod Immunol* 2007; 58: 425–433.

135. Piccinni M. Role of hormone-controlled Th1- and Th2-type cytokines in successful pregnancy. *Journal of Neuroimmunology* 2000; 109: 30–33.

136. Miyaura H, Iwata M. Direct and Indirect Inhibition of Th1 Development by Progesterone and Glucocorticoids. *J Immunol* 2002; 168: 1087–1094.

137. Piccinni MP, Giudizi MG, Biagiotti R, Beloni L, Sampognaro S, Parronchi P, Manetti R, Livi C. Progesterone favors the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established Th1 cell clones. *The Journal of Immunology* : 7.

138. Hall OJ, Limjunyawong N, Vermillion MS, Robinson DP, Wohlgemuth N, Pekosz A, Mitzner W, Klein SL. Progesterone-Based Therapy Protects Against Influenza by Promoting Lung Repair and Recovery in Females. Schultz-Cherry S, editor. *PLoS Pathog* 2016; 12: e1005840.

139. Nalbandian G, Kovats S. Understanding Sex Biases in Immunity: Effects of Estrogen on the Differentiation and Function of Antigen-Presenting Cells. *IR* 2005; 31: 091–106.

140. Paharkova-Vatchkova V, Maldonado R, Kovats S. Estrogen Preferentially Promotes the Differentiation of CD11c $^{+}$ CD11b $^{\text{intermediate}}$ Dendritic Cells from Bone Marrow Precursors. *J Immunol* 2004; 172: 1426–1436.

141. Siracusa MC, Overstreet MG, Housseau F, Scott AL, Klein SL. 17β-Estradiol Alters the Activity of Conventional and IFN-Producing Killer Dendritic Cells. *J Immunol* 2008; 180: 1423–1431.

142. Cunningham MA, Naga OS, Eudaly JG, Scott JL, Gilkeson GS. Estrogen receptor alpha modulates toll-like receptor signaling in murine lupus. *Clinical Immunology* 2012; 144: 1–12.

143. Karpuzoglu E, Fenaux JB, Phillips RA, Lengi AJ, Elvinger F, Ansar Ahmed S. Estrogen Up-Regulates Inducible Nitric Oxide Synthase, Nitric Oxide, and Cyclooxygenase-2 in Splenocytes Activated with T Cell Stimulants: Role of Interferon-γ. *Endocrinology* 2006; 147: 662–671.

144. Calippe B, Douin-Echinard V, Delpy L, Laffargue M, Lélu K, Krust A, Pipy B, Bayard F, Arnal J-F, Guéry J-C, Gourdy P. 17β-Estradiol Promotes TLR4-Triggered Proinflammatory Mediator Production through Direct Estrogen Receptor α Signaling in Macrophages In Vivo. *J.I.* 2010; 185: 1169–1176.

145. Dai R, Phillips RA, Zhang Y, Khan D, Crasta O, Ahmed SA. Suppression of LPS-induced Interferon-□ and nitric oxide in splenic lymphocytes by select estrogen-regulated microRNAs: a novel mechanism of immune modulation. 2008; 112: 7.

146. Lengi AJ, Phillips RA, Karpuzoglu E, Ahmed SA. Estrogen selectively regulates chemokines in murine splenocytes. *Journal of Leukocyte Biology* 2007; 81: 1065–1074.

147. Nakaya M, Tachibana H, Yamada K. Effect of Estrogens on the Interferon- Producing Cell Population of Mouse Splenocytes. : 7.

148. Messingham KAN, Heinrich SA, Kovacs EJ. Estrogen restores cellular immunity in injured male mice via suppression of interleukin-6 production. : 9.

149. Lang TJ. Estrogen as an immunomodulator. *Clinical Immunology* 2004; 113: 224–230.

150. Villa A, Rizzi N, Vegeto E, Ciana P, Maggi A. Estrogen accelerates the resolution of inflammation in macrophagic cells. *Sci Rep* 2015; 5: 15224.

151. Polanczyk MJ, Carson BD, Subramanian S, Afentoulis M, Vandenbark AA, Ziegler SF, Offner H. Cutting Edge: Estrogen Drives Expansion of the $CD4^+$ $CD25^+$ Regulatory T Cell Compartment. *J Immunol* 2004; 173: 2227–2230.

152. Ghisletti S, Meda C, Maggi A, Vegeto E. 17β-Estradiol Inhibits Inflammatory Gene Expression by Controlling NF-κB Intracellular Localization. *Mol Cell Biol* 2005; 25: 2957–2968.

153. Speyer CL, Rancilio NJ, McClintock SD, Crawford JD, Gao H, Sarma JV, Ward PA. Regulatory effects of estrogen on acute lung inflammation in mice. *American Journal of Physiology-Cell Physiology* 2005; 288: C881–C890.

154. Giraud SN, Caron CM, Pham-Dinh D, Kitabgi P, Nicot AB. Estradiol inhibits ongoing autoimmune neuroinflammation and NF B-dependent CCL2 expression in reactive astrocytes. *Proceedings of the National Academy of Sciences* 2010; 107: 8416–8421.

155. Sabahi F, Rola-Plesczcynski M, O'Connell S, Frenkel LD. Qualitative and Quantitative Analysis of T Lymphocytes During Normal Human Pregnancy. *American Journal of Reproductive Immunology* 1995; 33: 381–393.

156. Lü FX, Abel K, Ma Z, Rourke T, Lu D, Torten J, Mcchesney M, Miller CJ. The strength of B cell immunity in female rhesus macaques is controlled by $CD8^+$ T cells under the influence of ovarian steroid hormones: Ovarian hormones influence B cell immunity in menstrual primates. *Clinical & Experimental Immunology* 2002; 128: 10–20.

157. Mims JW. Asthma: definitions and pathophysiology: Asthma: definitions and pathophysiology. *International Forum of Allergy and Rhinology* 2015; 5: S2–S6.

158. Jackson DJ, Sykes A, Mallia P, Johnston SL. Asthma exacerbations: Origin, effect, and prevention. *Journal of Allergy and Clinical Immunology* 2011; 128: 1165–1174.

159. Global_Asthma_Report_2018.pdf. .

160. Papi A, Brightling C, Pedersen SE, Reddel HK. Asthma. *The Lancet* 2018; 391: 783–800.

161. Nunes C, Pereira AM, Morais-Almeida M. Asthma costs and social impact. *asthma res and pract* 2017; 3: 1.

162. Ismaila AS, Sayani AP, Marin M, Su Z. Clinical, economic, and humanistic burden of asthma in Canada: a systematic review. *BMC Pulm Med* 2013; 13: 70.

163. Bosonea A-M, Sharpe H, Wang T, Bakal JA, Befus AD, Svenson LW, Vliagoftis H. Developments in asthma incidence and prevalence in Alberta between 1995 and 2015. *Allergy Asthma Clin Immunol* 2020; 16: 87.

