The Role of Ovarian Hormones and Testosterone on Type 2 and IL-17A-Mediated Airway Inflammation

By

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To my incredibly supportive “Fathers”,

Abukari, Seidu and Osman

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

ABBREVIATIONS

AMφ	Alveolar macrophages
AHR	Airway hyperresponsiveness
AR	Androgen receptor
ATAC-Seq	Assay for Transposase-Accessible Chromatin using Sequencing
AR^{tfm}	Androgen receptor testicular femininized
AMPK	AMP-sensitive signaling kinase
BAL	Broncheoalveolar lavage
CAMP	Childhood Asthma Management Program
c-d-GMP	cyclic di-GMP
ChIP-Seq	Chromatin-Immunoprecipitation using DNA Sequencing
DCs	Dendritic cells
DEREG	Deletion of Regulatory T cells
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone-sulfate
DHT	Dihydrotestosterone
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERRα	Estrogen-related receptor alpha
ECRHS	European Community Respiratory Health Survey
FeNO	Forced nitric oxide
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GINA	Global Initiative for Asthma
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid
GPER1	G-protein coupled estrogen receptor 1
GWAS	Genome wide association study
HDM	House dust mite
HPG	Hypothalamus-pituitary-gonadal axis
HRT	Hormone replacement therapy
HRV	Human rhinovirus
IgE	Immunoglobulin E
IgG	Immunoglobulin G
iNOS	Inducible nitric oxide synthase
KO	Knockout
LH	Luteinizing hormone
MHC	Major Histocompatibility complex
mTOR	Mechanistic target of rapamycin
OVA	Ovalbumin
PRα	Progesterone receptor α
PRβ	Progesterone receptor β
RA	Rheumatoid arthritis

RSV	Respiratory syncytial virus
SARP	Severe Asthma Research Program
STAT	Signal transducer and activator of transcription
STING	Stimulator of Interferon Genes
SLE	Systemic lupus erythematosus
TLR	Toll-like Receptors
TSLP	Thymic Stromal Lymphopoietin
DAMPS	Damage-Associated Molecular Patterns
PAMPS	Pattern-Associated Molecular Patterns
WT	Wildtype

CHAPTER 1

Introduction

1.1 Overview

The prevalence of asthma has increased significantly in the United States over the last 30 years (1). While patients with mild-to-moderate asthma are generally responsive to current asthma therapies, patients with severe asthma have poorer responses to these therapeutics. Patients with severe asthma represent less than 10% of the total asthma population, however account for over 50% of the annual asthma-associated healthcare expenditures, estimated to be over 6 billion dollars yearly (1-3). In order to alleviate this economic burden, therapies best suited for patients with severe asthma are urgently needed. However, the underlying immunological mechanisms driving airway inflammation associated with severe asthma remain unclear.

Among patients with asthma, including severe asthma, there is a gender bias in asthma that changes throughout life. As children, boys are more likely to have asthma girls. However, after puberty, women become twice more likely than men to have asthma. After women reach menopause, the prevalence of asthma equalizes with respect to gender (4). The change in asthma prevalence in and around the time of puberty coincides with increasing levels of sex hormones in males and females, suggesting a potential role for sex hormones in asthma pathogenesis (5). The contents enclosed within this dissertation work focuses on understanding the foundational mechanisms by which ovarian hormones (estrogen and progesterone)

and androgens (testosterone and dihydrotestosterone (DHT)) influence asthma pathogenesis. Understanding how sex hormones regulate immunological mechanisms in asthma pathogenesis and severity will support the development of personalized asthma therapies for women and men with asthma at various life stages (pre-pubescent, child-bearing years, menopause, men with low testosterone). This dissertation begins with an outline of the significance and rationale for this work, followed by a chapter describing the literature upon which the studies in this dissertation were built on. Next, the data for three specific research aims are presented followed by an overall summation of the major findings and contributions of this dissertation work to this scientific field.

1.2 Significance and Rationale

Asthma is a chronic heterogenous disease that is characterized by different phenotypes and endotypes. While current asthma therapeutics are effective in some patients, others remain unresponsive to these therapies (6). Recently, non-biased cluster analyses performed in the United States, identified six unique clinical clusters of patients with asthma, based on varying asthma associated clinical outputs, including onset of asthma, atopy, lung function and medication dosage required to manage asthma symptoms (7). Patients with asthma grouped into clusters 1-3 accounted for asthmatics who required low to moderate medication to manage their asthma and were classified as patients with mild to moderate asthma. In contrast, patients grouped into clusters 4-6 required either high medication usage or were unresponsive to those therapies and were characterized as patients with moderate to severe asthma (7). While patients with severe asthma represent 5-10% of the total asthma population,

they account for over 50% of the total annual asthma healthcare expenditure (8). To improve asthma control and reduce morbidity and the healthcare economic burden, it is important to develop novel, personalized therapies for patients with severe asthma.

The unsupervised cluster analyses grouped asthma patients based on phenotypes associated with asthma severity, but also identified endotypes associated with varying pathophysiologies. Phenotypes of asthma are defined as observational clinical, physiological, morphological and biochemical characteristics as well as responses to different treatments. However, endotypes are defined by distinct pathophysiological mechanisms (9). Increased blood and/or sputum interleukin 4 (IL-4), 5 (IL-5) and 13 (IL-13) and eosinophils were usually evidence of a type 2-mediated inflammation. Alternatively, increased blood and/or sputum secretion of non-type 2 markers, including interleukin 17A (IL-17A) and/or interferon-γ (IFNγ) and increased neutrophils were evidence of non-type 2 mediated airway inflammation (10, 3). Patients with mild to moderate asthma often displayed more type 2 mediated inflammatory signatures while those with severe asthma displayed both type 2 and/or non-type 2 mediated inflammatory signatures (6, 7).

Across all six clusters identified, a female predominance was observed where women had increased levels of biomarkers associated with both type 2 and/or non-type 2 mediated airway inflammation. Moreover, the female predominance was statistically strongest among patients with more severe asthma ($p < 0.002$) (7). This finding supported previous studies that demonstrated that women of reproductive age have a higher prevalence of asthma compared to men of a similar age (4, 11-13). Mouse models that mimic asthma showed female sex hormones increased and male sex hormones decreased type 2 and non-type inflammatory responses

in the airway (14-19). These studies determined the role of sex hormones on type 2 OR non-type 2 inflammatory responses in isolation and it remained unclear the mechanisms by which sex hormones mediated both by type 2 and non-type 2 inflammation.

Severe asthma is also associated with dual type 2 and IL-17A mediated airway inflammation (3, 20). Thus, deciphering the role of sex hormones on dual type 2 and IL-17A mediated inflammatory responses is imperative for understanding the foundational mechanisms underlying the gender differences in asthma prevalence. Based on these previous findings, we hypothesize that during dual type 2 and IL-17A mediated airway inflammation, ovarian hormones promote inflammation by increasing cytokine expression of type 2 cytokines and IL-17A while testosterone attenuates inflammation by decreasing cell numbers of type 2 and IL-17A secreting cells. To test these hypotheses, we propose the following specific aims:

Aim 1: Delineate the effect of ovarian hormones and testosterone on house dust mite-induced type 2 and IL-17A-mediated airway inflammation, airway hyperresponsiveness and mucus production

Aim 2: Determine the mechanisms by which androgen receptor signaling attenuates Th2 and Th17 cell-mediated airway inflammation and cytokine expression

Aim 3: Determine the mechanisms by estrogen receptor α signaling enhances IL-17A production in Th17 cells.

1.3 Epidemiology of Asthma

Asthma is a chronic disease of the lower respiratory tract, associated with increased airway inflammation, mucus production and airway hyperresponsiveness (AHR). Airway exacerbations induced by these hallmarks of asthma can lead to episodic or persistent cough, wheezing and dyspnea (21). Over 25 million individuals in the United States are affected by asthma and this number has steadily increased by 3% each annually since the 1970s (1). Globally, over 300 million individuals suffer from asthma and the Global Initiative for Asthma further estimates that there will be an additional 100 million people with asthma by 2025 (1).

While asthma symptoms are controlled with current medications for most patients with asthma, there are some patients with severe asthma phenotypes that require high systemic corticosteroid usage or are refractory to current asthma therapeutics (22). According to the European Respiratory Society and the American Thoracic Society guidelines, asthma is characterized as intermittent to persistent, severe asthma based on two criteria: (i) whether the patient is on high-dose inhaled corticosteroids and one additional controller medication (including long-acting β2 agonists) or (ii) is currently on oral corticosteroids medications with the past 6-12 months. In additional to one of these criteria, a patient must also present one of the following clinical symptoms or responses in response to reduced treatment: (iii) low asthma control scores, including an asthma control questionnaire (ACQ) > 1.5 or (iv) an Asthma Control Test (ACT) < 20, (2) at least 2 exacerbations in the last 12 months, at least exacerbation treated in hospital or (v) requiring mechanical ventilation in the last 12 months or (Forced expiratory volume in 1 second) FEV_1 less than 80% (23). Patients with more severe asthma symptoms account for only 5-10% of the total US asthma population but represent over 50% of annual

asthma associated healthcare expenditure (6). Annually, this amounts to an estimated six billion dollars, with the largest costs associated with emergency visits and hospitalizations (3, 24). This disproportionally high health care burden is due to increased mortality and morbidity among this subgroup of asthmatics due in part poorer responses to current asthma therapeutics. To lower this high economic burden on the state, new therapies better suited for patients with asthma who are nonresponsive to current asthma therapies are urgently needed. However, efforts to develop these therapeutics are compounded by the heterogeneity that exits in the clinical and pathological manifestations of asthma. Below is a review of the different classification systems that been utilized to identify asthma patients into subgroups, based on individual or multivariate variables.

1.4 Phenotypes and Endotypes of Asthma

Asthma is heterogenous disease and studies report that patients with asthma can be further separated into subgroups based on clinical (phenotype) or pathophysiological (endotype) characteristics (25) Asthma phenotype influences the types of diagnostic tests performed and how responsive a patient will be to different to pharmacotherapies. Many asthma phenotypes have been discovered, where patients with asthma are divided into broad subgroups based on an individual variable. Utilizing this phenotyping strategy patients with asthma have been divided in to the following subgroups,

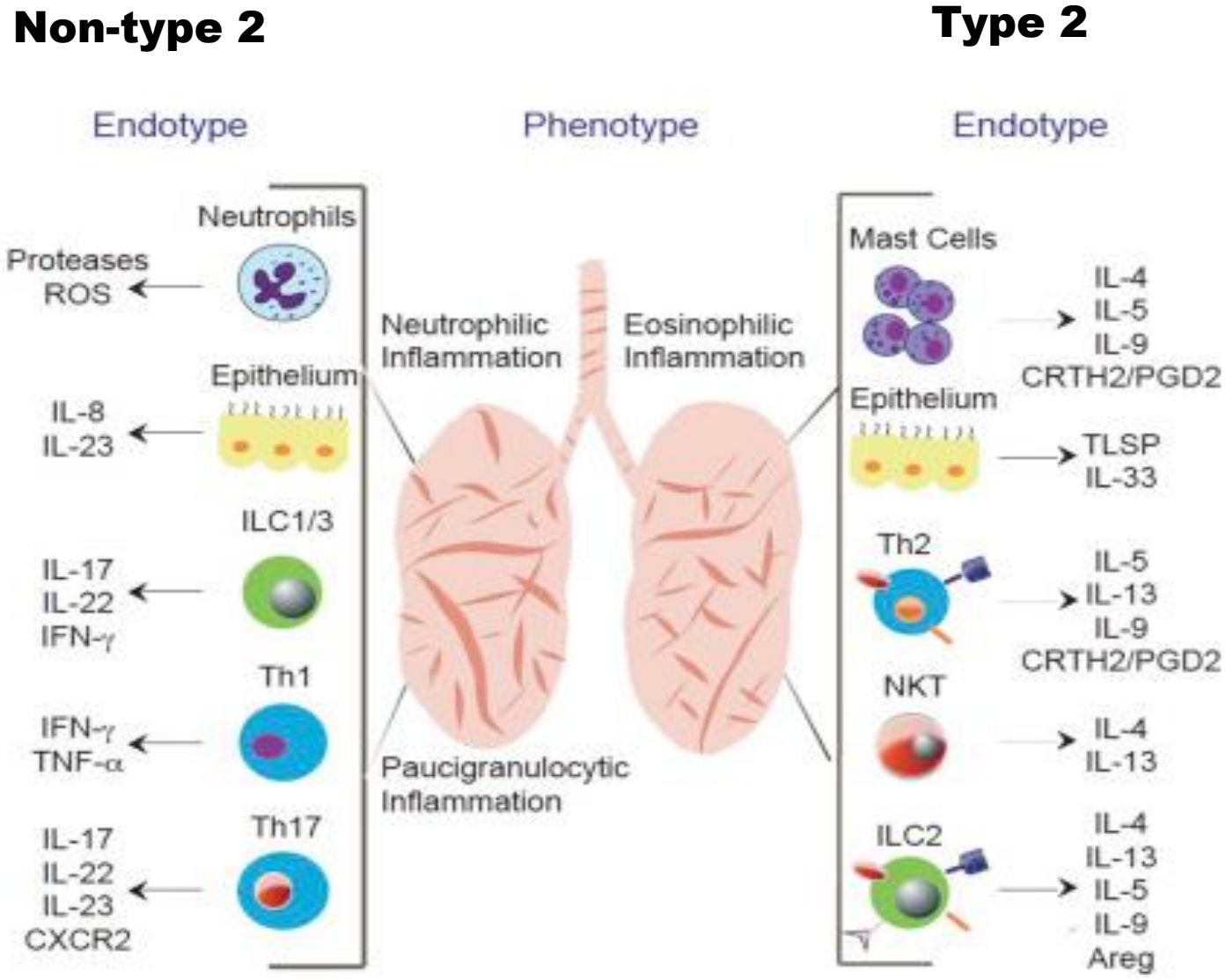

Figure 1-1: Type 2 and Non-Type 2-Mediated Inflammation Pathways in Asthma. Different immunological mechanisms drive asthma pathogenesis. In Non-Type 2 mediated airway inflammation, increased neutrophils numbers or paucigranuloytic-mediated inflammation is predominant and patients with these phenotypes are less likely to respond to corticosteroids. In neutrophilic-predominant disease, patients might respond to antibodies that block TNF-α, IL-17, IL-23. Innate lymphoid cells group 1 and group 3 are more predominant in non-type 2 mediated asthma. These cells produce IFNγ, IL-17, and IL-22. Type 2 mediated airway inflammation is associated with eosinophilic phenotypes and increased responsiveness to corticosteroids. Many cytokines contribute to type 2 immunity but targeting specific type 2 cytokines have been effective in the development of biologics for patients with persistent eosinophilic mediated asthma, including antagonists against including antagonists of IgE, IL-5, IL-13, and CRTH2. IFNγ, interferon gamma; ILC1, type 1 innate lymphoid cells; ILC2, group 2 innate lymphoid cells; ILC3, group 3 innate lymphoid cells; NKT, natural killer cells; PGD_2, prostaglandin D_2; ROS, reactive oxygen species. *Adapted* from Tabatabaian et al. 2017. "Biologic and New Therapies of Asthma". *Immunology and Allergy Clinics of North America*. 37 (2): Pages 329-34.

including (1) Allergy phenotypes (allergic or non-allergic (intrinsic)), (2) Treatment-responsiveness phenotype (steroid responsive and resistant), and (3) Pathology-defined phenotypes (25). This strategy of phenotyping has been successful in identifying patients with asthma who respond well to current asthma therapies, including allergen immunotherapy (26) and anti-IgE antibody therapy as well as individuals with more severe asthma who have varying responses to these therapies (27, 28).

While these asthma phenotypes and endotypes give a general idea about the heterogeneity in asthma, these broad classifications disregard overlaps that may exist between different phenotypes and endotypes of asthma. To better classify patients with asthma based on clinical characteristics and pathobiology, unsupervised statistical cluster analyses were performed in three cohorts of patients with asthma in the Europe and the United States. Clusters were identified based on different factors, including the age of onset of asthma, gender, allergic status, asthma symptoms, and lung function (29). Despite differences in patient populations, these three cluster analysis studies identified clusters that were disproportionality women, in particular a cluster with late onset, nonatopic asthma and with higher levels of neutrophils in the sputum (30, 31) (32). Combined, these cluster analysis studies showed that a predominance of women was seen in the more severe asthma phenotypes.

Patients with asthma can also be divided into subgroups based on their asthma endotype, or the specific pathological mechanisms driving their asthma (29). Prior studies characterized two distinct inflammatory endotypes of asthma, based on the presence of type 2 cytokines, including interleukin 4 (IL-4), 5 (IL-5), 13 (IL-13) and eosinophils or the presence of non -type 2 cytokines, including interleukin IL-17A (IL-17A), interferon-γ, (IFNγ) and

neutrophils in the sputum and lavage. From these studies, patients with asthma, including those with severe asthma, are often categorized into two endotypes; Type 2 or Non-type 2 mediated airway inflammation (33). Type 2 or non-type 2 cytokines are produced by various cell types in the lung, including T cells, innate lymphoid cells and other immune cells (34, 35, 3). These immunological pathways are implicated in the pathogenesis of asthma and cause the immunological hallmarks of asthma, including increased airway inflammation, airway remodeling, airway reactivity and mucus production, leading to the clinical symptoms associated with asthma (36, 37, 20). These immunological mechanisms are reviewed below the next section and depicted in Figure 1-1.

1.5 Immunopathogenesis of Asthma

The lung mucosa is continuously exposed to inhaled foreign particles, where normal functioning respiratory systems are able to distinguish innocuous particles from pathogenic ones (38). However, in asthma, underlying host susceptibility to allergic disease coupled with an immune response is mounted to inhaled allergens (39). Aeroallergens that cause allergic asthma, including house dust mite and pollen, have pattern recognition receptors , including Toll-like receptors (TLR), Damage-associated molecular patterns (DAMPS) and (Pattern-associated molecular patterns (PAMPS) by innate immune cells, including airway epithelial cells, leading to increased secretion of TSLP, IL-25 and IL-33 (40).

The release of these cytokines can stimulation the activation and proliferation of innate cells, including innate lymphoid group 2 (ILC2) cells, mast cells and basophils, leading to production of type 2 cytokines, including IL-4, IL-5 and IL-13 and other pro-inflammatory proteins (41). IL-4 secretion is important for promoting of CD4 T helper (Th2) cells) and B cell

isotype switching for the immunoglobulin (IgE) production (42). IL-5 secretion results in the maturation and recruitment of eosinophils from the peripheral blood into the airways. Eosinophils contain multiple granules proteins that proinflammatory and cytotoxic properties, including major basic protein (MBP), and peroxidase (43). Finally, IL-13 secretion promotes the differentiation of airway epithelial cells into goblet cells, leading to mucus production. Furthermore, IL-13 secretion promotes contraction of the airway smooth muscle cells, leading to increased airway hyperresponsiveness (44, 45).

Airway epithelial cells also produce cytokines that promote non-type 2 mediated allergen airway responses, including IL-23 and IL-1β, leading to the activation and proliferation of innate, including γδ T cells, to produce IL-17A and IFNγ (46). Production of IL-17A and INF-γ recruit neutrophils into the airway, leading to increased secretion of neutrophil granule proteins, including elastase, myeloperoxidase, cathepsins and defensins (47). Similar to IL-13, IL-17A production has also been shown to promote mucus production but it remains unclear whether IL-17A promotes airway hyperresponsiveness (48, 49).

The release of epithelial derived cytokines results in a "leaky" airway epithelium, permitting the entry of allergens into the lamina propria of the airways (50), where they are further recognized and taken up by antigen presenting cells, including dendritic cells (DCs) (51). Furthermore, DCs may protrude through the airway lumen to further survey for foreign particles. The update of allergens induces DC activation and maturation, as well as the proteolytic processing and presentation of allergen derived peptide on the DC cell surface. DCs then migrate to the lung draining lymph nodes where they present antigen to naïve T cells (51). The naïve T helper cells subsequently differentiate into various T helper subsets, under the influence of inflammatory cytokines in the microenvironment, including T helper Th1, Th2, Th17 and

regulatory T cells (Tregs) (52). Following differentiation, these cells migrate back to the lung, where upon further antigen exposure, they produce proinflammatory cytokines that modulate adaptive immune responses, including further promotion of type 2 and non-type 2 mediated inflammatory responses, leading to increased eosinophils and/or neutrophil mediated airway inflammation, airway hyperresponsiveness and mucus production (52).

In parallel, antigen is presented to naïve B cells in the draining lymph nodes where the presence of pro-inflammatory cytokines like IL-4, induce isotype class switching in activated B cells from IgE molecules, to IgE molecules in an allergic environment (53). These antibodies are released into the peripheral blood and lung tissue in preparation for future allergen exposures. Allergen sanitization results from the initial allergen exposure and subsequent exposures results in an allergic reaction. These allergic reactions are characterized by the recognition of allergen derived peptides by IgE which bind to the FcεR1 receptor on mast cells and induce degranulation and release of various lipid mediators, including histamines, leukotrienes and prostaglandins (54). Combined, the immunological mechanisms driving asthma pathogenesis are complex and varied mediated by type 2 and non-type 2 cytokines. The following section discusses the effects of type 2 and non-type 2 inflammatory pathways in the pathogenesis of asthma.

1.6 Type 2 Eosinophilic-Mediated Airway Inflammation

Type 2 eosinophilic mediated airway inflammation is an immunological pathway involving cells that secret primarily IL-4, IL-5 and IL-13 to stimulate type 2 immunity, leading to increased serum IgE levels, and increased eosinophil recruitment into the airways (35). Secretion of type 2 cytokines from various cellular sources, including Th2 cells, ILC2, eosinophils, mast cells, basophils and IgE producing B cells (55), drives a cascade of

downstream effects that subsequently lead to increased airway inflammation, airway hyperresponsiveness, mucus production and remodeling of the epithelium (Figure 1-2). These inflammatory and pathological changes in the lung can predispose subjects to increased allergic reactions to inhaled agents (35).

Type 2-mediated airway inflammation is associated with an increased risk for exacerbations (55) . Genetic studies show that mutations in the IL-4 receptor gene, IL4Rα, is associated with a severe asthma exacerbation, and decreased lung function. Furthermore, these mutations were associated with increased tissue resident mast cells numbers and increased IgE bound to mast cells (56). Clinical studies of treatments targeting type 2 mediated airway inflammation have further revealed that blockade of type 2 cytokine secretion and/or signaling suppress type 2 mediating airway inflammation, further showing the role of type 2 cytokines in driving asthma exacerbations. Patients with airway eosinophils typically respond to corticosteroids, however the degree of the responsiveness is variable thus biological targets for type 2 cytokines, have bene identified for inhibition of type 2 responses in patients with poorer responses just corticosteroids alone, including those that target IgE, IL-5 and IL-13.

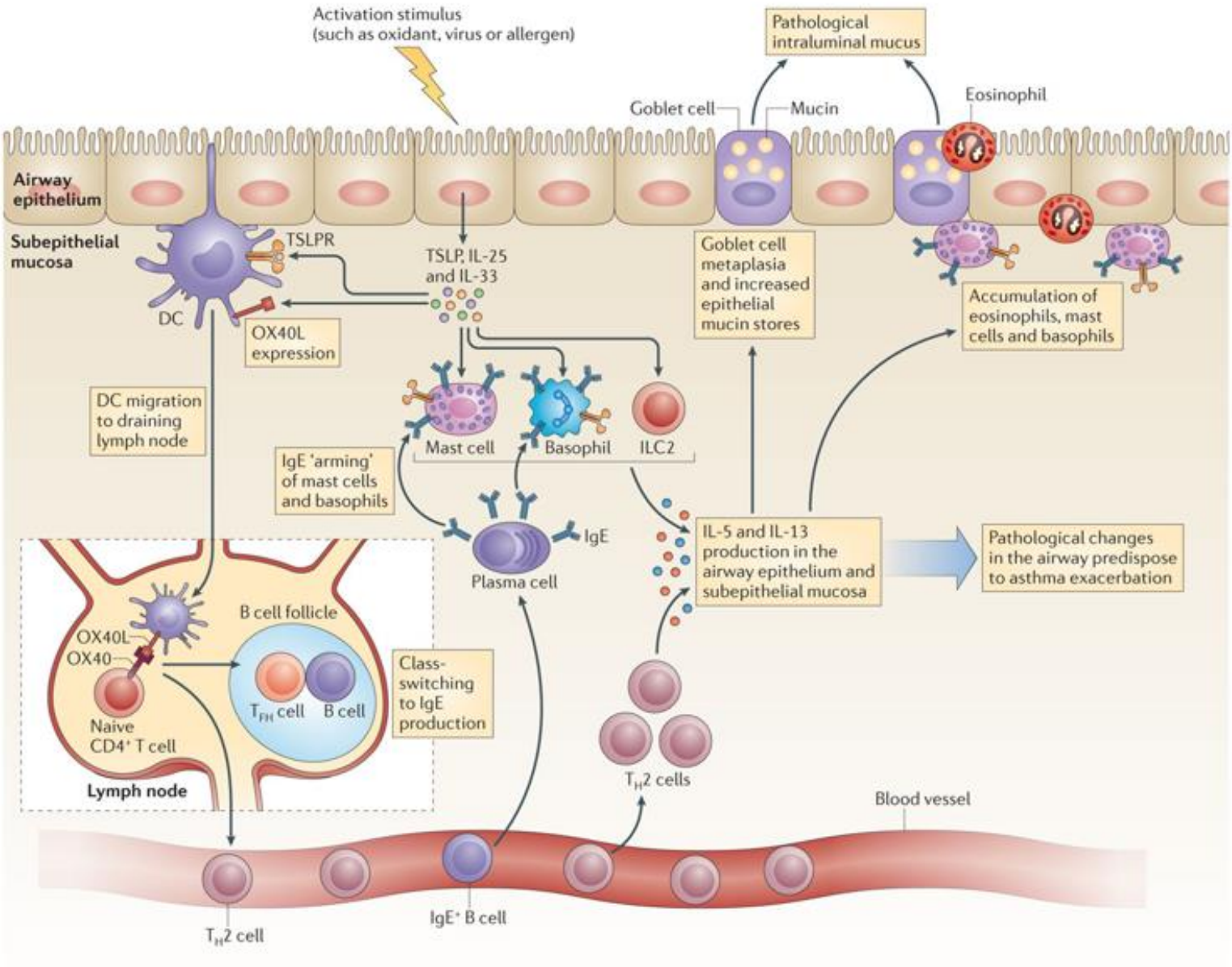

Figure 1-2: Type 2 Eosinophilic-Mediated Airway Inflammation. Activation stimuli, including allergens of epithelial cells leads to increased secretion of TSLP, IL-25, and IL-33, which promotes activation and effector functions of mast cast cells, basophil and ILC2, leading to increased secretion of type 2 cytokines, including IL-4, IL-5 and IL-13. DC migration to lymph nodes leads to activation of allergen-specific Th2 cells and activation of IgE producing B cells. Th2 cells migrate back to the lung to produce further IL-4, IL-5 and IL-13, leading to increased type 2 mediated airway inflammation, mucus production, AHR and eosinophil recruitment into the airways. Serum IgE levels crosslinked on mast cells, basophils and eosinophils lead to release of pro-inflammatory mediators that further promote airway inflammation. Fahy, V. J., (2015).“Type 2 inflammation in asthma -present in most, absent in many”. *Nature Reviews: Immunology*. **15** (57-65).

The anti-IgE antibody omalizumab has been shown to reduce asthma exacerbations by blocking the binding of IgE to the FcεR1α, leading to IgE neutralization, and inhibition of IgE-mediated mast cell and basophil degranulation (57). Clinical trials of omalizumab demonstrated a 50% reduction in asthma symptoms, reduction in maintenance dose of inhaled corticosteroids, modest but statistically increases in FEV_1 scores (58, 59). Omalizumab is currently used on many patients with difficult to control asthma.

IL-5 is another major therapeutic target of type 2 immunity, due to its central role in regulating eosinophil maturation and recruitment into the lung (35). Mepolizumab is an anti-IL-5 antibody that binds to IL-5 and prevents its interaction with the IL-5 receptor (IL-5Rα) on target cells, leading to decreased eosinophil maturation and recruitment (60). Initially, mepolizumab failed to show an improvement in asthma control and exacerbations (61) however, subsequent studies in selected patients with persistent eosinophilia, (> 300 cells/μL despite steroid treatment), showed a 50% reduction in the rate of exacerbations and asthma control (62). Similar results were reported with other IL-5 biologics, including reslizumab, another anti-IL-5 neutralizing antibody, with a 50% reduction in exacerbation rate (63). Furthermore, an anti-IG1 antibody that binds to the IL-5Rα called benralizumab has been FDA approved for the treatment of patients with uncontrolled eosinophilic asthma.

IL-13 plays a central role in type 2 immunity with roles in regulation of airway hyperresponsiveness, mucus production and airway remodeling (45). IL-13 signals through two receptors, the first is a heterodimer of the IL-13 receptor α1(IL-13Rα1) and the IL-4Rα and the second is a monomeric receptor IL-13 receptor α2 (IL-13Rα2)(64). Several monoclonal antibodies that inhibit IL-13 binding to these receptors have been studied in clinical trials of

asthma. Dupilumab, is a monoclonal antibody that binds to IL-4Rα to block both IL-4 and IL-13 signaling. Attenuation of these two signaling pathways in patients with type 2 driven asthma with persistent eosinophilia been shown to reduce rate of exacerbations by 87% and significantly improve lung function and asthma control (65). Another antibodies specifically targeting IL-13, including lebrikizumab and tralokinumab, did not show improvements in rates of asthma exacerbations, asthma control, and lung function (66, 27). Anti-IL-13 therapeutics are currently not undergoing next phase clinical trials for use in patients with asthma. Combined these the usage of these type 2 therapeutic biologics in combination with corticosteroid therapies has been efficacious in patients with persistent eosinophilia. However, only 50% of patients with asthma have the type 2 high eosinophilic mediated airway inflammation(67) and alternative immunological mechanisms drive non-type 2 mediated inflammatory responses that are currently the subject of ongoing research.