164. Postma DS. Gender Differences in Asthma Development and Progression. *Gender Medicine* 2007; 4: S133–S146.

165. Dharmage SC, Perret JL, Custovic A. Epidemiology of Asthma in Children and Adults. *Front. Pediatr.* 2019; 7: 246.

166. Leynaert B, Sunyer J, Garcia-Esteban R, Svanes C, Jarvis D, Cerveri I, Dratva J, Gislason T, Heinrich J, Janson C, Kuenzli N, de Marco R, Omenaas E, Raherison C, Gómez Real F, Wjst M, Zemp E, Zureik M, Burney PGJ, Anto JM, Neukirch F. Gender differences in prevalence, diagnosis and incidence of allergic and non-allergic asthma: a population-based cohort. *Thorax* 2012; 67: 625–631.

167. Fuseini H, Newcomb DC. Mechanisms Driving Gender Differences in Asthma. *Curr Allergy Asthma Rep* 2017; 17: 19.

168. Vrieze A, Postma DS, Kerstjens HAM. Perimenstrual asthma: A syndrome without known cause or cure. *Journal of Allergy and Clinical Immunology* 2003; 112: 271–282.

169. Graziottin A, Serafini A. Perimenstrual asthma: from pathophysiology to treatment strategies. *Multidiscip Respir Med* 2016; 11: 30.

170. Brenner BE. Relation between phase of the menstrual cycle and asthma presentations in the emergency department. *Thorax* 2005; 60: 806–809.

171. Zimmerman JL, Woodruff PG, Clark S, Camargo CA. Relation between Phase of Menstrual Cycle and Emergency Department Visits for Acute Asthma. *Am J Respir Crit Care Med* 2000; 162: 512–515.

172. Kuruvilla ME, Lee FE-H, Lee GB. Understanding Asthma Phenotypes, Endotypes, and Mechanisms of Disease. *Clinic Rev Allerg Immunol* 2019; 56: 219–233.

173. Svenningsen S, Nair P. Asthma Endotypes and an Overview of Targeted Therapy for Asthma. *Front. Med.* 2017; 4: 158.

174. Chapman KR, Ernst P, Grenville A, Dewland P, Zimmerman S. Control of Asthma in Canada: Failure to Achieve Guideline Targets. *Canadian Respiratory Journal* 2001; 8: 35A-40A.

175. Peters SP, Ferguson G, Deniz Y, Reisner C. Uncontrolled asthma: A review of the prevalence, disease burden and options for treatment. *Respiratory Medicine* 2006; 100: 1139–1151.

176. Day A, Ernst P, Glick L, Zimmerman S, Chapman KR. Women and Asthma: Lessons from a Gender Analysis of the Asthma in Canada Survey. *Journal of Asthma* 2006; 43: 169–173.

177. Bønnelykke K, Ober C. Leveraging gene-environment interactions and endotypes for asthma gene discovery. *Journal of Allergy and Clinical Immunology* 2016; 137: 667–679.

178. Marshall CL, Hasani K, Mookherjee N. Immunobiology of Steroid-Unresponsive Severe Asthma. *Front. Allergy* 2021; 2: 718267.

179. Fitzpatrick AM, Chipps BE, Holguin F, Woodruff PG. T2-"Low" Asthma: Overview and Management Strategies. *The Journal of Allergy and Clinical Immunology: In Practice* 2020; 8: 452–463.

180. Kim Y-M, Kim Y-S, Jeon SG, Kim Y-K. Immunopathogenesis of Allergic Asthma: More Than the Th2 Hypothesis. *Allergy Asthma Immunol Res* 2013; 5: 189.

181. Holgate ST, Wenzel S, Postma DS, Weiss ST, Renz H, Sly PD. Asthma. *Nat Rev Dis Primers* 2015; 1: 15025.

182. Doerschuk CM. Leukocyte trafficking in alveoli and airway passages. *Respir Res* 2000; 1: 4.

183. Kumar V. Pulmonary Innate Immune Response Determines the Outcome of Inflammation During Pneumonia and Sepsis-Associated Acute Lung Injury. *Front. Immunol.* 2020; 11: 1722.

184. Davis JD, Wypych TP. Cellular and functional heterogeneity of the airway epithelium. *Mucosal Immunol* 2021; 14: 978–990.

185. Porsbjerg CM, Sverrild A, Lloyd CM, Menzies-Gow AN, Bel EH. Anti-alarmins in asthma: targeting the airway epithelium with next-generation biologics. *Eur Respir J* 2020; 56: 2000260.

186. Morianos I, Semitekolou M. Dendritic Cells: Critical Regulators of Allergic Asthma. *IJMS* 2020; 21: 7930.

187. Salazar F, Ghaemmaghami AM. Allergen Recognition by Innate Immune Cells: Critical Role of Dendritic and Epithelial Cells. *Front. Immunol.* [Internet] 2013 [cited 2021 Nov 22]; 4Available from: http://journal.frontiersin.org/article/10.3389/fimmu.2013.00356/abstract.

188. Hammad H, Lambrecht BN. The basic immunology of asthma. *Cell* 2021; 184: 1469–1485.

189. Greenfeder S, Umland SP, Cuss FM, Chapman RW, Egan RW. Th2 cytokines and asthma The role of interleukin-5 in allergic eosinophilic disease. 2: 9.

190. Kips JC. Cytokines in asthma. *European Respiratory Journal* 2001; 18: 24–33.

191. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol* 2015; 16: 45–56.

192. Ngoc LP, Gold DR, Tzianabos AO, Weiss ST, Celedón JC. Cytokines, allergy, and asthma. *Current Opinion in Allergy & Clinical Immunology* 2005; 5: 161–166.

193. Bax HJ, Keeble AH, Gould HJ. Cytokinergic IgE Action in Mast Cell Activation. *Front. Immun.* [Internet] 2012 [cited 2021 Nov 19]; 3Available from: http://journal.frontiersin.org/article/10.3389/fimmu.2012.00229/abstract.

194. Deo S, Mistry K, Kakade A, Niphadkar P. Role played by Th2 type cytokines in IgE mediated allergy and asthma. *Lung India* 2010; 27: 66.

195. McBrien CN, Menzies-Gow A. The Biology of Eosinophils and Their Role in Asthma. *Front Med (Lausanne)* 2017; 4: 93.

196. Calhoun WJ, Sedgwick J, Busse WW. The role of eosinophils in the pathophysiology of asthma. *Ann N Y Acad Sci* 1991; 629: 62–72.

197. Kariyawasam HH, Robinson DS. The role of eosinophils in airway tissue remodelling in asthma. *Curr Opin Immunol* 2007; 19: 681–686.

198. Byrne AJ, Mathie SA, Gregory LG, Lloyd CM. Pulmonary macrophages: key players in the innate defence of the airways. *Thorax* 2015; 70: 1189–1196.

199. Hirayama D, Iida T, Nakase H. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. *IJMS* 2017; 19: 92.

200. Rubins JB. Alveolar Macrophages. : 2.

201. Kopf M, Schneider C, Nobs SP. The development and function of lung-resident macrophages and dendritic cells. *Nat Immunol* 2015; 16: 36–44.

202. Hu G, Christman JW. Editorial: Alveolar Macrophages in Lung Inflammation and Resolution. *Front. Immunol.* 2019; 10: 2275.

203. Morales-Nebreda L, Misharin AV, Perlman H, Budinger GRS. The heterogeneity of lung macrophages in the susceptibility to disease. *Eur Respir Rev* 2015; 24: 505–509.

204. Balhara J, Gounni AS. The alveolar macrophages in asthma: a double-edged sword. *Mucosal Immunol* 2012; 5: 605–609.

205. Draijer C, Peters-Golden M. Alveolar Macrophages in Allergic Asthma: the Forgotten Cell Awakes. *Curr Allergy Asthma Rep* 2017; 17: 12.

206. van der Veen TA, de Groot LES, Melgert BN. The different faces of the macrophage in asthma. *Current Opinion in Pulmonary Medicine* 2020; 26: 62–68.

207. Rosenberg HF, Dyer KD, Foster PS. Eosinophils: changing perspectives in health and disease. *Nat Rev Immunol* 2013; 13: 9–22.

208. Kovalszki A, Weller PF. Eosinophilia. *Primary Care: Clinics in Office Practice* 2016; 43: 607–617.

209. Diny NL, Rose NR, Čiháková D. Eosinophils in Autoimmune Diseases. *Front. Immunol.* 2017; 8: 484.

210. Nakagome K, Nagata M. Involvement and Possible Role of Eosinophils in Asthma Exacerbation. *Front. Immunol.* 2018; 9: 2220.

211. Frigas E, Gleich GJ. The eosinophil and the pathophysiology of asthma. *J Allergy Clin Immunol* 1986; 77: 527–537.

212. Kay AB, Phipps S, Robinson DS. A role for eosinophils in airway remodelling in asthma. *Trends Immunol* 2004; 25: 477–482.

213. Venge P. The eosinophil and airway remodelling in asthma. *Clin Respir J* 2010; 4 Suppl 1: 15–19.

214. Simmons SR, Bhalla M, Herring SE, Tchalla EYI, Bou Ghanem EN. Older but Not Wiser: the Age-Driven Changes in Neutrophil Responses during Pulmonary Infections. Richardson AR, editor. *Infect Immun* [Internet] 2021 [cited 2021 Nov 19]; 89Available from: https://journals.asm.org/doi/10.1128/IAI.00653-20.

215. Bordon J, Aliberti S, Fernandez-Botran R, Uriarte SM, Rane MJ, Duvvuri P, Peyrani P, Morlacchi LC, Blasi F, Ramirez JA. Understanding the roles of cytokines and neutrophil activity and neutrophil apoptosis in the protective versus deleterious inflammatory response in pneumonia. *International Journal of Infectious Diseases* 2013; 17: e76–e83.

216. Pechous RD. With Friends Like These: The Complex Role of Neutrophils in the Progression of Severe Pneumonia. *Front. Cell. Infect. Microbiol.* 2017; 7: 160.

217. Radermecker C, Louis R, Bureau F, Marichal T. Role of neutrophils in allergic asthma. *Curr Opin Immunol* 2018; 54: 28–34.

218. Monteseirín J. Neutrophils and asthma. *J Investig Allergol Clin Immunol* 2009; 19: 340–354.

219. Macdowell AL, Peters SP. Neutrophils in asthma. *Curr Allergy Asthma Rep* 2007; 7: 464–468.

220. Ray A, Kolls JK. Neutrophilic Inflammation in Asthma and Association with Disease Severity. *Trends in Immunology* 2017; 38: 942–954.

221. Gao H, Ying S, Dai Y. Pathological Roles of Neutrophil-Mediated Inflammation in Asthma and Its Potential for Therapy as a Target. *J Immunol Res* 2017; 2017: 3743048.

222. Bajbouj K, Ramakrishnan RK, Hamid Q. Role of Matrix Metalloproteinases in Angiogenesis and Its Implications in Asthma. Pirozzi CJ, editor. *Journal of Immunology Research* 2021; 2021: 1–12.

223. Klein SL, Schwarz JM. Sex-Specific Regulation of Peripheral and Central Immune Responses. *Oxford Research Encyclopedia of Neuroscience* [Internet] Oxford University Press; 2018 [cited 2021 Oct 25].Available from: https://oxfordre.com/neuroscience/view/10.1093/acrefore/9780190264086.001.0001/acrefore-9780190264086-e-223.

224. Silveyra P, Tigno XT, editors. Sex-Based Differences in Lung Physiology [Internet]. Cham: Springer International Publishing; 2021 [cited 2021 May 2].Available from: https://link.springer.com/10.1007/978-3-030-63549-7.

225. Lachowicz-Scroggins ME, Vuga LJ, Laposky AD, Brown M, Banerjee K, Croxton TL, Kiley JP. The intersection of women's health, lung health, and disease. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 2021; 321: L624–L627.

226. Han MK, Arteaga-Solis E, Blenis J, Bourjeily G, Clegg DJ, DeMeo D, Duffy J, Gaston B, Heller NM, Hemnes A, Henske EP, Jain R, Lahm T, Lancaster LH, Lee J, Legato MJ, McKee S, Mehra R, Morris A, Prakash YS, Stampfli MR, Gopal-Srivastava R, Laposky AD, Punturieri A, Reineck L, Tigno X, Clayton J. Female Sex and Gender in Lung/Sleep Health and Disease. Increased Understanding of Basic Biological, Pathophysiological, and Behavioral Mechanisms Leading to Better Health for Female Patients with Lung Disease. *Am J Respir Crit Care Med* 2018; 198: 850–858.

227. Pradhan A, Olsson P-E. Sex differences in severity and mortality from COVID-19: are males more vulnerable? *Biol Sex Differ* 2020; 11: 53.

228. Majdic G. Could Sex/Gender Differences in ACE2 Expression in the Lungs Contribute to the Large Gender Disparity in the Morbidity and Mortality of Patients Infected With the SARS-CoV-2 Virus? *Front. Cell. Infect. Microbiol.* 2020; 10: 327.

229. Dehingia N, Raj A. Sex differences in COVID-19 case fatality: do we know enough? *The Lancet Global Health* 2021; 9: e14–e15.

230. Gadi N, Wu SC, Spihlman AP, Moulton VR. What's Sex Got to Do With COVID-19? Gender-Based Differences in the Host Immune Response to Coronaviruses. *Front. Immunol.* 2020; 11: 2147.

231. Kynyk JA, Mastronarde JG, McCallister JW. Asthma, the sex difference. *Current Opinion in Pulmonary Medicine* 2011; 17: 6–11.