1.7 Non-Type 2 Neutrophilic-Mediated Airway Inflammation

Although Type 2 immunity drives asthma exacerbations and eosinophilic mediated airway inflammation, some patients exhibit pathophysiologies mediated by non-type 2 inflammatory pathways. Patients with asthma, particularly those with severe asthma phenotypes, can have airway inflammation mediated by increased secretion of non-type 2 cytokines, including IL-17A and INF-γ, from CD4 T cells, γδ T cells and NK cells, leading to increased infiltration of neutrophils into the airways and increased mucus production (49).

Patients with asthma, including severe asthma have increased sputum IL-17A levels and are more likely to have steroid resistant asthma with overexpression of IL-17A (68). IL-17A

promotes neutrophil recruitment into the airways by binding to the IL-17A receptor (IL-17R), composed of the subunits A (IL-17RA) and C (IL-17RC) on structural cells, including airway epithelial cells and fibroblasts, leading to secretion of neutrophilic specific chemoattractants, including chemokine ligand 8 or (CXCL8, IL-8) (humans) or keratinocyte chemoattractant (KC) in mice (47). In primary human epithelial cells, IL-17A as well as IL-1β, increased mucin (MUC)5 mRNA and protein expression via an NF-κβ dependent mechanism, leading to increased mucus cell metaplasia (69, 70). IL-17A stimulation of isolated human fibroblasts from patients with allergen-induced asthma exacerbations increased CXCL1, CXCL8 and TNF-α secretion compared to stimulation of fibroblasts form healthy control subjects. Surprisingly, fibroblasts cultured in the presence of IL-17A increased expression of α-smooth muscle action mRNA and protein expression, but this effect was completely inhibited by a neutralizing antibody against CXCL8 (71). CXL8 is potent mediator of neutrophil recruitment into the airways via the chemokine receptor CXCR2 and CXCR2 antagonists have been studied for potentially inhibiting neutrophilic mediated airway inflammation in asthma (46). Preliminary studies targeting CXCL8 in 12 patients with increased sputum neutrophils showed that a CXCR2 blocker reduced the numbers of neutrophils in the sputum but did improve asthma control or increase FEV_1 scores (72).

Patient with severe asthma respond poorly to current asthma therapies, including corticosteroids. Studies in mice demonstrated that Th17-mediated airway inflammation and airway hyperresponsiveness were resistant to the corticosteroid dexamethasone (73). Furthermore, corticosteroid therapies are known to promote neutrophil survival but induce cell death of eosinophils, further driving neutrophilic mediated inflammation in these patients (74). These finding suggest that targeting IL-17A signaling may decrease persistent neutrophilia in

asthma. Therapeutics for blockade of IL-17A or the IL-17Rα signaling have undergone clinical trials in patients with asthma, including brodalumab is a humanized monoclonal IgG2 antibody against IL-17Rα and secukinumab, an IgG1 monoclonal antibody against IL-17A (49). A phase 2 clinical trial of brodalumab in subjects with moderate to severe asthma showed no improved in asthma control scores nor increases in lung function scores and a preliminary study of secukinumab in 46 patients with asthma not adequately controlled with corticosteroids was prematurely terminated supposedly due to a lack of efficacy (identifier NCT01478360) (75). Currently Secukinumab, is being investigated for the treatment of allergic contact dermatitis (identifier NCT02778711)(76, 49).

While IL-17A is important in driving neutrophils into the airway, IFNγ production can also promote neutrophilia as well as contribute to non-type 2 mediated airway inflammation in asthma (3). Genetic studies of different asthmatic cohorts showed increased mRNA expression of *IFNG* in the lung and sputum samples of patients with severe asthma (77, 78). Furthermore, GWAS studies identified Single nucleotide polymorphism (SNPS) in four genes associated with Type 1 immunity, including *IL-12A, IL12RB1, STAT4, and IRF2,* that positively correlated with increased asthma severity but negatively correlated with FEV_1 scores (79). In cluster analyses, patients with severe asthma had increased mRNA levels of corticosteroid resistant fractional exhaled nitric oxide (FeNO) and induced nitro oxide synthase signatures ,both of which are induced by IFNγ as well as type 2 cytokines (80) Analysis of cells from BAL fluid of subjects with mild, moderate and severe asthma showed increased *IFNG* mRNA expression in patients with severe asthma compared to patients with mild/moderate asthma (81). Furthermore, immunophenotyping analyses of cells in the BAL fluid revealed increased percentages and total

numbers of IFNγ+ CD4+ T cells in the BAL fluid of patients with severe asthma compared to those with mild/moderate asthma (81).

The findings of increased IFNG mRNA levels in the airways of patients with severe asthma has raises the question of the trigger for the type 1-mediated inflammation in asthma. IFNγ secretion and type 2 responses can be induced by viruses and bacteria and studies show that continuous infections by viruses and bacteria are known in patients with severe asthma and contribute to increased asthma exacerbations (82, 83). These findings suggest a causal link between chronic viral infections and severe asthma. Viruses and bacteria infections induce the expression of intracellular cyclic-di-GMP (c-di-GMP, leading to the induction of type 1 interferons dependent on the stimulator of interferon genes (STING) pathway (84, 85). Mouse models of steroid-resistant severe asthma have been investigated using a combination of house dust mite (HDM) and c-di-GMP to mimic IL-17A and IFNγ induced allergic airway inflammation. In experiments where WT or knockout IFNγ (IFNγ KO) and IL-17A (IL-17A KO) mice were subjected to the this mouse model, methacholine-induced airway hyperresponsiveness was significantly decreased in IFNγ KO mice compared to WT and IL-17A KO mice but BAL neutrophil numbers were significantly decreased in IL-17A KO mice compared to WT and IFNγ KO mice (81). Other mouse models of asthma have found that IFNγ increased airway hyperresponsiveness and decreased steroid responsiveness (86).

Studies using computer-associated gene analyses have identified secretory leukocyte protease inhibitor (SLPI) in airway epithelial cells, as a potential link between IFNγ and airway hyperresponsiveness (87). Paired analyses of BAL fluid cells showed that IFNγ expression was inversely correlated with SLPI expression in patients with severe asthma . Furthermore, expression of SLPI was decreased in the airways of WT mice compared to IFNγ KO mice

subjected to HDM/c-di-GMP severe asthma model (81). Combined, these studies suggest that while IFNγ responses may mediate airway hyperresponsiveness and contribute to decreased lung function in asthma. In contrast, IL-17A promotion of neutrophilia in the airways may indirectly promote neutrophil recruitment and degranulation, leading to the release of neutrophil-derived products that promote airway remodeling and mucus cell metaplasia.

1.8 Role of Helper T cell Subsets in the Immunopathogenesis of Asthma

CD 4 T helper (Th) are central players in allergic disease as they orchestrate adaptive immune responses and have been shown to regulate type 2 and non-type airway inflammatory responses (88). Below is a review of the generation and roles of allergen-specific CD4+ T cells in the pathogenesis of allergic type 2 and non-type 2 mediated airway inflammation (Figure 1-3).

CD4 T cells originate from a common lymphoid hematopoietic stem cell in the bone marrow and migrate to the thymus for further maturation. The thymus is an integral part of T cell-development due to the combination of stromal cells, cytokines and chemokines in this environment that support the generation of functional T cell from T cell precursors called thymocytes. T cell development in the thymus involves the migration of thymocytes through specific thymic microenvironment and contact with peptide-MHC complexes on thymic antigen presenting cells that play a pivotal role in T cell antigen recognition, negative and positive selection and then the expression of surface molecules, including CD4 and CD8 (89-91) .

The selection process tests for thymocytes with an intermediate affinity of their T cell receptor (TCR) to the peptide-MHC complex, where thymocytes expressing TCR with low or high are destroyed in a process called negative selection.

Following negative selection, thymocytes with TCR of intermediate affinity to peptide-MHC then undergo positive selection into naïve CD4+ and CD8+ T lymphocytes (92, 90). Mature naïve CD4 T cells then migrate from the thymus to the secondary lymphoid organs, including the spleen, lymph nodes and the mucosal-associated lymphoid tissue, where they consistently survey for peptide-MHC class II molecules for antigen recognition (93).

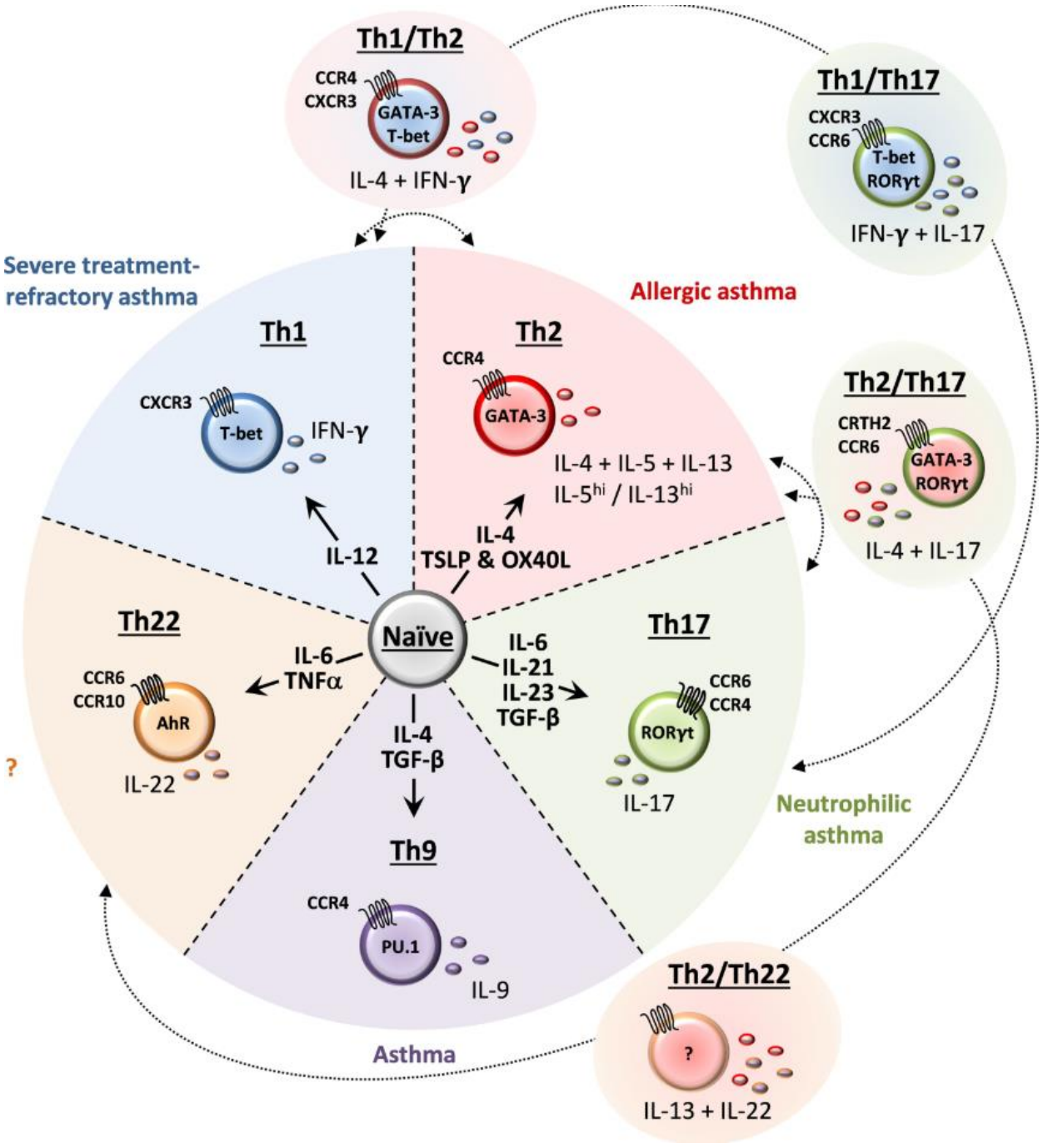

Figure 1-3: CD4 T cell Subsets Linked to Asthma Pathogenesis. Various Th1 subsets are implicated in the immunopathogenesis of asthma. Each subset has a unique inflammatory signature with distinct transcription regulators, surface markers and secreted cytokines. The transcriptional regulator of the Th2/Th22 types remain unclear. A typical Th1 subset are possible due to the genetic plasticity of some subsets, where specific epigenetic mechanisms allow for secretion of cytokines not typically attributed to a specific subset. *Muehling,et al,* "Pathogenic CD4+ T cells in patients with asthma'. *J Allergy Clin. Immunol.(2017).* 140 (6). Pages 1523-1540.

1.9 Th2 cells in Asthma

Allergen-specific CD4 T helper 2 (Th2) cells are highly implicated in allergic asthma, specifically in type 2-mediated airway inflammation (88). After antigen recognition, activated T cells differentiate into Th2 cells in the presence of IL-4, leading to increased signal transducer and activator of transcription 6 (STAT6) activation, increased expression of *Gata3* and production of type 2 cytokines, including IL-4, IL-5, and IL-13. (94-96). Th2 cells are increased in bronchoalveolar lavage fluid of patients with atopic and nonatopic asthma compared to normal control and Th2 gene signatures are increased in the BAL fluid of asthmatics after allergen inhalation challenge.(97-99). Corticosteroids inhibit Th2 cytokine production and patients with mild-moderate type 2 asthma are generally responsive to current asthma therapeutics, including anti-IL-4 (dupilumab), IL-5 (mepolizumab) and IL-13 (lebrikizumab and tralokinumab) monoclonal therapies (100). However, in patients with severe asthma, Th2 cytokines remain elevated despite high dose corticosteroid usage, and new therapeutics targets for patients with severe type 2 asthma remain are needed. The transcription factor *Gata3* is required for Th2 cytokine secretion, (101) and is increased in the BAL fluid of patients with asthma compared to health control subjects(102). Furthermore, studies showed that inhibiting Gata3 activity in an experimental model of allergic asthma attenuates allergic airway inflammation (103). These studies demonstrated that importance of Gata3 expression in regulating Th2 cytokine production and suggests that drug therapeutics that target Gata3 may potentially help the management of asthma in patients with within this group.

Other CD T cells-derived cytokines are implicated in type 2 mediated airway inflammation, including interleukin 9 (IL-9). IL-9 expression is significantly increased

in the BAL of patients with asthma compared to healthy control subjects (104) and has been shown to increase T cell proliferation (105) . Through IL-9 production can be generated by conventional Th2 cells (106), T helper 9 (Th9) cells can be generated from activated naïve T cells in the presence of IL-4 and TGF-β (107). Similar to Th2 cells, Th9 cells require Gata3 expression as well as other transcription factors, including STAT6, IRF4 and the tyrosine kinase Itk (108). Prior studies demonstrated a role for IL-9 in promotion IL-4 mediated IgE production in B cells and in promoting mast cell proliferation (109). In allergic models of asthma, Th2 and Th9 cells were found to co-exist in the lungs following allergen challenge (110), but studies in ovalbumin (OVA) models demonstrate that adoptive transfer of OVA-specific Th9 cells but not Th2 cells T cell deficient RAG knockout mice increased mast cell-mediated airway inflammation (111).