232. Senna G, Latorre M, Bugiani M, Caminati M, Heffler E, Morrone D, Paoletti G, Parronchi P, Puggioni F, Blasi F, Canonica GW, Paggiaro P, on behalf of SANI Network. Sex Differences in Severe Asthma: Results From Severe Asthma Network in Italy-SANI. *Allergy Asthma Immunol Res* 2021; 13: 219.

233. Ricciardolo FLM, Levra S, Sprio AE, Bertolini F, Carriero V, Gallo F, Ciprandi G. Asthma in the Real-World: The Relevance of Gender. *Int Arch Allergy Immunol* 2020; 181: 462–466.

234. Boezen HM, Jansen DF, Postma DS. Sex and gender differences in lung development and their clinical significance. *Clinics in Chest Medicine* 2004; 25: 237–245.

235. Cohen J, Douma WR, ten Hacken NHT, Oudkerk M, Postma DS. Physiology of the small airways: A gender difference? *Respiratory Medicine* 2008; 102: 1264–1271.

236. Torday JS, Nielsen HC. The Sex Difference in Fetal Lung Surfactant Production. *Experimental Lung Research* 1987; 12: 1–19.

237. Han S, Mallampalli RK. The Role of Surfactant in Lung Disease and Host Defense against Pulmonary Infections. *Annals ATS* 2015; 12: 765–774.

238. Chakraborty M, Kotecha S. Pulmonary surfactant in newborn infants and children. *Breathe* 2013; 9: 476–488.

239. Carey MA, Card JW, Voltz JW, Arbes SJ, Germolec DR, Korach KS, Zeldin DC. It's all about sex: gender, lung development and lung disease. *Trends in Endocrinology & Metabolism* 2007; 18: 308–313.

240. LoMauro A, Aliverti A. Sex differences in respiratory function. *Breathe* 2018; 14: 131–140.

241. Seaborn T, Simard M, Provost PR, Piedboeuf B, Tremblay Y. Sex hormone metabolism in lung development and maturation. *Trends in Endocrinology & Metabolism* 2010; 21: 729–738.

242. Bellemare F, Jeanneret A, Couture J. Sex Differences in Thoracic Dimensions and Configuration. *Am J Respir Crit Care Med* 2003; 168: 305–312.

243. Johnson CC, Peterson EL, Ownby DR. Gender Differences in Total and Allergen-specific Immunoglobulin E (IgE) Concentrations in a Population-based Cohort from Birth to Age Four Years. *American Journal of Epidemiology* 1998; 147: 1145–1152.

244. Uekert S, Akan G, Evans M, Li Z, Roberg K, Tisler C, Dasilva D, Anderson E, Gangnon R, Allen D. Sex-related differences in immune development and the expression of atopy in early childhood. *Journal of Allergy and Clinical Immunology* 2006; 118: 1375–1381.

245. Manfreda J, Sears MR, Becklake MR, Chan-Yeung M, Dimich-Ward H, Siersted HC, Ernst P, Sweet L, Van Til L, Bowie DM, Anthonisen NR. Geographic and Gender Variability in the Prevalence of Bronchial Responsiveness in Canada. *Chest* 2004; 125: 1657–1664.

246. Leynaert B, Bousquet J, Henry C, Liard R, Neukirch F. Is Bronchial Hyperresponsiveness More Frequent in Women than in Men?: A Population-based Study. *Am J Respir Crit Care Med* 1997; 156: 1413–1420.

247. Schwartz J, Schindler C, Zemp E, Perruchoud AP, Zellweger J-P, Wu¨thrich B, Leuenberger P, Ackermann-Liebrich U. Predictors of Methacholine Responsiveness in a General Population. *Chest* 2002; 122: 812–820.

248. Card JW, Carey MA, Bradbury JA, DeGraff LM, Morgan DL, Moorman MP, Flake GP, Zeldin DC. Gender Differences in Murine Airway Responsiveness and Lipopolysaccharide-Induced Inflammation. *J Immunol* 2006; 177: 621–630.

249. Dijkstra A, Howard TD, Vonk JM, Ampleford EJ, Lange LA, Bleecker ER, Meyers DA, Postma DS. Estrogen receptor 1 polymorphisms are associated with airway hyperresponsiveness and lung function decline, particularly in female subjects with asthma. *Journal of Allergy and Clinical Immunology* 2006; 117: 604–611.

250. Aravamudan B, Goorhouse KJ, Unnikrishnan G, Thompson MA, Pabelick CM, Hawse JR, Prakash YS, Sathish V. Differential Expression of Estrogen Receptor Variants in Response to Inflammation Signals in Human Airway Smooth Muscle: EXPRESSION OF ESTROGEN RECEPTOR VARIANTS IN THE AIRWAY. *J. Cell. Physiol.* 2017; 232: 1754–1760.

251. Bhallamudi S, Connell J, Pabelick CM, Prakash YS, Sathish V. Estrogen receptors differentially regulate intracellular calcium handling in human nonasthmatic and asthmatic airway smooth muscle cells. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 2020; 318: L112–L124.

252. Bradding P, Walls A, Holgate S. The role of the mast cell in the pathophysiology of asthma. *Journal of Allergy and Clinical Immunology* 2006; 117: 1277–1284.

253. Zaitsu M, Narita S-I, Lambert KC, Grady JJ, Estes DM, Curran EM, Brooks EG, Watson CS, Goldblum RM, Midoro-Horiuti T. Estradiol activates mast cells via a non-genomic estrogen receptor-α and calcium influx. *Molecular Immunology* 2007; 44: 1977–1985.

254. Kalidhindi RSR, Ambhore NS, Bhallamudi S, Loganathan J, Sathish V. Role of Estrogen Receptors α and β in a Murine Model of Asthma: Exacerbated Airway Hyperresponsiveness and Remodeling in ERβ Knockout Mice. *Front. Pharmacol.* 2020; 10: 1499.

255. Tan KS. Premenstrual Asthma: Epidemiology, Pathogenesis and Treatment. *Drugs* 2001; 61: 2079–2086.

256. Oguzulgen IK, Turktas H, Erbas D. Airway Inflammation in Premenstrual Asthma. *Journal of Asthma* 2002; 39: 517–522.

257. Rao CK, Moore CG, Bleecker E, Busse WW, Calhoun W, Castro M, Chung KF, Erzurum SC, Israel E, Curran-Everett D, Wenzel SE. Characteristics of Perimenstrual Asthma and Its Relation to Asthma Severity and Control. *Chest* 2013; 143: 984–992.

258. Matteis M, Polverino F, Spaziano G, Roviezzo F, Santoriello C, Sullo N, Bucci MR, Rossi F, Polverino M, Owen CA, D'Agostino B. Effects of sex hormones on bronchial reactivity during the menstrual cycle. *BMC Pulm Med* 2014; 14: 108.

259. O'Connor BJ. Premenstrual asthma: still poorly understood. *Thorax* 1997; 52: 591–592.

260. Lee PY, Bazar KA, Joon Yun A. Menstrual variation of autonomic balance may be a factor in exacerbations of certain diseases during the menstrual cycle. *Medical Hypotheses* 2004; 63: 163–167.