A quarter of patients with asthma do not have atopy to known allergens (112), thus it likely that other environmental triggers may induce type 2 allergic airway inflammatory responses that are independent of Th2 cells. Studies show that innate immune cells, including innate lymphoid group 2 (ILC2) cells produce large amounts of type 2 cytokines following upregulation of Gata3 expression (113, 102). ILC2 are elevated in the BAL of patients with asthma, including severe asthma (114). Furthermore, in both human and mouse studies, ILC2 were shown to produce large amounts of type 2 cytokines in the presence of epithelial derived cytokines, including thymic stromal lymphopoietin (TSLP), Interleukin 33 (IL-33) and 25 (IL-25)(115). While ILC2 are one-tenth that of T cells in the lung following allergen or viral challenge, they produce 40-fold more IL-5 and 10-fold more IL-13 than Th2 cells (116). More over these cells are not restricted by antigen specificity like T cells, thus following allergen insult

and upregulation of IL-33, these cells have the ability to promote type 2 mediated airway inflammation prior to induction of Th2 cells. Recent studies show that Th2 cells are able to respond to these epithelial derived cytokines, thus current therapeutics are heavily focused on targeting the IL-33 and TSLP pathway to modulate both Th2 and ILC2 cell-mediated responses (117). In various mouse models of allergic asthma, ILC2s were required for the induction of allergen-specific Th2 cells. Similar to DCs, ILC2s express costimulatory molecules and can present allergen-derived peptides to naïve T cells and IL-13 production from ILC2s further promotes DCs migration to the lymph nodes (118).

1.10 Th1 and Th17 cells in Asthma

Other Th subsets are associated with asthma pathogenesis, including Th1 and Th17 cells. Interleukin 27 (IL-27) and 12 (IL-12) are important cytokines for the differentiation of activated T cells towards the Th1 lineage (119). Upon contact with aeroallergens and other foreign agents, DCs and other APCs become activated through recognition of specific antigens via TLRs and other pattern recognition receptors, leading to increased secretion of IL-27 and IL-12 from these cells (120). IL-27 promotes commitment to the Th1 lineage by activating signal transducer and activator of transcription 1 (STAT1) signaling, leading to increased leading to expression T-bet, the master transcriptional regulatory of Th1 cells, inhibition of the expression of Gata3, the transcriptional regulatory of Th2 cells. T-bet then promotes the expression of IL-12Rβ2, one of the subunits of the IL-12 receptor. Naïve T cells expression IL-12β1, the other subunit of the IL-12R. IL-12Rβ1 and IL-12Rβ2 dimerize to form a functional IL-12R, rendering the cell responsive to IL-12. IL-12 binding to the IL-12R receptor stimulates activation of signal transducer and activator of transcription 4 (STAT4) signaling, leading to IFNγ production. IL-12 and IFNγ further stimulate STAT1 signaling to maintain T-bet expression and Th1-effector responses (121, 122).

Studies with MHC Class II tetramers have demonstrated increased IFNγ expression in allergen-specific CD4 T cells; however, this increase has been attributed to T cells responding to inert inhaled particles that promote allergen sensitization early on in life, including persistent viral and bacterial infections (123). The role of respiratory viruses, including respiratory syncytial virus (RSV) and human rhinovirus (HRV) is well established in the development of early onset childhood asthma (124) and viruses such as RSV are known to increase IL-33 and TSLP, leading to the induction of Th1 inflammatory responses (125, 126). Increased frequencies of IFNγ CD4+ T cells and increased expression of Th1-specific chemokines were detected in the BAL fluid of patients with asthma following experimental infection with HRV (127, 128). Furthermore, recent studies show that patients with severe asthma have increased mRNA and protein levels of Th1-associated genes, which was correlated with increased predominance corticosteroid resistance and decreased lung function compared to patients with mild asthma phenotypes (81, 3).

Recent studies also support a role for IgE in promoting Th1 responses following allergen sanitization. In some circumstances, IgE binding to antigen can enhance secretion of interferon-α by plasmacytoid DCs, which has been predicted to promote Th1 responses(129). Alternatively, current studies further suggest that blocking IgE-mediated inflammation in vivo may potentially promote IFNα responses in children with asthma(130). These findings suggest that studying the role of respiratory viruses on induction of Th1 responses in early on in life, could potentially provide insight into the role of Th1 cells in the pathogenesis of asthma.

Th17 cells are also associated with asthma pathogenesis. IL-6, TGF-β, IL-1β, and IL-23 are important cytokines involved in Th17 cell differentiation. These cells are secreted by structural cells and antigen presenting cells follow allergen exposure. In humans, IL-6, TGF-β

and IL-1β are important for the early commitment of the activated T cell towards the Th1 lineage. These cytokines promote activation of signal transducer and activator of transcription 3 (STAT3) signaling, leading to increased IL-21 protein expression (131). IL-21 acts in an autocrine fashion to further promote STAT3 expression and upregulate the RORγT, the master transcriptional regulator of Th17 cell differentiation and IL-17A protein expression. IL-21 autocrine signaling also increases IL-23 receptor (IL-23R) expression, which then heterodimerizes with IL-12Rβ1 to form a functional IL-23R. IL-23 signaling via the IL-23R maintains STAT3 and RORγT expression and promote optimal IL-17A, IL-17F and IL-22 protein expression we well as maintenance of the Th17 phenotype (132, 133). In contrast to human T cell differentiation, differentiation of mouse T cells requires IL-6 and TGF-β and may be less dependent on IL-1β (134).

IL-17A is increased in sputum of patients with asthma and positively correlates with increased neutrophilic mediate airway inflammation, increased airway remodeling and increased mucus cell metaplasia (135, 136). In allergic asthma models, adoptive transfer of OVA-specific D011.10 Th17 cells into WT recipient mice followed by OVA challenge increased neutrophils and Th17 infiltration in the lungs of recipient mice. Unlike Th2 cells, Th17 cells were more resistant to dexamethasone-induced cell death and promoted steroid resistant inflammation and airway hyperresponsiveness (73). Th17 cells are regulated by the transcript factor RORγT and OVA sensitization and challenge of RORγT transgenic mice increased IL-17A protein expression neutrophilic-mediated airway inflammation and induced steroid-insensitive AHR compared to similarly sensitized and challenged GATA3 transgenic mice with increased Th2 mediated inflammation or WT control mice. (137) OVA sensitization and challenge of RORγT transgenic mice treated with an anti-IL-17A antibody or a CXC2 antagonist, for a chemokine

receptor on neutrophils, significantly decreased IL-17A mediated airway inflammation and AHR compared to isotype control antibody or vehicle controls (137).

IL-17A has also been associated with increased airway inflammation and AHR following viral during chronic airway inflammation (138). In asthma, viral infections are known to trigger asthma exacerbations and underlying mechanisms of increased airway inflammation and asthma symptoms from viral infection are currently under investigation. IL-17A protein expression is increased in the lung of mice following RSV infection during ongoing OVA-induced allergic airway inflammation compared to after RSV infection alone or following OVA-induced allergic airway inflammation (138, 48). Prior studies further showed that RSV infection during ongoing OVA-induced airway inflammation increased AHR, IL-13 protein expression in the lung and BAL eosinophil numbers in IL-17A KO mice compared to WT mice. (48) These findings suggested that IL-17A inhibits IL-13 protein expression and AHR following RSV infection and ongoing OVA-induced allergic airway inflammation.

Other Th17 cytokines, including IL-22 and IL-17F are associated with increased airway remodeling associated with increased mucus cell metaplasia and increased smooth muscle cell mass (49). In human airway epithelial cells, these Th17 cytokines were shown to increase mRNA expression of mucus proteins, including (MUC)5AC (69, 70). Furthermore, they increased airway smooth muscle proliferation and migration (139).

Airway smooth muscle cells from non-asthmatics and asthmatic patients stimulated with IL-17A, IL-17E or IL-22 lead to increased cell proliferation (140). IL-17A and IL-17F increased cell proliferation via an ERK1/2 dependent mechanism and migration via a p38 dependent manner (139). In contrast, IL-22 increased cell proliferation via both ERK1/2 and NF-κβ dependent

manners and migration dependent on NF-κβ (141). IL-17A was also shown to increase smooth muscle actin expression in fibrocytes, however unlike IL-4 and IL-13, IL-17A did not increase collagen expression (142). Smooth muscle constriction is associated with increased AHR and IL-17A, but not IL-17F and IL-22 were shown to increase smooth muscle cell contractility via an NF-κβ, RhoA, RORC2-dependent mechanism (143).

Th17, Th22 and Th1 cells also secret other cytokines, including IL-22. IL-22 is a member of the IL-10 family of proteins and can have immunopathogenic or suppressive effects on airway inflammation (141). IL-22 binds to the IL-22 receptor (IL-22R), expressed on structural cells, including airway epithelial cells and smooth muscle cells (144). Patients with severe asthma have increased serum IL-22 levels and increased cells numbers of IL-22+ Th17 cells compared to healthy control subjects (141, 145). In mice, OVA-induced allergic airway inflammation was decreased BAL eosinophil numbers, IL-13 protein levels in the lung, AHR and mucus production in IL-22 knockout (IL-22 KO) mice compared to WT mice (145). In contrast, other studies demonstrated antibody neutralization of IL-22 at the time of antigen challenge increased IL-25 and IL-13 protein expression as well allergic airway inflammation (146). Similar to IFNγ, IL-22 has also been shown to inhibit Th2 responses, where intranasal administration of recombinant IL-22 during OVA-induced allergic airway inflammation decreased Th2 cell-mediated airway inflammation, potentially by decreased type 2 cytokine expression, including IL-25 and IL-13 levels (141).

1.11 T-regulatory cells in Asthma

The pathogenies of asthma results from an ineffective tolerogenic immune response to allergens.

Regulatory T cells (T regs) play an important role in maintaining immune tolerance to allergens, and therefore recent studies have focused on determining the underlying mechanism by which T regs fail to maintain tolerance in patients with asthma and other allergic diseases(147). T regs are a subset of CD4 T cells that express the IL-2 receptor α chain (CD25) and express the transcription factor Forkhead box P3 (Foxp3)(148). Foxp3 is required for the generation of T regs and a major population arise from the thymus known as CD4+Foxp3+ natural Tregs (n Tregs)(149). A second population of CD4+Foxp3 T regs cells called inducible T regs (iTregs) arise in peripheral lymphoid organs from naive CD4 T cells following exposure to antigens and in the presence of TGF-β(149). The primary role of nTregs is to mediate tolerance to self-antigens, while iTregs are associated increased in chronic lung and gut inflammation (147).

In the lung, alveolar macrophages expressing TFG-β and retinoic-acid drive iTreg differentiation from naïve T cells. T reg cell suppressive functions are induced via various methods (150). During asthma pathogenesis, T regs can suppress allergic airway inflammation via various mechanisms. T regs have been shown to decrease IgE-mediated mast cell degranulation and effector molecule release OX40-OX40 ligand interactions with mast cells, leading to increased cyclic AMP levels and blockade of calcium influx to inhibit mast cell granule release (151). In food allergic diseases IL-4 production from ILC2 has been shown to promote Th2 cell differentiation but inhibit T reg cell responses and promotes mast cell activation. Alternatively, T regs cells can interact directly block ILC2 proliferation and suppress ILC2-derived IL-4 production(152). T regs are also known to suppress allergen-specific B and T cells responses (147). *In vitro* studies showed that isolated iTregs from health subjects inhibited B-cell production of IgE production by promoting class switching to IgG_4 as opposed to IgE (153).

In allergic mouse studies, co-adoptive transfer of effector OVA-specific Th2 cells and n Tregs followed by OVA challenged did not suppress Th2-cell mediated airway inflammation. However, OVA-specific nTregs were effective at inhibiting the differentiation of naïve T cells into Th2 cells. In separate experiments, nTregs did not suppress Th1 cell-mediated airway inflammation but effectively decreased Th17 cell-mediated airway inflammation but not AHR. The suppression of Th17 cell-mediated airway inflammation was associated with decreased BAL neutrophils and decreased frequencies of IL-17+ Th17 cells. These studies suggest that Tregs inhibit Th differentiation and that Th17 cell are particularly sensitive to the immunosuppressive responses of Tregs (154).

Patients with asthma have decreased frequencies of CD4+CD25^{high} Tregs in the BAL fluid compared to control subjects (147). Genetic studies also show that in patients with asthma, loss of function mutations in *FOXP3* is associated with increased asthma severity, increased serum IgE levels and persistent eosinophilia. Similarity in mice, *Foxp3* mutant mice spontaneously exhibit allergic airway inflammation, increased serum IgE levels independent of genetic background. In a murine genetic model, DEREG mice were used to investigate the role of T regs on central tolerance. DEGREG mice express the diphtheria toxin receptor under the control of *Foxp3,*allowing for depletion of Foxp3+ Tregs by diphtheria toxin (DT). Injection of DT in OVA-sensitized and challenged DEREG mice was sufficient to break tolerance, leading to increased serum levels of OVA-specific IgE and IgG1 antibodies as well as increased diphtheria-induced inflammatory responses (155). Combined these findings, support a role of T regs in regulating allergies immune responses by cell-dependent and independent mechanisms.

1.12 T-cell Plasticity

In additional to their signature cytokines, Th subsets can secret broad non-subset specific cytokines dependent on the specific immunological context. Furthermore, novel T-cell subsets with mixed/intermediate phenotypes continue to be identified , which appear to be heavily influenced by T-cell genetic reprogramming or plasticity. The plasticity of T cells is most likely relevant in asthma due to heterogeneity of cytokines produced in the inflamed lung. A classic example of this plastic nature of T cells is exemplified by a scenario where Th1 differentiated cells when stimulated in an IL-4 rich environment, repolarize towards making type 2 cytokines. In contrast, a Th2 differentiated cell subject to an IL-12 rich environment will repolarize to produce IFNγ (156, 157). These reprogramming events have been suggested to explain the surprisingly high numbers of IL-4+IFNγ+CD4 memory T cells from patients with asthma (157). This notion of plasticity extends to other T cell subsets, where Th2 cells can repolarize into the Th9 cells in an IL-4 and TFG-β rich environment (158). Similarly, Th17 cells have been shown to secrete IFNγ in the presence of IL-12 or IL-23 (158).

T cell-plasticity might also affect Tregs and the immunosuppressive functions in patients with asthma.(124) Human T regs can be reprogrammed to expression Th subset cytokines, including IFNγ and IL-17A, where depending on the circumstance these cells can maintain Foxp3 expression (159, 147). Recent studies suggest that miRNAs may regulate Treg reprogramming towards effector T cell phenotypes. Micro RNA miR-17 was shown to decrease expression of a T reg-phenotype stabilizing transcription factor called Eos, leading to increased expression of effector T cell gene signatures (160), however it remains unclear if this phenomenon contributes to persistent inflammation in asthma pathogenesis.