261. Jenkins MA, Dharmage SC, Flander LB, Douglass JA, Ugoni AM, Carlin JB, Sawyer SM, Giles GG, Hopper JL. Parity and decreased use of oral contraceptives as predictors of asthma in young women. *Clin Exp Allergy* 2006; 36: 609–613.

262. Macsali F, Real FG, Omenaas ER, Bjorge L, Janson C, Franklin K, Svanes C. Oral contraception, body mass index, and asthma: A cross-sectional Nordic-Baltic population survey. *Journal of Allergy and Clinical Immunology* 2009; 123: 391–397.

263. Erkoçoğlu M, Kaya A, Azkur D, Özyer Ş, Özcan C, Beşli M, Civelek E, Kocabaş CN. The effect of oral contraceptives on current wheezing in young women. *Allergologia et Immunopathologia* 2013; 41: 169–175.

264. Salam MT, Wenten M, Gilliland FD. Endogenous and exogenous sex steroid hormones and asthma and wheeze in young women. *Journal of Allergy and Clinical Immunology* 2006; 117: 1001–1007.

265. Dratva J, Schindler C, Curjuric I, Stolz D, Macsali F, Gomez FR, Zemp E. Perimenstrual increase in bronchial hyperreactivity in premenopausal women: Results from the population-based SAPALDIA 2 cohort. *Journal of Allergy and Clinical Immunology* 2010; 125: 823–829.

266. Murphy VE, Gibson PG. Premenstrual Asthma: Prevalence, Cycle-to-Cycle Variability and Relationship to Oral Contraceptive Use and Menstrual Symptoms. *Journal of Asthma* 2008; 45: 696–704.

267. Mauvais-Jarvis F, Bairey Merz N, Barnes PJ, Brinton RD, Carrero J-J, DeMeo DL, De Vries GJ, Epperson CN, Govindan R, Klein SL, Lonardo A, Maki PM, McCullough LD, Regitz-Zagrosek V, Regensteiner JG, Rubin JB, Sandberg K, Suzuki A. Sex and gender: modifiers of health, disease, and medicine. *The Lancet* 2020; 396: 565–582.

268. Casimir GJ, Lefèvre N, Corazza F, Duchateau J. Sex and inflammation in respiratory diseases: a clinical viewpoint. *Biol sex dif* 2013; 4: 16.

269. Boonpiyathad T, Sözener ZC, Satitsuksanoa P, Akdis CA. Immunologic mechanisms in asthma. *Seminars in Immunology* 2019; 46: 101333.

270. Hamid Q, Tulic M. Immunobiology of Asthma. *Annu. Rev. Physiol.* 2009; 71: 489–507.

271. Pignataro FS, Bonini M, Forgione A, Melandri S, Usmani OS. Asthma and gender: The female lung. *Pharmacological Research* 2017; 119: 384–390.

272. Zein JG, Erzurum SC. Asthma is Different in Women. *Curr Allergy Asthma Rep* 2015; 15: 28.

273. Wang Y-X, editor. Lung Inflammation in Health and Disease, Volume II [Internet]. Cham: Springer International Publishing; 2021 [cited 2021 Oct 25].Available from: https://link.springer.com/10.1007/978-3-030-68748-9.

274. Townsend EA, Miller VM, Prakash YS. Sex Differences and Sex Steroids in Lung Health and Disease. *Endocrine Reviews* 2012; 33: 1–47.

275. Whitehead GS, Walker JKL, Berman KG, Foster WM, Schwartz DA. Allergen-induced airway disease is mouse strain dependent. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 2003; 285: L32–L42.

276. Ulland TK, Jain N, Hornick EE, Elliott EI, Clay GM, Sadler JJ, Mills KAM, Janowski AM, Volk APD, Wang K, Legge KL, Gakhar L, Bourdi M, Ferguson PJ, Wilson ME, Cassel SL, Sutterwala FS. Nlrp12 mutation causes C57BL/6J strain-specific defect in neutrophil recruitment. *Nat Commun* 2016; 7: 13180.

277. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *Osteoarthritis and Cartilage* 2012; 20: 256–260.

278. Pascoe CD, Jha A, Basu S, Mahood T, Lee A, Hinshaw S, Falsafi R, Hancock REW, Mookherjee N, Halayko AJ, Canadian Respiratory Research Network. The importance of reporting house dust mite endotoxin abundance: impact on the lung transcriptome. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 2020; 318: L1229–L1236.

279. Piyadasa H, Altieri A, Basu S, Schwartz J, Halayko AJ, Mookherjee N. Biosignature for airway inflammation in a house dust mite-challenged murine model of allergic asthma. *Biology Open* 2016; 5: 112–121.

280. Schluger NW, Koppaka R. Lung Disease in a Global Context. A Call for Public Health Action. *Annals ATS* 2014; 11: 407–416.

281. Nials AT, Uddin S. Mouse models of allergic asthma: acute and chronic allergen challenge. *Disease Models and Mechanisms* 2008; 1: 213–220.

282. Aun M, Bonamichi-Santos R, Arantes-Costa FM, Kalil J, Giavina-Bianchi P. Animal models of asthma: utility and limitations. *JAA* 2017; Volume10: 293–301.

283. Chapman DG, Tully JE, Nolin JD, Janssen-Heininger YM, Irvin CG. Animal Models of Allergic Airways Disease: Where Are We and Where to Next?: A NIMAL M ODELS OF A LLERGIC A IRWAYS D ISEASE. *J. Cell. Biochem.* 2014; 115: 2055–2064.

284. Okuyama K, Wada K, Chihara J, Takayanagi M, Ohno I. Sex-related splenocyte function in a murine model of allergic asthma. *Clinical & Experimental Allergy* 2008; 38: 1212–1219.

285. Takeda M, Tanabe M, Ito W, Ueki S, Konnno Y, Chihara M, Itoga M, Kobayashi Y, Moritoki Y, Kayaba H, Chihara J. Gender difference in allergic airway remodelling and immunoglobulin production in mouse model of asthma: Severe airway remodelling in female mice. *Respirology* 2013; 18: 797–806.

286. Antunes MA, Abreu SC, Silva AL, Parra-Cuentas ER, Ab'Saber AM, Capelozzi VL, Ferreira TPT, Martins MA, Silva PMR, Rocco PRM. Sex-specific lung remodeling and inflammation changes in experimental allergic asthma. *Journal of Applied Physiology* 2010; 109: 855–863.

287. Doras C, Petak F, Bayat S, Baudat A, Von Garnier C, Eigenmann P, Habre W. Lung responses in murine models of experimental asthma: Value of house dust mite over ovalbumin sensitization. *Respiratory Physiology & Neurobiology* 2018; 247: 43–51.

288. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends in Immunology* 2011; 32: 402–411.

289. Gueders MM, Paulissen G, Crahay C, Quesada-Calvo F, Hacha J, Van Hove C, Tournoy K, Louis R, Foidart J-M, Noël A, Cataldo DD. Mouse models of asthma: a comparison between C57BL/6 and BALB/c strains regarding bronchial responsiveness, inflammation, and cytokine production. *Inflamm. Res.* 2009; 58: 845–854.

290. Galli SJ, Tsai M. IgE and mast cells in allergic disease. *Nat Med* 2012; 18: 693–704.

291. Piyadasa H, Hemshekhar M, Altieri A, Basu S, van der Does AM, Halayko AJ, Hiemstra PS, Mookherjee N. Immunomodulatory innate defence regulator (IDR) peptide alleviates airway inflammation and hyper-responsiveness. *Thorax* 2018; 73: 908–917.

292. Virchow JC, Kroegel C, Walker C, Matthys H. Inflammatory determinants of asthma severity: Mediator and cellular changes in bronchoalveolar lavage fluid of patients with severe asthma. 98: 14.

293. Fuseini H, Yung JA, Cephus JY, Zhang J, Goleniewska K, Polosukhin VV, Peebles RS, Newcomb DC. Testosterone Decreases House Dust Mite–Induced Type 2 and IL-17A–Mediated Airway Inflammation. *J.I.* 2018; 201: 1843–1854.

294. Sellers RS, Clifford CB, Treuting PM, Brayton C. Immunological Variation Between Inbred Laboratory Mouse Strains: Points to Consider in Phenotyping Genetically Immunomodified Mice. *Vet Pathol* 2012; 49: 32–43.

295. Zhu W, Gilmour MI. Comparison of allergic lung disease in three mouse strains after systemic or mucosal sensitization with ovalbumin antigen. *Immunogenetics* 2009; 61: 199–207.

296. Nakajima H, Takatsu K. Role of Cytokines in Allergic Airway Inflammation. *Int Arch Allergy Immunol* 2007; 142: 265–273.

297. Lambrecht BN, Hammad H, Fahy JV. The Cytokines of Asthma. *Immunity* 2019; 50: 975–991.

298. Pawankar R, Hayashi M, Yamanishi S, Igarashi T. The paradigm of cytokine networks in allergic airway inflammation. *Current Opinion in Allergy & Clinical Immunology* 2015; 15: 41–48.

299. Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J. Clin. Invest.* 2008; 118: 3546–3556.

300. Hansbro PM, Kaiko GE, Foster PS. Cytokine/anti-cytokine therapy - novel treatments for asthma?: Anti-cytokine asthma therapies. *British Journal of Pharmacology* 2011; 163: 81–95.

301. Hayashi T, Adachi Y, Hasegawa K, Morimoto M. Less Sensitivity for Late Airway Inflammation in Males than Females in BALB/c Mice. *Scand J Immunol* 2003; 57: 562–567.

302. Corteling R, Trifilieff A. Gender comparison in a murine model of allergen-driven airway inflammation and the response to budesonide treatment. *BMC Pharmacology* 2004; : 9.

303. Blacquière MJ, Hylkema MN, Postma DS, Geerlings M, Timens W, Melgert BN. Airway Inflammation and Remodeling in Two Mouse Models of Asthma: Comparison of Males and Females. *Int Arch Allergy Immunol* 2010; 153: 173–181.

304. Garcia G, Taille C, Laveneziana P, Bourdin A, Chanez P, Humbert M. Anti-interleukin-5 therapy in severe asthma. *European Respiratory Review* 2013; 22: 251–257.

305. Pelaia C, Paoletti G, Puggioni F, Racca F, Pelaia G, Canonica GW, Heffler E. Interleukin-5 in the Pathophysiology of Severe Asthma. *Front. Physiol.* 2019; 10: 1514.

306. Li BWS, de Bruijn MJW, Tindemans I, Lukkes M, KleinJan A, Hoogsteden HC, Hendriks RW. T cells are necessary for ILC2 activation in house dust mite-induced allergic airway inflammation in mice. *Eur. J. Immunol.* 2016; 46: 1392–1403.

307. Martinez-Gonzalez I, Steer CA, Takei F. Lung ILC2s link innate and adaptive responses in allergic inflammation. *Trends in Immunology* 2015; 36: 189–195.

308. Laffont S, Blanquart E, Guéry J-C. Sex Differences in Asthma: A Key Role of Androgen-Signaling in Group 2 Innate Lymphoid Cells. *Front. Immunol.* 2017; 8: 1069.

309. Laffont S, Blanquart E, Savignac M, Cénac C, Laverny G, Metzger D, Girard J-P, Belz GT, Pelletier L, Seillet C, Guéry J-C. Androgen signaling negatively controls group 2 innate lymphoid cells. *Journal of Experimental Medicine* 2017; 214: 1581–1592.

310. Kadel S, Ainsua-Enrich E, Hatipoglu I, Turner S, Singh S, Khan S, Kovats S. A Major Population of Functional KLRG1 ⁻ ILC2s in Female Lungs Contributes to a Sex Bias in ILC2 Numbers. *IH* 2018; 2: 74–86.

311. Pease JE, Sabroe I. The Role of Interleukin-8 and its Receptors in Inflammatory Lung Disease: Implications for Therapy. *Am J Respir Med* 2002; 1: 19–25.

312. Morishima Y, Ano S, Ishii Y, Ohtsuka S, Matsuyama M, Kawaguchi M, Hizawa N. Th17-Associated Cytokines as a Therapeutic Target for Steroid-Insensitive Asthma. *Clinical and Developmental Immunology* 2013; 2013: 1–9.

313. Hynes GM, Hinks TSC. The role of interleukin-17 in asthma: a protective response? *ERJ Open Res* 2020; 6: 00364–02019.

314. Elderman M, de Vos P, Faas M. Role of Microbiota in Sexually Dimorphic Immunity. *Front. Immunol.* 2018; 9: 1018.

315. Elderman M, Hugenholtz F, Belzer C, Boekschoten M, van Beek A, de Haan B, Savelkoul H, de Vos P, Faas M. Sex and strain dependent differences in mucosal immunology and microbiota composition in mice. *Biol Sex Differ* 2018; 9: 26.

316. Valeri F, Endres K. How biological sex of the host shapes its gut microbiota. *Frontiers in Neuroendocrinology* 2021; 61: 100912.

317. Beauruelle C, Guilloux C-A, Lamoureux C, Héry-Arnaud G. The Human Microbiome, an Emerging Key-Player in the Sex Gap in Respiratory Diseases. *Front. Med.* 2021; 8: 600879.

318. Zijlstra GJ, ten Hacken NHT, Hoffmann RF, van Oosterhout AJM, Heijink IH. Interleukin-17A induces glucocorticoid insensitivity in human bronchial epithelial cells. *European Respiratory Journal* 2012; 39: 439–445.

319. Melgert BN, Ray A, Hylkema MN, Timens W, Postma DS. Are there reasons why adult asthma is more common in females? *Curr Allergy Asthma Rep* 2007; 7: 143–150.