1.13 Dual Type 2 and Non-Type 2-Mediated Airway Inflammation

Patients with asthma can also have airway inflammation mediated by both type 2 and non-type cytokines, with increased eosinophils and neutrophils (3). Prior studies show patients with severe asthma have increased frequencies of dual positive Th2/Th17 cells in the BAL fluid compared to patients with mild asthma (145) . Increased prevalence of these Th2/Th17 cells was associated with increased IL-17A production in the BAL fluid and increased resistance to dexamethasone-induced apoptosis(145). These findings identified three new sub-phenotypes of asthma, including Th2 dominant, Th2/Th17 dominant, and Th2/Th17 low asthma. Patients with asthma with Th2/Th17 dominant sub-phenotype exhibited more severe asthma and were more resistant to exhibited increased airway obstruction and hyperreactivity compared to the other groups (161). Since patients with Th2/Th17 dominant asthma exhibit increased eosinophils and neutrophils, additional studies were conducted, investigating the effect of type 2 cytokine suppression in promoting Th17-mediated inflammatory responses in an HDM-induced preclinical model of Th2 and Th17 cell-mediated airway inflammation. These studies showed found reciprocal crosstalk between the IL-13 and IL-17A signaling pathways in the lung, where IL-13 suppressed the expression of Th17-signature genes (162). In additional studies, IL-13-gene signatures were significantly increased in human nasal epithelial cells when stimulated with IL-13 and IL-17A as compared to IL-13 alone, suggesting that IL-17A promotes the effect of IL-13 stimulation on Th2 associated genes (163). Combined, these studies suggest that combinations targeting both type 2 cytokines and IL-17A may be more effective in patients with airway inflammation mediated by both Th2 and Th17 cells.

CHAPTER 2

The Role of Sex Hormones in Asthma Prevalence and Pathogenesis

2.1 Sex Hormones and Asthma

A gender disparity is well established in asthma and changes throughout life (164). As children, boys have an increased prevalence of asthma compared to girls (11.9 versus 7.5%, respectively) (165), and boys are also twice as likely as girls to be hospitalized for an asthma exacerbation (166). However, during adolescence, there is a decline in asthma prevalence and morbidity in males concurrent with an increase in females. By adulthood, women have increased asthma prevalence compared to men (9.6 versus 6.3%, respectively) (165, 167) and women are three times more likely than men to be hospitalized for an asthma-related even (168, 169, 12). This increase in asthma prevalence in women compared men is maintained until around the time of menopause, when a decrease in asthma prevalence is noted in women (170). Shifts in asthma prevalence based on gender coincide with changes in sex hormones and suggests that sex hormones modulate pathways associated with asthma pathogenesis (166, 11). Many studies have demonstrated that generally female sex hormones worsen asthma exacerbations, potentially by increasing lung inflammation, while male sex hormones have a protective role by suppressing immune cell function and proliferation (171, 172). Dehydroepiandrosterone (DHEA), is a metabolic intermediate in the biosynthesis of androgen and estrogen, is upregulated with the onset of puberty more in males compared to females. Increased sulfonated DHEA (DHEA-S) was recently associated with decreased asthma symptoms and increased lung function in boys but not girls from the Severe Asthma Research Program (SARP) (173).

Further, in a separate study of adult men, higher testosterone and DHT, but not oestradiol levels, were associated with higher FEV1 and FVC (174). Combined, these studies showed that male sex hormones, including DHEA and DHT may play a protective role in asthma, however, additional studies are needed to determine the mechanisms by which androgens decrease airway inflammation associated with asthma. Additionally, sex hormones levels vary during throughout childhood and in adulthood, specifically during pregnancy in women, therefore several studies reported below have further characteristics the gender differences in asthma prevalence in the context of puberty, menstruation and pregnancy.

2.2 Asthma Prevalence Changes during Puberty

Allergic, atopic asthma is associated with onset during childhood, and boys have increased allergic inflammation and serum immunoglobulin E (IgE) levels compared to girls (175, 176). Boys also have dysanapsis, smaller airway diameters relative to lung volumes compared to girls, making boys more likely to have asthma symptoms than girls (177). As children age, the switch in asthma prevalence from highest in males to highest in females coincided with the age of puberty onset (166, 178). The Childhood Asthma Management Program (CAMP) study longitudinally tracked the average asthma symptom score as well as progression through puberty, using the Tanner stage metric, in boys and girls ages 4–17 (179). At approximately age 10, when the Tanner scores start increasing in girls, the average asthma symptom score also increased in girls and declined in boys (179). Further, asthma symptoms continued to increase in girls as Tanner stages increased (179).

Additional studies have also shown that early-aged menarche (≤11 years old) increased the incidence of asthma (180). Collectively, these studies present strong epidemiological evidence that the prevalence of asthma in females increased in adolescence and that early menarche further increases the risk of developing asthma.

2.3 Pre or Peri-Menstrual Worsening of Asthma

Changes in asthma symptoms through the menstrual cycle are well established, and approximately 30–40% of women with asthma report pre or peri-menstrual worsening of asthma. (181-187). Decreased peak expiratory flow rates, increased asthma symptoms, and increased use of rescue mediations were determined during the pre or peri-menstrual phase of the cycle (181, 185, 187). Additional studies determined women with pre-menstrual asthma symptoms had increased fractional exhaled nitric oxide (FeNO), a non-invasive measure of epithelial-induced nitric oxide that correlates with eosinophilic inflammation (188), and eosinophils in the sputum in the pre-menstrual phase compared to those of the seventh day of their cycle (189). In the Severe Asthma Research Program (SARP) study, peri-menstrual worsening of asthma had increased oral corticosteroid bursts and increased emergency department visits compared to those of women without peri-menstrual worsening of asthma (186). However, other studies including multiple phenotypes of asthma found no differences in the phase of the menstrual cycle of women requiring emergency department visits for asthma symptoms (182, 189). While pre-menstrual asthma impacts many women with asthma, the molecular mechanisms driving the cyclic increase in symptoms are poorly understood. The pre-menstrual phase of the cycle occurs

after the peaks of serum estrogen and progesterone. Therefore, it is unknown how estrogen, progesterone, or potentially other hormones which are differentially regulated through the menstrual cycle affect airway inflammation.

To delineate if ovarian hormones increased airway inflammation, studies tracking asthma symptoms in women taking hormonal oral contraceptives versus women not taking contraceptives were conducted. Cross-sectional surveys in menstruating women determined that women using oral contraceptives had increased asthma risk compared to women not on oral contraceptives (190, 191). However, Macsali and colleagues found the association between increased asthma risk and oral contraceptive use was not seen in lean (underweight) women (191). Oral contraceptives were associated with increased wheezing in women with asthma in some studies (192, 191), but decreased wheezing and/or asthma symptoms in others (193, 194) . In another study, 28 women with asthma were followed for 12 weeks (2–4 menstrual cycles) and no differences were determined in asthma symptoms in women taking oral contraceptives versus women not taking oral contraceptives (195). These discordant findings from various studies may be due to small sample sizes (for some studies) or the many different forms of birth control medications used by women, including monophasic, multiphasic, progesterone-only, hormonal vaginal or intrauterine devices, or extended-cycle pills. Additional studies with increased sample size and women on similar types of birth control medications should be conducted over several cycles and seasons to determine if hormonal contraceptives affect asthma symptoms.

2.4 Pregnancy and Asthma

During pregnancy, women with asthma are known to have decreased asthma symptoms, increased asthma symptoms, or maintain similar asthma symptoms as prior to pregnancy (196, 197). Women with more severe phenotypes of asthma are more likely to have asthma worsening during pregnancy (198). However, for mild and moderate asthma patients, it is difficult to predict if asthma symptoms will increase, decrease, or stay the same during pregnancy. The National Heart, Lung, and Blood Institute and the Global Initiative for Asthma (GINA) guidelines indicate that pregnant women should maintain their current regimen of asthma medications, including inhaled corticosteroids, long-acting beta agonists, leukotriene modifiers, theophylline, and oral corticosteroids (199, 200). Maintaining asthma control during pregnancy is important as severe asthma, poorly controlled asthma, and asthma exacerbations during pregnancy are associated with increased risk for development of pre-eclampsia and gestational diabetes in the mother and pre-term birth, low birth weight, and peri-natal mortality for the baby (199, 201, 195). Future longitudinal studies are needed to track women with asthma prior to pregnancy and throughout the pregnancy to determine if any biomarker or clinical lung function test can be used to predict if asthma symptoms will change during pregnancy. This information would be beneficial in educating the patient on the importance of taking or increasing use of asthma medication during pregnancy.

2.5 Menopause and Asthma

The age-adjusted risk of asthma decreases in postmenopausal women compared to that of pre-menopausal women (181). However, variable findings are reported in the literature regarding menopause and asthma.

No difference in self-reported asthma between pre-menopausal and postmenopausal women not taking hormone replacement therapies (HRTs) was reported by the European Community Respiratory Healthy Survey I (ECRHS I) (202). However, the US Nurses' Health Study determined postmenopausal women not taking HRT had decreased risk of developing asthma compared to pre-menopausal women (181), and the ECRHS II cross-sectional study reported increased asthma symptoms in women during the menopause transition (amenorrhea for 6+ months) compared to those in pre-menopausal women (203). Further, a new phenotype of asthma with onset after menopause has been recently described for a subset of women (204-206), but the mechanisms that initiate and regulate postmenopausal asthma remain largely unknown.

2.6 Androgen, Estrogen and Progesterone Synthesis and Action

Since sex differences in asthma prevalence are seen throughout life, the role of sex hormones in modulating airway inflammation has been an area of active investigation. Sex hormones are glandular secretions that regulate various biological processes, including reproduction, aging, and immune responses (207) . Synthesis of sex hormones is regulated by the hypothalamus-pituitary-gonadal (HPG) axis, where release of hypothalamic gonadotropin-releasing hormone (GnRH) stimulates the release of luteinizing hormone (LH) into peripheral blood (208). Circulating LH binds to sex hormone producing cells (Leydig cells in males and Theca cells in females), leading to increased expression of steroidogenic acute regulatory protein (*STAR*) (208). *STAR* increases the transfer of cholesterol to the inner mitochondrial membrane of target cells, and initiates steroidogenesis and at the inner mitochondrial membrane, where cholesterol is converted to pregnenolone by a cholesterol side-chain cleavage enzyme called P450ssc (208).

Pregnenolone synthesis is important for the production of androgens, estrogen and progesterone. Below is a recap of how these sex hormones are further synthesis from this precursor and how the mechanisms by which they regulate target cells (209, 207) .

Male sex hormones, or androgens, regulate various biological processes, including the development and maintenance of male sexual characteristics, adipogenesis, angiogenesis, enlargement of skeletal muscle and immune responses (210). Synthesized pregnenolone can be further converted into various androgens, including testosterone, DHEA and dihydrotestosterone (DHT) (210, 211). While testosterone is more abundant than either DHT or DHEA, these testosterone metabolites have a higher binding affinity to the androgen receptor (AR) in target cells, and accounts for most of testosterone's biological action (212) . Androgens, including aldosterone and androstenedione can be synthesized from pregnenolone in Zona reticularis of the adrenal glands and converted to testosterone (210). Androgens circulate at higher levels in men compared to men but are also important in females by regulating estrogen synthesis (213). Circulating androgen precursors such as DHEA sulfate (DHEAS), DHEA and androstenedione are present females and important for the production of over 50% of the total amount of testosterone produced by females (213) . The remaining 50% is synthesized in the zona reticularis of the adrenal gland. Furthermore, testosterone can be converted into estradiol via the enzyme *aromatase* in certain peripheral tissues and is an important source of estrogens in postmenopausal women (213, 214).

Female sex hormones, including estrogen and progesterone, are important for the development and maintenance of female sexual characteristics, as well as pregnancy and menopause (215). Synthesis of estrogen occurs is the granulosa and theca cells ovaries,

and to a lesser amount in the corpus luteum (216). LH and FSH leads to pregnenolone synthesis and several signaling events that lead to the conversion of androgen precursors such as androstenedione into estrogens *aromatase* (216). *Aromatase* is expressed in non-gonadal tissues, including the adrenal glands and promote the conversion of androgens to estrogens (213). Synthesis of progesterone from pregnenolone occurs via the actions of a 3β-HSD enzyme in the corpus luteum of the ovaries, by the placenta during pregnancy and by the adrenal glands during androgen and mineralocorticoid synthesis (216). In menstruating women, the levels of estrogen and progesterone vary, where estrogens are high during follicular maturation while progesterone are high in the luteal phase after ovulation (217).

Androgens and female sex hormones both regulate biological processes by altering gene expression in target cells (207). These actions are mediated via slow genomic signaling mechanisms via intracellular hormone receptors, or fast non-genomic signaling cascades via membrane-bound receptors (215) . For genomic signaling, androgens all signal via the androgen receptor (AR), estrogens through either estrogen receptor α (ERα) and/or β (ERβ) and progesterone through progesterone receptor α (PRα) and PRβ (218). In the classical genomic signaling pathway, circulating sex hormones are transported to target tissue and penetrate target cells through passive or facilitated diffusion. Upon entry, the hormone binds to its cognate receptor, localizes to the nucleus in an activated confirmation, where this complex then binds to hormone response elements in the target cell genome regulate the recruitment of transcription machinery that modulate the activity of Pol II, including co-regulators such as the p160/steroid receptor coactivator (SRC) (219, 220, 211, 221). Recent studies also report that membrane-bound hormone receptors can induce non-nuclear effects of sex hormones by functioning

as G-coupled protein receptors upon ligation, leading to downstream signaling cascades that regulate calcium influx, cyclic AMP production and NF-κβ signaling in target cells (215). It is thought that these membrane-embedded receptors are actually the classical cytoplasmic receptors, however, novel membrane bound receptors have also been reported as the mediators of non-genomic hormone-induced signaling (222). Prior studies demonstrated that a novel membrane bound estrogen-related-receptor α, (ERRα), was important for promoting T cell proliferation and glucose update following TCR activation. Additional ERRα, promotes glycolysis and mitochondrial respiration in T effectors (223). Studies also show that sex hormones are able regulate signaling through cell surface receptors. This was demonstrated in a study where progesterone was directly able to bind to cell surface oxytocin-receptors to inhibit uterine contractions (224), suggesting that in some cases, sex hormones can modulate signaling cascades independent of binding to cognate receptors. Combined, androgens and female sex hormone genomic and non-genomic mediated mechanisms are important in regulation gene expression in target cells, including CD4 T cells.