320. Becklake MR, Kauffmann F. Gender differences in airway behaviour over the human life span. *Thorax* 1999; 54: 1119–1138.

321. Lin RY, Lee GB. The Gender Disparity in Adult Asthma Hospitalizations Dynamically Relates to Age. *Journal of Asthma* 2008; 45: 931–935.

322. Zijlstra GJ, Fattahi F, Rozeveld D, Jonker MR, Kliphuis NM, van den Berge M, Hylkema MN, ten Hacken NHT, van Oosterhout AJM, Heijink IH. Glucocorticoids induce the production of the chemoattractant CCL20 in airway epithelium. *European Respiratory Journal* 2014; 44: 361–370.

323. Li Q, Laumonnier Y, Syrovets T, Simmet T. Recruitment of CCR6-expressing Th17 cells by CCL20 secreted from plasmin-stimulated macrophages. *Acta Biochimica et Biophysica Sinica* 2013; 45: 593–600.

324. Fröhlich A, Marsland BJ, Sonderegger I, Kurrer M, Hodge MR, Harris NL, Kopf M. IL-21 receptor signaling is integral to the development of Th2 effector responses in vivo. *Blood* 2007; 109: 2023–2031.

325. Sjöberg LC, Nilsson AZ, Lei Y, Gregory JA, Adner M, Nilsson GP. Interleukin 33 exacerbates antigen driven airway hyperresponsiveness, inflammation and remodeling in a mouse model of asthma. *Sci Rep* 2017; 7: 4219.

326. Chan BCL, Lam CWK, Tam L-S, Wong CK. IL33: Roles in Allergic Inflammation and Therapeutic Perspectives. *Front. Immunol.* 2019; 10: 364.

327. Piyadasa H, Lloyd D, Lee AHY, Altieri A, Hemshekhar M, Osawa N, Basu S, Blimkie T, Falsafi R, Halayko AJ, Hancock REW, Mookherjee N. Characterization of immune responses and the lung transcriptome in a murine model of IL-33 challenge. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 2020; 1866: 165950.

328. Coquet JM, Schuijs MJ, Smyth MJ, Deswarte K, Beyaert R, Braun H, Boon L, Hedestam GBK, Nutt SL, Hammad H, Lambrecht BN. Interleukin-21-Producing CD4+ T Cells Promote Type 2 Immunity to House Dust Mites. *Immunity* 2015; 43: 318–330.

329. Gong F, Su Q, Pan YH, Huang X, Shen WH. The emerging role of interleukin-21 in allergic diseases (Review). *Biomedical Reports* 2013; 1: 837–839.

330. Liu Y, Shao Z, Shangguan G, Bie Q, Zhang B. Biological Properties and the Role of IL-25 in Disease Pathogenesis. *Journal of Immunology Research* 2018; 2018: 1–8.

331. Zhou Y, McLane M, Levitt RC. Th2 cytokines and asthma Interleukin-9 as a therapeutic target for asthma. 2: 5.

332. Temann U-A, Laouar Y, Eynon EE, Homer R, Flavell RA. IL9 leads to airway inflammation by inducing IL13 expression in airway epithelial cells. *International Immunology* 2006; 19: 1–10.

333. Soussi-Gounni A, Kontolemos M, Hamid Q. Role of IL-9 in the pathophysiology of allergic diseases. *Journal of Allergy and Clinical Immunology* 2001; 107: 575–582.

334. Martin RA, Hodgkins SR, Dixon AE, Poynter ME. Aligning mouse models of asthma to human endotypes of disease: Asthma endotypes inform mouse models. *Respirology* 2014; 19: 823–833.

335. Shah R, Newcomb DC. Sex Bias in Asthma Prevalence and Pathogenesis. *Front. Immunol.* 2018; 9: 2997.

336. Lee Y, Hwang Y-H, Kim K-J, Park A-K, Paik M-J, Kim SH, Lee SU, Yee S-T, Son Y-J. Proteomic and transcriptomic analysis of lung tissue in OVA-challenged mice. *Arch. Pharm. Res.* 2018; 41: 87–100.

337. Chang DW, Hayashi S, Gharib SA, Vaisar T, King ST, Tsuchiya M, Ruzinski JT, Park DR, Matute-Bello G, Wurfel MM, Bumgarner R, Heinecke JW, Martin TR. Proteomic and Computational Analysis of Bronchoalveolar Proteins during the Course of the Acute Respiratory Distress Syndrome. *Am J Respir Crit Care Med* 2008; 178: 701–709.

338. Lee EJ, In KH, Kim JH, Lee SY, Shin C, Shim JJ, Kang KH, Yoo SH, Kim CH, Kim H-K, Lee SH, Uhm CS. Proteomic Analysis in Lung Tissue of Smokers and COPD Patients. *Chest* 2009; 135: 344–352.

339. Mookherjee N, Piyadasa H, Ryu MH, Rider CF, Ezzati P, Spicer V, Carlsten C. Inhaled diesel exhaust alters the allergen-induced bronchial secretome in humans. *Eur Respir J* 2018; 51: 1701385.

340. Mahood TH, Pascoe CD, Karakach TK, Jha A, Basu S, Ezzati P, Spicer V, Mookherjee N, Halayko AJ. Integrating Proteomes for Lung Tissues and Lavage Reveals Pathways That Link Responses in Allergen-Challenged Mice. *ACS Omega* 2021; 6: 1171–1189.

341. Nair P, Ochkur SI, Protheroe C, Radford K, Efthimiadis A, Lee NA, Lee JJ. Eosinophil peroxidase in sputum represents a unique biomarker of airway eosinophilia. *Allergy* 2013; : n/a-n/a.

342. Zhao H, Moarbes V, Gaudreault V, Shan J, Aldossary H, Cyr L, Fixman ED. Sex Differences in IL-33-Induced STAT6-Dependent Type 2 Airway Inflammation. *Front. Immunol.* 2019; 10: 859.

343. Bresnick AR. S100 proteins as therapeutic targets. *Biophys Rev* 2018; 10: 1617–1629.

344. Xia C, Braunstein Z, Toomey AC, Zhong J, Rao X. S100 Proteins As an Important Regulator of Macrophage Inflammation. *Front. Immunol.* 2018; 8: 1908.

345. Ma J. S100A8/A9 in Inflammation. *Frontiers in Immunology* 2018; 9: 14.

346. Pruenster M, Vogl T, Roth J, Sperandio M. S100A8/A9: From basic science to clinical application. *Pharmacology & Therapeutics* 2016; 167: 120–131.

347. Sattar Z, Lora A, Jundi B, Railwah C, Geraghty P. The S100 Protein Family as Players and Therapeutic Targets in Pulmonary Diseases. Kuwano K, editor. *Pulmonary Medicine* 2021; 2021: 1–20.