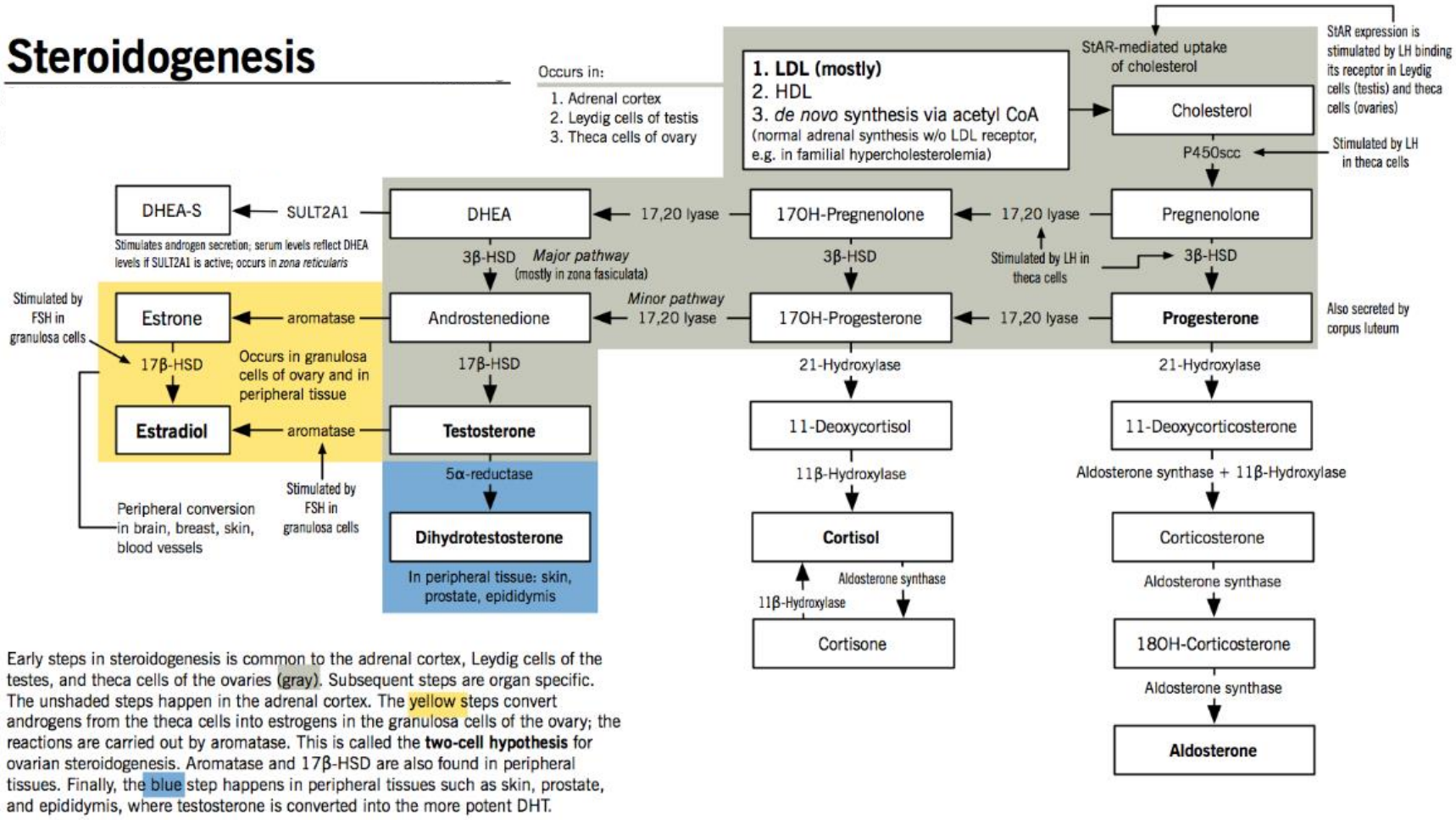

Figure 2-1: Sex hormone synthesis pathways in the gonad and adrenal glands. The conversation cholesterol (mostly LDL) pregnenolone by both gonadal and adrenal tissue leads to the productive of androgens, estrogen and progesterone. Progesterone, testosterone, including testosterone metabolites, including DHEA and androstenedione can further be synthesized into estrogens, including estradiol. Adapted from Rotstein, A., and Wong. E., (2013, March 13). "Sex Hormone Synthesis, Regulation and Function". *McMaster Pathophysiology Review.* Retrieved from http:pathophys.org/sexhormones/

2.7 Role of Sex Hormones in Type-High and Type-Low Mediated Airway Inflammation

Clinical and epidemiological data have provided insight into the role of sex hormones in driving asthma symptoms, prevalence, and severity, but mouse models of asthma have elucidated some of the mechanism by which sex hormones regulate airway inflammation. As summarized in Table 2-1 and Figure 2-2, ovarian hormones, including estrogen and progesterone, enhanced while androgens, including, testosterone and 5-alpha dihydrotestosterone (DHT), suppressed the innate and adaptive immune responses driving airway inflammation in asthma. In the following subsections, we will describe the findings from these animal studies.

2.8 The Role of Sex Hormones on Type 2 Immune Cells

In chapter 1, we have discussed the inflammatory pathways important in asthma pathogenesis and how sex hormones signal and are regulated in females and male, In this section, we will discuss previous studies that have examined how sex hormone signaling impacts airway inflammation in asthma. In multiple studies, female mice had increased ovalbumin (OVA)-induced infiltration of eosinophils, serum IgE concentrations, and IL-13 protein expression in the lungs compared to male mice (14, 17, 225). However, OVA-challenged females had increased airway remodeling and mucus production compared to male mice in some reports, but no differences were found in others. Presence of ovarian hormones during the sensitization phase is required for maximal Th2-mediated airway inflammation in female mice (226). In the OVA sensitization and challenge model of allergic airway inflammation, compared

to sham-operated female mice, female mice ovariectomized prior to OVA sensitization had decreased IL-5 and eosinophil bronchioalveolar (BAL) levels as well as decreased AHR to methacholine (19). However, if female mice were ovariectomized after sensitization, but before OVA challenge, there was no difference in BAL IL-5 protein expression or eosinophils as well as no effect on the percent increase in AHR (19) Further, addition of estradiol benzoate, a synthetic, steroidal estrogen, before OVA sensitization increased eosinophils in the BAL compared to those of vehicle-treated ovariectomized OVA-challenged female mice, albeit not to levels observed in OVA-challenged sham-operated female mice (19). These data show that estrogen signaling is important for OVA-induced allergic airway inflammation prior to sensitization, but that other ovarian hormones, potentially progesterone, are also important in establishing OVA-induced allergic airway inflammation.

Other studies have looked at the direct action of estrogen or testosterone on allergic airway inflammation. Estrogen can signal through multiple receptors including the nuclear receptors, estrogen receptor α (ERα) and β (ERβ), as well as the membrane-bound G protein-couple estrogen receptor 1 (GPER1). Estrogen signaling through ERα increased OVA-induced allergic airway inflammation, as mice deficient in ERα had decreased AHR compared to that of (wild type) WT and ERβ deficient mice (227). However, additional studies showed that ERα signaling increased OVA-induced AHR but had no effect on airway inflammation (227). Testosterone is also important in type 2-mediated airway inflammation. Castrated male mice had a significant increase in OVA-induced eosinophil and lymphocyte infiltration as well as IL-13 protein expression compared to sham-operated male mice. Further, addition of dehydroepiandrosterone (DHEA), a hormone upstream of testosterone, to mouse chow decreased

house dust mite (HDM)-induced allergic airway inflammation compared to that of mice on control diet (228). DHEA-treated mice undergoing the HDM protocol had decreased serum eosinophils, IL-5, IL-4, and IFNγ levels but no change in serum IgE concentrations compared to HDM-stimulated mice on normal chow (228). Combined, these data showed sex hormones were important in regulating type 2 inflammation and that many pathways are affected by ovarian hormone and/or testosterone signaling.

Group 2 innate lymphoid cells are also important for the allergic response in the lungs (229-231, 226). Recently, IL-5 and IL-13 were increased in IL-33-stimulated ILC2 from OVA sensitization and challenge female BALB/c mice compared to those of IL-33-stimulated ILC2 from OVA sensitization and challenge male mice (232). Our group and others have also published that the ILC2 numbers are increased in women compared to men with asthma and that testosterone, via androgen receptor signaling, intrinsically and extrinsically attenuates allergen-induced allergic airway inflammation, ILC2 proliferation and cytokine expression of IL-5 and IL-13 (233, 234). Furthermore, additional studies demonstrated that both lung and uterine ILC2 populations expression ERα, further supporting the idea that female sex hormones regulate ILC2 function and proliferation (235). Combined, these data suggest that sex hormones may also be important in ILC2-mediated airway inflammation, and future studies need to be conducted to determine the underlying mechanisms by which steroid hormone receptors, including estrogen, progesterone and testosterone regulate ILC2-mediated airway inflammation.

Other immune cells, including mast cells, dendritic cells (DCs), and macrophages (Mφ), are also important for type 2-mediated airway inflammation. Allergens increase antigen-specific IgE production by B cells, and IgE binds to the high-affinity IgE receptor, FcεR1, on tissue mast cells, and peripheral blood basophils. Antigen crosslinking of the antigen-specific IgE/FcεR1

complex ignites degranulation of the mast cells and basophils, causing release of soluble mediators, including histamines, cytokines, prostaglandins, and proteases (236, 237). The role of sex hormones on mast cell degranulation or cytokine expression is unclear. As mentioned previously, IgE is increased in the serum of OVA-sensitized and OVA-challenged female mice compared to that of male mice (14, 19, 225). Other studies showed that peritoneal mast cells (PMCs) from female, but not male, Sprague-Dawley rats treated with estradiol, progesterone, testosterone, or DHT at the time of ex vivo stimulation with substance P had decreased histamine release compared to PMCs treated with vehicle (238). Further, histamine release from PMCs stimulated with IgE from female, but not male rats, increased with estradiol treatment and decreased with progesterone, testosterone, or 5α-DHT (238). Therefore, sex hormones regulate activation of mast cells, but the mechanisms are not clear and additional investigation is needed. the role of sex hormones on mast cell degranulation or cytokine expression during airway inflammation is unclear, studies in isolated peritoneal mast cells (PMCs) from female Sprague-Dawley rats stimulated with substance P showed decreased histamine release in PMCs from female rats administered with estradiol, progesterone, testosterone, or DHT.

DCs and M2 alveolar macrophages also play a central role in allergic airway inflammation, and sex hormones affect cytokine expression and antigen presentation in DCs and alveolar macrophages. Female mice that were OVA sensitized and challenged had increased myeloid DCs and plasmacytoid DCs migrating to the lung-draining lymph nodes as well as increased the percentages of alveolar macrophages (AAMφ) compared to those of male BALB/c mice (239). Further, 17β-estradiol (E2) treatment of LPS-stimulated DCs increased T cell activation, proliferation, and protein expression of IL-6, IL-8, and MCP-1 compared to that of

vehicle-treated, LPS-stimulated DCs (240). 17β-E2 administration to GM-CSF-stimulated bone marrow cells also increased CD11b+, CD11c+ DCs. ERα deficiency (esr1−/− mice) significantly decreased the number of CD11b+, CD11c+ DCs generated from GM-CSF and IL-4 treated-bone marrow cells compared to WT mice (241). Other studies reported that bone marrow macrophages from female mice treated with IL-4 and an ERα agonist, PPT, have an increased capacity to polarize toward the M2 phenotype compared to bone marrow macrophages from male mice (242) . Studies on macrophages from other tissues have shown that the administration of E2 to ovariectomized LysM-Cre Erαflox mice significantly reduced numbers of peritoneal macrophages in the peritoneal cavity and increased protein levels of IL-1β and IL-6 as well as mRNA expression of iNOS, compared to ovariectomized LysM-Cre Erαflox mice administered placebo pellets (243). The role of androgens and androgen receptor signaling on alveolar macrophage polarization has also been investigated. These studies reported that DHT administration to castrated male mice reduced OVA-induced allergic airway inflammation and but enhanced M2 polarization of alveolar macrophages (244) . All together, these findings demonstrate that sex hormones regulate lung DCs and AAMφ differentiation, function, and cytokine expression in airway inflammation associated with asthma.

2.9 The Role of Sex Hormones on IL-17A Secreting Cells

While type 2 immune-mediated airway inflammation is found in many patients with asthma, increased IL-17A is associated with more severe phenotypes of asthma (34, 18).

IL-17A is increased in the bronchoalveolar lavage fluid of patients with asthma, leading to increased mucus production and increased neutrophils in the airways (245, 48, 246, 18). In the lungs, IL-17A is secreted by CD4+ T helper 17 (Th17) cells, γδ T cells, and neutrophils and

innate lymphoid group 3 (ILC3) cells. Our group showed women with severe asthma had significantly increased IL-17A-producing Th17 cells compared to men with severe asthma (18). Using an adoptive transfer mouse model of OVA-specific Th17-mediated inflammation, we also showed that the OVA-specific Th17 cells from female mice had increased IL-17A production and caused increased neutrophilic inflammation in recipient mice compared to OVA-specific Th17 cells from male mice (18). Further, we showed that in vivo administration of 17β-estradiol and progesterone increased ex vivo Th17 cell differentiation and increased IL-17A protein expression in Th17 cells by increasing IL-23/IL-23R signaling through a let-7f miRNA expression-dependent pathway (18).

IL-17A-producing γδ T cells and ILC3 also augment the IL-17A-mediated airway inflammatory response, and the role of sex hormones on these cell types has also been studied (247, 248). Estradiol treatment of γδ T cells decreased numbers of IL-17+ T cells in the draining lymph nodes, suggested an estradiol-mediated regulation of γδ T cell migration from the lymph nodes to various tissues (249). The role of sex hormones on ILC3 function has not been investigated but is an important area for future studies. Additional studies need to be conducted to determine how sex hormones regulate IL-17A-mediated airway inflammation or both type 2 and IL-17A-mediated inflammation, as many patients with asthma have increased eosinophils and neutrophils in the airway (7).

2.10 The Role of Sex Hormones in Airway Hyperresponsiveness and Mucus Production

Non-immunological mechanisms, such as gender differences in AHR, smooth muscle contractility, and mucus production, may also drive the gender differences in asthma. Baseline

AHR is increased in male mice compared to that of female mice for both BALB/c and C57BL/6 strains of mice (15). Increased basal AHR in male mice is potentially caused by fewer numbers of alveoli and decreased alveolar surface area compared to that on female mice as ovariectomized female mice have similar alveoli structures as male mice (250). Additionally, ER-α and ER-β signaling is important for alveolar development since ER-α- and ER-β-deficient mice have decreased alveoli (251). A gender difference in smooth muscle contractility is also reported. Vagal nerve responses in response to methacholine or carbachol challenge were increased in male mice compared to those of female mice and gonadectomized male mice had similar levels of vagal nerve responses as female mice (16). Further restoring androgens to gonadectomized mice increased vagal nerve responses and increased AHR in these mice (16). In guinea pigs, testosterone signaling has been shown to decrease airway smooth muscle cell by inhibiting IP_3 signaling (252). These findings show that androgens are important in increasing vagal nerve responses and AHR.

Estrogen and progesterone are also important in mucus production and mucociliary clearance. Administration of ethynyl estradiol to guinea pigs significantly increased mucus cell hyperplasia compared to that of vehicle-treated animals (251). Further, administration of estrogen or progesterone increased to cultured human airway or nasal epithelial cells the expression of the mucus proteins, Muc5AC and Muc5B, as well as mucus production when compared to those of vehicle treated-cells (253, 251). However, progesterone decreased the cilia beat frequency from cultured primary human airway epithelial cells, but cells that were co-administered 17β-E2 with progesterone had cilia beat frequency that was similar to vehicle-treated cells (254). In summary, sex hormones regulate baseline airway responsiveness to methacholine, smooth muscle contractility, and mucus production.