348. Halayko AJ, Ghavami S. S100A8/A9: a mediator of severe asthma pathogenesis and morbidity?This article is one of a selection of papers published in a special issue celebrating the 125th anniversary of the Faculty of Medicine at the University of Manitoba. *Can. J. Physiol. Pharmacol.* 2009; 87: 743–755.

349. Kim DH, Gu A, Lee J-S, Yang EJ, Kashif A, Hong MH, Kim G, Park BS, Lee SJ, Kim IS. Suppressive effects of S100A8 and S100A9 on neutrophil apoptosis by cytokine release of human bronchial epithelial cells in asthma. *Int. J. Med. Sci.* 2020; 17: 498–509.

350. Palmer LD, Maloney KN, Boyd KL, Goleniewska AK, Toki S, Maxwell CN, Chazin WJ, Peebles RS, Newcomb DC, Skaar EP. The Innate Immune Protein S100A9 Protects from T-Helper Cell Type 2–mediated Allergic Airway Inflammation. *Am J Respir Cell Mol Biol* 2019; 61: 459–468.

351. Manni ML, Alcorn JF. Calprotectin-g the Lung during Type 2 Allergic Airway Inflammation. *Am J Respir Cell Mol Biol* 2019; 61: 405–407.

352. Wang Y, Miwa T, Ducka-Kokalari B, Redai IG, Sato S, Gullipalli D, Zangrilli JG, Haczku A, Song W-C. Properdin Contributes to Allergic Airway Inflammation through Local C3a Generation. *J.I.* 2015; 195: 1171–1181.

353. Chan KT, Creed SJ, Bear JE. Unraveling the enigma: progress towards understanding the coronin family of actin regulators. *Trends in Cell Biology* 2011; 21: 481–488.

354. Siegmund K, Klepsch V, Hermann-Kleiter N, Baier G. Proof of Principle for a T Lymphocyte Intrinsic Function of Coronin 1A. *Journal of Biological Chemistry* 2016; 291: 22086–22092.

355. Punwani D, Pelz B, Yu J, Arva NC, Schafernak K, Kondratowicz K, Makhija M, Puck JM. Coronin-1A: Immune Deficiency in Humans and Mice. *J Clin Immunol* 2015; 35: 100–107.

356. Dasgupta A, Chakraborty R, Saha B, Suri H, Singh P, Raj A, Taneja B, Dash D, Sengupta S, Agrawal A. Sputum Protein Biomarkers in Airway Diseases: A Pilot Study. *International Journal of Chronic Obstructive Pulmonary Disease* : 13.

357. Liu KA, DiPietro Mager NA. Women's involvement in clinical trials: historical perspective and future implications. *Pharm Pract (Granada)* 2016; 14: 708–708.

358. Keselman A, Heller N. Estrogen Signaling Modulates Allergic Inflammation and Contributes to Sex Differences in Asthma. *Front. Immunol.* [Internet] 2015 [cited 2020 Oct 9]; 6Available from: http://journal.frontiersin.org/Article/10.3389/fimmu.2015.00568/abstract.

359. Oettgen HC, Geha RS. IgE regulation and roles in asthma pathogenesis. *Journal of Allergy and Clinical Immunology* 2001; 107: 429–441.

360. Froidure A, Mouthuy J, Durham SR, Chanez P, Sibille Y, Pilette C. Asthma phenotypes and IgE responses. *Eur Respir J* 2016; 47: 304–319.

361. Aghasafari P, George U, Pidaparti R. A review of inflammatory mechanism in airway diseases. *Inflamm. Res.* 2019; 68: 59–74.

362. Larsen GL, Holt PG. The Concept of Airway Inflammation. *Am J Respir Crit Care Med* 2000; 162: S2–S6.

363. Fahy JV. Eosinophilic and Neutrophilic Inflammation in Asthma: Insights from Clinical Studies. *Proceedings of the American Thoracic Society* 2009; 6: 256–259.

364. Ray A, Kolls JK. Neutrophilic Inflammation in Asthma and Association with Disease Severity. *Trends in Immunology* 2017; 38: 942–954.

365. Kamath AV. Is the neutrophil the key effector cell in severe asthma? *Thorax* 2005; 60: 529–530.

366. Wang Y-H, Wills-Karp M. The Potential Role of Interleukin-17 in Severe Asthma. *Curr Allergy Asthma Rep* 2011; 11: 388–394.

367. Ramakrishnan RK, Al Heialy S, Hamid Q. Role of IL-17 in asthma pathogenesis and its implications for the clinic. *Expert Review of Respiratory Medicine* 2019; 13: 1057–1068.

368. Gautam Y, Afanador Y, Abebe T, López JE, Mersha TB. Genome-wide analysis revealed sex-specific gene expression in asthmatics. *Human Molecular Genetics* 2019; 28: 2600–2614.

369. Fuentes N, Cabello N, Nicoleau M, Chroneos ZC, Silveyra P. Modulation of the lung inflammatory response to ozone by the estrous cycle. *Physiol Rep* 2019; 7: e14026.

370. Taylor AE, Keevil B, Huhtaniemi IT. Mass spectrometry and immunoassay: how to measure steroid hormones today and tomorrow. *European Journal of Endocrinology* 2015; 173: D1–D12.

371. Handelsman DJ, Wartofsky L. Requirement for Mass Spectrometry Sex Steroid Assays in the Journal of Clinical Endocrinology and Metabolism. *The Journal of Clinical Endocrinology & Metabolism* 2013; 98: 3971–3973.

372. Byers SL, Wiles MV, Dunn SL, Taft RA. Mouse Estrous Cycle Identification Tool and Images. Singh SR, editor. *PLoS ONE* 2012; 7: e35538.

373. Kudo M, Ishigatsubo Y, Aoki I. Pathology of asthma. *Front. Microbiol.* [Internet] 2013 [cited 2021 Oct 25]; 4Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2013.00263/abstract.

374. Hough KP, Curtiss ML, Blain TJ, Liu R-M, Trevor J, Deshane JS, Thannickal VJ. Airway Remodeling in Asthma. *Front. Med.* 2020; 7: 191.

375. Locke NR, Royce SG, Wainewright JS, Samuel CS, Tang ML. Comparison of Airway Remodeling in Acute, Subacute, and Chronic Models of Allergic Airways Disease. *Am J Respir Cell Mol Biol* 2007; 36: 625–632.

376. Krishnamoorthy N, Douda DN, Brüggemann TR, Ricklefs I, Duvall MG, Abdulnour R-EE, Martinod K, Tavares L, Wang X, Cernadas M, Israel E, Mauger DT, Bleecker ER, Castro M, Erzurum SC, Gaston BM, Jarjour NN, Wenzel S, Dunican E, Fahy JV, Irimia D, Wagner DD, Levy BD, National Heart, Lung, and Blood Institute Severe Asthma Research Program-3 Investigators. Neutrophil cytoplasts induce T_H 17 differentiation and skew inflammation toward neutrophilia in severe asthma. *Sci. Immunol.* 2018; 3: eaao4747.