Table 2-1: The role of sex hormones in animal models of airway inflammation associated with asthma

Protocol for airway inflammation	Gender effects on endpoints	Endpoints	References
OVA sensitization and challenge	Females > males	BAL or lung eosinophils, Serum IgE, IL-13 protein expression, AHR, airway remodeling and mucus production	(17, 225, 14)
OVA sensitization and challenge	Females = males	Mucus production	(14)
HDM	Female > males	HDM-specific IgE levels in serum IL-17A protein expression AHR	(14)
OVA sensitization and challenge	Castrated males > intact male	BAL eosinophils and lymphocytes	(17)
OVA sensitization and challenge	Intact females > ovariectomized females	Numbers of eosinophils IL-5 protein expression Total IgE serum levels AHR	(19)
OVA sensitization and challenge	ERα KO > ERβ KO and WT mice	AHR	(5)
OVA sensitization and challenge	Females > males	IL-5 and IL-13 protein expression in ILC2 isolated from whole lung homogenates	(232)
Alternaria and HDM challenge	AR^{tfm} > WT male	IL-5+ and IL-13+ ILC2 in the lungs	(233, 234)
Transfer of OVA-specific Th17 cells followed by OVA challenge	Females > males	OVA-specific IL-17+ T cells Neutrophils	(18)
OVA sensitization and challenge	Females > males	Myleoid dendritic cells (DCs) Plasmacytoid DCS Alveolar macrophages (AAMφ)	(239)
OVA sensitization and challenge	Ovariectomized female mice: Estradiol (E2) hormone pellet > vehicle hormone pellet	M2 gene expression analysis following IL-4 stimulation of Bone-marrow derived macrophages	(242)
OVA sensitization and challenge	Castrated male mice: dihydrotestosterone (DHT) hormone pellet > vehicle hormone pellet	M2 polarization	(244)

AHR, airway hyperresponsiveness, ERα/β; Estrogen receptor α/β, AR^{tfm}, Androgen receptor testicularized mice

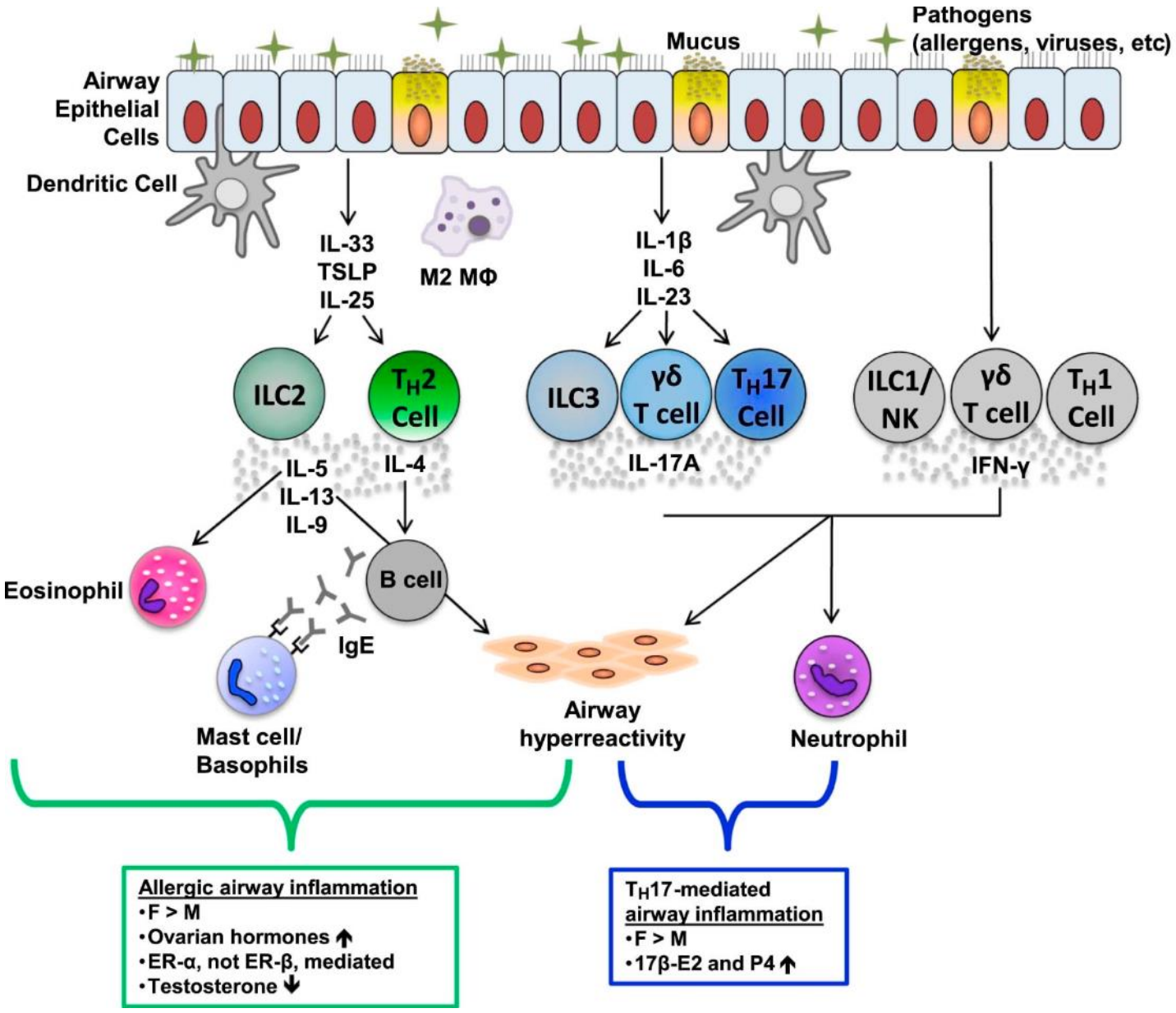

Figure 2-2: Gender differences and the Role of Sex Hormone Signaling on Airway Inflammatory Pathways Associated with Asthma. Schematic of allergen-induced type 2 and non-type (IL-17A and INF-γ mediated airway inflammation. Summary of sex differences and the role of sex hormone signaling on these inflammatory pathways is depicted below. E2 indicates estradiol; ER, estrogen receptor, F, female, ILC, innate lymphoid cell, Mφ, macrophage; M, male, P4, progesterone, TLSP, thymic stromal-lymphopoietin.

2.21 Summary of Background

The heterogeneity in asthma, had resulted in various phenotypes and endotypes in patients with asthma. Clustering analytical studies of asthma patients based on asthma severity revealed that patients with more severe asthma phenotypes tend to have poor responses to current asthma therapies and are more likely to display a dual type 2 and IL-17A mediated airway inflammation, an inflammatory signature associated with refractory asthma (3, 33, 7). Type 2 cytokines and IL-17A have been shown to have signaling feedback loops where type 2 cytokines, including IL-4 and IL-13, have been shown to inhibit IL-17A expression (255, 256). However, in contrast, IL-17A has been shown to promote type 2 cytokine secretion (257). Several studies show that women are twice more likely to have asthma, including severe asthma compared to men (258, 178). Further studies have demonstrated that the sex hormones signaling may regulate the observed female predominance in asthma. Additionally, sex hormones have been shown to regulate both the type 2 and IL-17A inflammatory pathways (171, 259, 172). However, it remains unclear how sex hormones regulate airway inflammation mediated both by type 2 cytokines and IL-17A. Additionally, it is not known how these specific interactions may modulate the signaling feedback loops that have been demonstrated between the type 2 and IL-17A inflammatory pathways. The focus of this dissertation work is to understand the molecular mechanisms by which ovarian hormones and testosterone regulate dual type 2 and IL-17A-mediated airway inflammation as well as the reciprocal feedback loop between type 2 cytokines and IL-17A.

CHAPTER 3

Testosterone decreases and Ovarian Hormones increase HDM-induced Type 2 and IL-17A-Mediated Airway Inflammation

3.1 Introduction

A sexual dimorphism exits in asthma, and as adults, women have an increased prevalence of asthma compared with men. Epidemiological and clinical studies showed women with asthma had increased exacerbations, decreased forced expiratory volume in 1 s (FEV1) after bronchodilator use, and increased eosinophils and neutrophils in the bronchoalveolar lavage (BAL) fluid and sputum compared with men with asthma (260, 261, 7). Premenstrual worsening of asthma symptoms and changes in asthma control during pregnancy were also reported by women (181, 195, 186, 196, 187, 197). Combined, these findings suggested that sex hormones are important in regulating mechanisms that drive airway inflammation in asthma.

Asthma is a heterogeneous disease that is regulated by different pathophysiologies. Increased eosinophils and neutrophils are found in the sputum of patients with more severe phenotypes of asthma (261, 3, 20, 8). Type 2 inflammation is mediated by increased secretion of IL-4, IL-5, and IL-13 from CD4+ Th2 cells, group 2 innate lymphoid cells (ILC2), and other cells (262, 101), resulting in increased airway eosinophils, mucus production, IgE production, and airway hyperresponsiveness (AHR) (263, 55, 262). Increased secretion of IL-17A, a cytokine secreted by CD4+ Th17 cells, γδ T cells, group 3 innate lymphoid cells (ILC3), and other cell types, leads to increased airway neutrophils and mucus production (34, 264). However, it remains unclear how sex hormones regulate

inflammatory pathways that increase airway eosinophils and neutrophils.

Mouse models of allergic, type 2–mediated airway inflammation showed that testosterone decreased eosinophils and lymphocyte infiltration of the airways (17, 225) and ovarian hormones (estrogen and progesterone) increased IL-5 protein expression, airway eosinophils, AHR, and total serum IgE levels (171, 19). Recently, our group and others showed testosterone signaling through the androgen receptor (AR) attenuated both *Alternaria alternata* extract and house dust mite (HDM)–induced, ILC2–mediated airway inflammation (233, 234). Our group has also showed that ovarian hormones increased Th17 cell differentiation and IL-17A production through an IL-23R–dependent mechanism in mice and humans as well as increasing neutrophilic airway inflammation in mice (18). However, how testosterone regulates dual type 2– and IL-17A–mediated airway inflammation remains unknown. We hypothesized that testosterone signaling attenuates type 2– and IL-17A–mediated airway inflammation. Our results showed that testosterone decreased HDM-induced total numbers of IL-13+ Th2 and IL-17A+ Th17 cells in the lung.

3.2 Methods

3.2.1 Mice

Wild-type (WT) BALB/cJ 6- to 8-wk-old female and male were purchased from Charles River Laboratories (Wilmington, MA). WT female, WT male, and AR testicular feminized (AR^{tfm}) male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME), and breeding colonies were established at Vanderbilt University Medical Center. CD90.1/CD90.2 mice were bred in-house using homozygous CD90.1 and CD90.2 C57BL/6J mice from Jackson Laboratory. Gonadectomy or sham surgeries were conducted at 3–4 weeks of age by Charles

River laboratories veterinary staff, and experiments were started when gonadectomized or sham-operated mice were 6–8 weeks old. All animal experiments were conducted in adherence to the rules and regulations of the Association for Assessment and Accreditation of Laboratory Animal Care and were approved by the International Animal Care and Use Committee at Vanderbilt University Medical Center.

3.2.2 House dust mite (HDM) Challenge Protocol

Mice were intranasally administered 40 μg of HDM from Greer Laboratories (Lenoir, NC) or PBS as vehicle control in a 100-μl total volume for four times a week for 3 wk as shown in Figure 3A.

3.2.3 BAL and Analysis of Inflammatory cell Infiltration in the Airway

BAL was performed by instilling 800 μl of saline solution through a tracheostomy tube and then withdrawing the fluid with gentle suction through a syringe, as previously described (18). The total cell count in the BAL fluid was counted using a hemocytometer and 0.04% trypan blue exclusion dye (Sigma-Aldrich). The cells from the BAL were then fixed to a slide and stained using the Three-Step Stain system (Richard-Allan Scientific, Waltham, MA). Two hundred BAL cells were classified as eosinophils, neutrophils, lymphocytes, or macrophages using standard morphologic criteria, and percentages of these inflammatory cells were determined. Total numbers of inflammatory cells were determined by multiplying the percentage of the inflammatory cells by the total numbers of viable cells in the BAL fluid.

3.2.4 Cytokine and IgE Measurements

Cytokine levels were measured from BAL fluid, lung homogenates, and/or Th0, Th2, and Th17 cell culture supernatants by ELISA using Quantikine and Duoset kits (R&D Systems). Total serum IgE levels were determined using an ELISA (BioLegend). All experiments were performed according to the manufacturer's instructions. Any OD450 value less than the lower limit of detection was assigned half the value of the lowest detectable standard.

3.2.5 AHR Measurements

Mice were anesthetized with pentobarbital sodium (85 mg/kg), and an 18-gauge tracheostomy tube was placed in the trachea of the mice. Mice were then mechanically ventilated using the SCIREQ flexiVent machine with 150 breaths/min and a tidal volume of 10 ml/kg body weight. Airway resistance was determined at baseline and after administration of increasing doses of nebulized acetyl-β-methacholine (0–50 mg/ml) as previously described (265).

3.2.6 Flow Cytometric Analysis of Lung IL-13 and IL-17A Production

Lungs were harvested and digested, and lung single-cell suspensions were restimulated with 50 ng/ml of PMA (Sigma-Aldrich), 1 μg/ml of ionomycin (Sigma-Aldrich), and 0.07% Golgi Stop (BD Biosciences) for 5 h at 37°C in RPMI 1640 plus 10% FBS. Following restimulation, cells were stained with viability dye (Ghost Dye UV 450; Tonbo Biosciences), blocked with an anti-FcR Ab (clone 2.4G2), and surface stained with biotin-labeled anti-CD3 (clone 17A2), PE-Cy5 anti-CD4 (clone 129.19), BV786 anti-CD90.2 (clone 53-2.1), Alexa Fluor 700 anti-CD45 (clone 30-F11), Alexa Fluor 488 anti-CD25 (clone OC61), PE-Cy7 anti-CD127 (clone A7R34), and FITC anti-γδTCR (clone GL3)

Abs, followed by allophycocyanin-Cy7 streptavidin staining (1:250). Cells were then fixed, permeabilized using the Foxp3/transcription factor staining kit (Tonbo Biosciences), and intracellularly stained with PE–IL-13 (clone eBio13A), PE-Cy7 anti–IL-17A (clone eBio17B7). Flow cytometry analysis was conducted on LSR II flow cytometer, and all data were processed using FlowJo software version 10.

3.2.7 Statistical Analysis

Unless otherwise indicated, data are represented as mean ± SEM where groups were compared by one-way ANOVA with Tukey post hoc analysis. For AHR experiments, ANOVA of repeated measures and Bonferroni post hoc analysis were used to calculate values. For all analysis, $p < 0.05$ was considered significant.

3.3 Results

3.3.1 HDM-induced BAL Eosinophils and Neutrophils were decreased in Male compared to Female Mice

Type 2 and IL-17A inflammatory pathways are important in regulating airway eosinophils and neutrophils, respectively, in asthma. Because asthma prevalence is increased in women compared with men (4, 7), we hypothesized that allergen-induced airway inflammation is increased in female mice compared with male mice. To test this hypothesis, male and female WT BALB/c mice were intranasally challenged four times per week for 3 wk with 40 μg of HDM or vehicle (PBS) (Figure 3-1A). Twenty-four hours after the last challenge, on day 18, lung and BAL fluid were collected to measure cytokine expression and infiltration of inflammatory cells into the BAL fluid. In male and female mice, HDM significantly increased whole lung

homogenate IL-13 and IL-17A protein expression as well as significantly increased total BAL inflammatory cells, eosinophils, neutrophils, macrophages, and lymphocytes compared with PBS-challenged mice (Figure 3-1B–H). However, male mice had significantly decreased HDM-induced lung IL-13 and IL-17A protein expression and total BAL cell numbers, eosinophils, neutrophils, macrophages, and lymphocytes compared with HDM-challenged female mice (Figure 3-1B–H). These data showed female mice had increased HDM-induced IL-13 and IL-17A protein expression and BAL inflammatory cells compared with male mice.

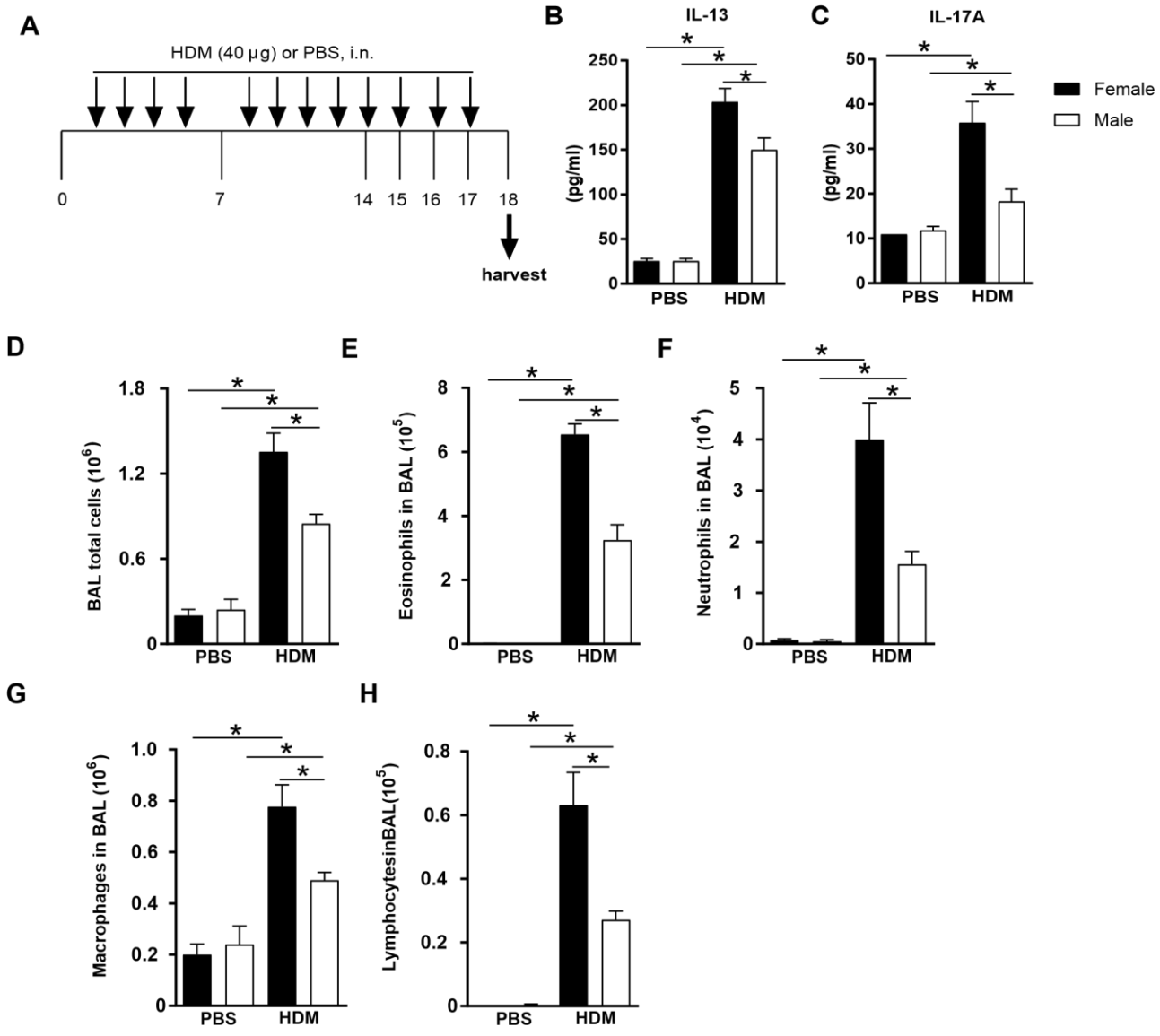

Figure 3-1: HDM-induced BAL Eosinophils and Neutrophils were decreased in Male compared to Female mice. (A) Experimental design for HDM-induced airway inflammation. **(B-C)** IL-13 and IL-17A protein expression in the lung homogenates as measured by ELISA. **(D-H)** Total BAL cells and total BAL eosinophils, neutrophils, macrophages, and lymphocytes. * p< 0.05, 1-way ANOVA with Tukey post-hoc analysis, n= 6-9 mice/group. Data pooled from 2 independent experiments.

3.3.2 Testosterone decreased and Ovarian Hormones increased HDM-induced Eosinophilic and Neutrophilic Airway Inflammation, IgE production, and AHR

Because there was a gender difference in HDM-induced airway eosinophils and neutrophils (14), we hypothesized that endogenous sex hormones were important in regulating eosinophilic and neutrophilic-mediated airway inflammation. To test our hypothesis, male and female WT BALB/c mice underwent gonadectomy or sham operation at 3–4 wk of age. This age is prior to sexual maturity and abundant production of sex hormones. When the mice were 6–8 wk old, the mice underwent the HDM or PBS challenge protocol. Twenty-four hours after the last challenge, BAL eosinophils and neutrophils were significantly increased in HDM-challenged sham-operated female mice and gonadectomized male mice compared with sham-operated male and gonadectomized female mice.

HDM has been shown to increase IgE serum levels, AHR, and mucus production (266, 267). Thus, we wanted to determine how testosterone and ovarian hormones regulated these hallmarks of asthma. We measured total serum IgE levels by ELISA and AHR to increasing concentration of methacholine challenge using the flexiVent plethysmography system. Total serum IgE levels were increased in HDM-challenged sham-operated female mice and gonadectomized male mice compared with sham-operated male mice and gonadectomized female mice (Figure 3-2C). HDM also significantly increased methacholine-induced AHR in the lungs of HDM-challenged mice from all groups (Figure 3-2D), but no increase in AHR was seen with PBS-challenged mice (Figure 3-2E). Furthermore, HDM-induced AHR was increased in sham-operated female mice and gonadectomized male mice compared with sham-operated male mice and gonadectomized female mice (Figure 3-2D). Forty-eight hours after the last challenge, when mucus production levels are upregulated (268), lungs were harvested, and mucus cell

metaplasia was determined by PAS staining . HDM-challenged mice in all groups had significantly increased mucus production compared with PBS-challenged mice, but no significant differences in periodic acid–Schiff staining were detected between HDM-challenged experimental groups (Figure 3-3A-B) Combined, these results showed that HDM-induced eosinophilic and neutrophilic inflammation, IgE production, and AHR were decreased by testosterone and increased by ovarian hormones.

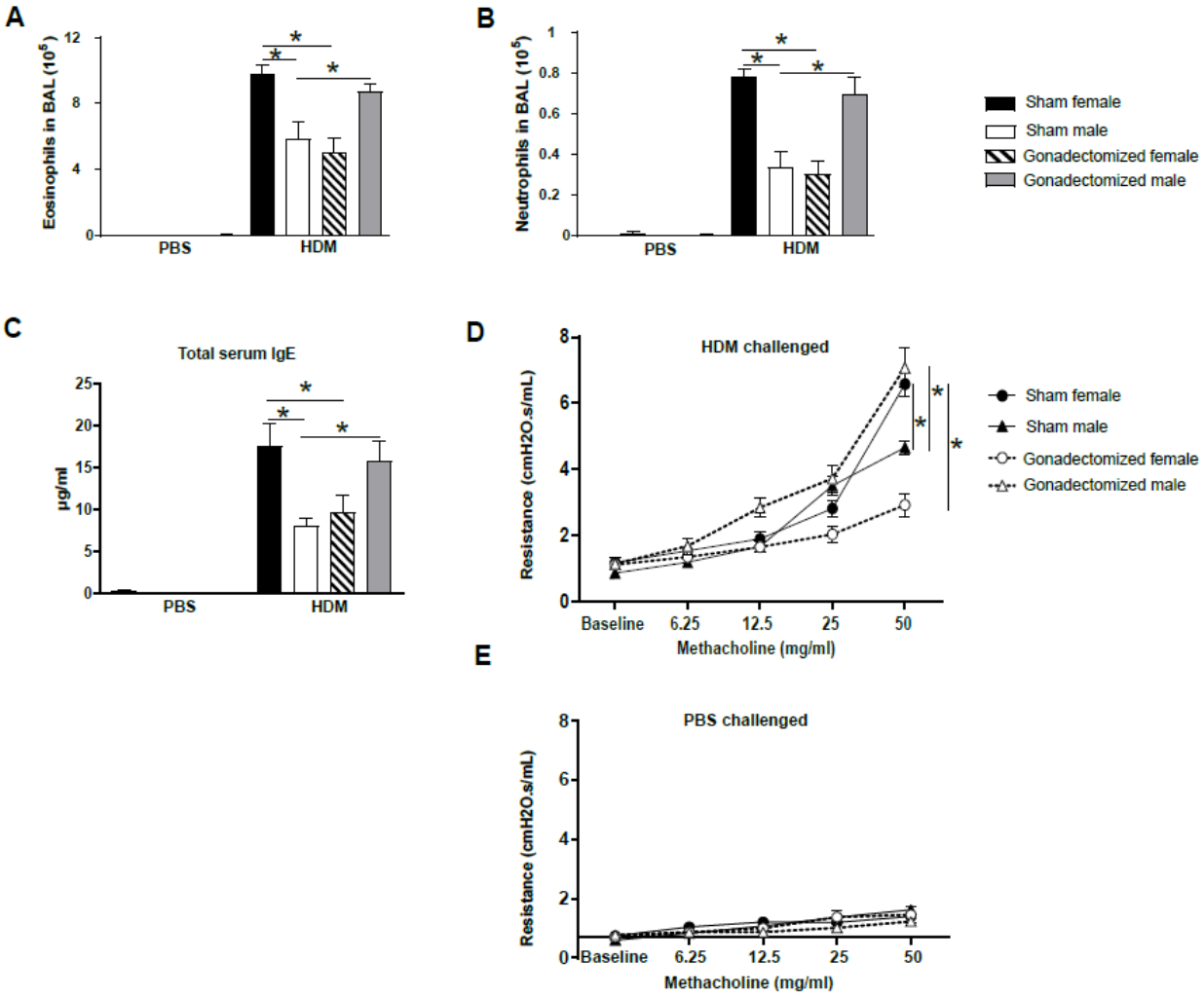

Figure 3-2: Testosterone decreased and Ovarian Hormones increased HDM-induced BAL Eosinophilic and Neutrophilic Airway Inflammation, IgE production, and AHR. WT gonadectomized and sham-operated male and female BALB/c mice were challenged with HDM or PBS. **(A-B)** Total BAL eosinophils and neutrophils. **(C)** Total serum IgE levels. **(A-C)** *p<0.05, 1-way ANOVA with Tukey post hoc analysis, n=3-7 mice/group. Representative sample of 3 independent experiments. **(D-E)** AHR in response to increasing concentrations of methacholine 24 hours after the last HDM or PBS challenge. * p<0.05 ANOVA of repeated measures with Bonferroni post hoc analysis. n=12-14/group. Data pooled from 2 independent experiments.

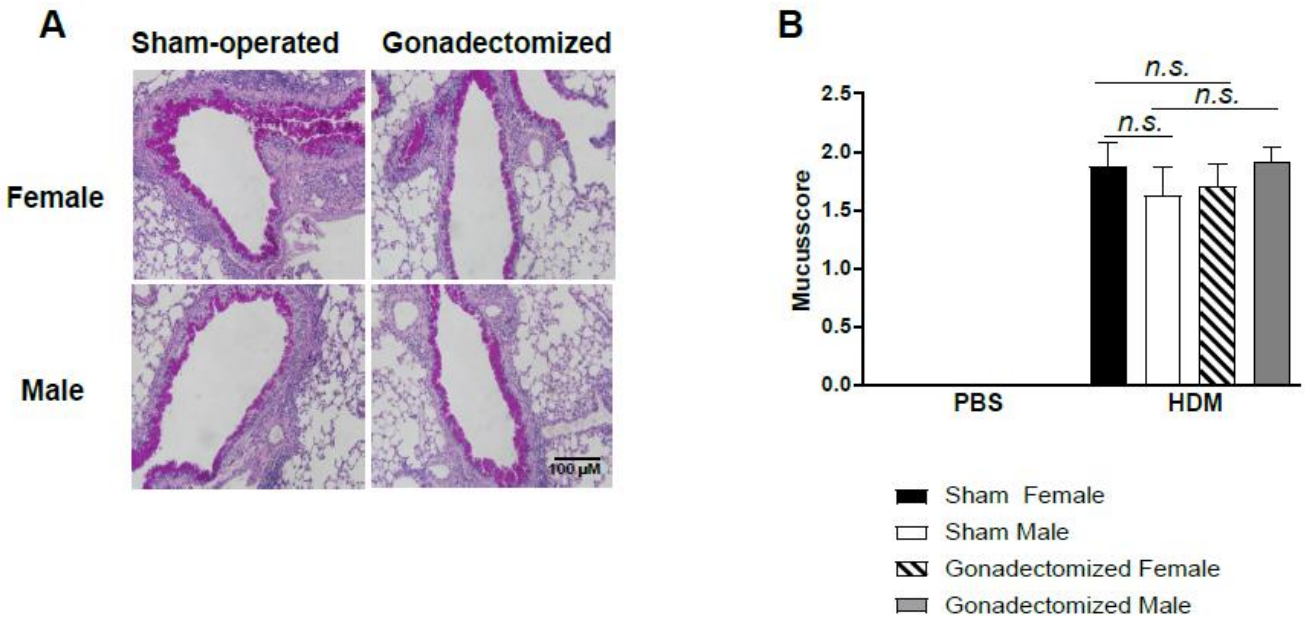

Figure 3-3: The Role of Testosterone and Ovarian Hormones on Mucus Production following HDM-induced Type 2 and IL-17A-Mediated Airway Inflammation: (A-B) Representative 20X magnification and quantification of lung histology sections in PAS positive staining in HMD challenged mice 48 hours after last HDM. n=6-10 mice per experimental group. *n.s.* means not statistically significant.

3.3.3 Testosterone decreased and Ovarian Hormones increased HDM-induced Total Numbers of IL-13$^+$ Th2 and IL-17A$^+$ Th17 cells

IL-13 and IL-17A are increased with HDM exposure, and we next determined if sex hormones regulated IL-13– and IL-17A–producing CD45+ cells. On day 18, IL-13– and IL-17A–producing CD45+ cells were determined by flow cytometry using the gating strategy detailed in Figure 3-4. The total number, but not frequency, of IL-13+ CD45+ cells was increased in HDM-challenged sham-operated female mice and gonadectomized male mice compared with the HDM-challenged sham-operated male mice and gonadectomized female mice (Figure 3-5A-C). Furthermore, the total number of IL-17A+ CD45+ cells was increased in HDM-challenged sham-operated female mice and gonadectomized male mice compared with the HDM-challenged sham-operated male mice and gonadectomized female mice (Figure 3-5E). The frequency of IL-17A+ CD45+ was also increased in the HDM-challenged sham-operated female mice compared gonadectomized female mice, but the frequency of IL-17A+ CD45+ was similar in sham-operated and gonadectomized male mice (Figure 3-5D). We did not detect a substantial population of dual IL-13– and IL-17A–producing cells in our model.

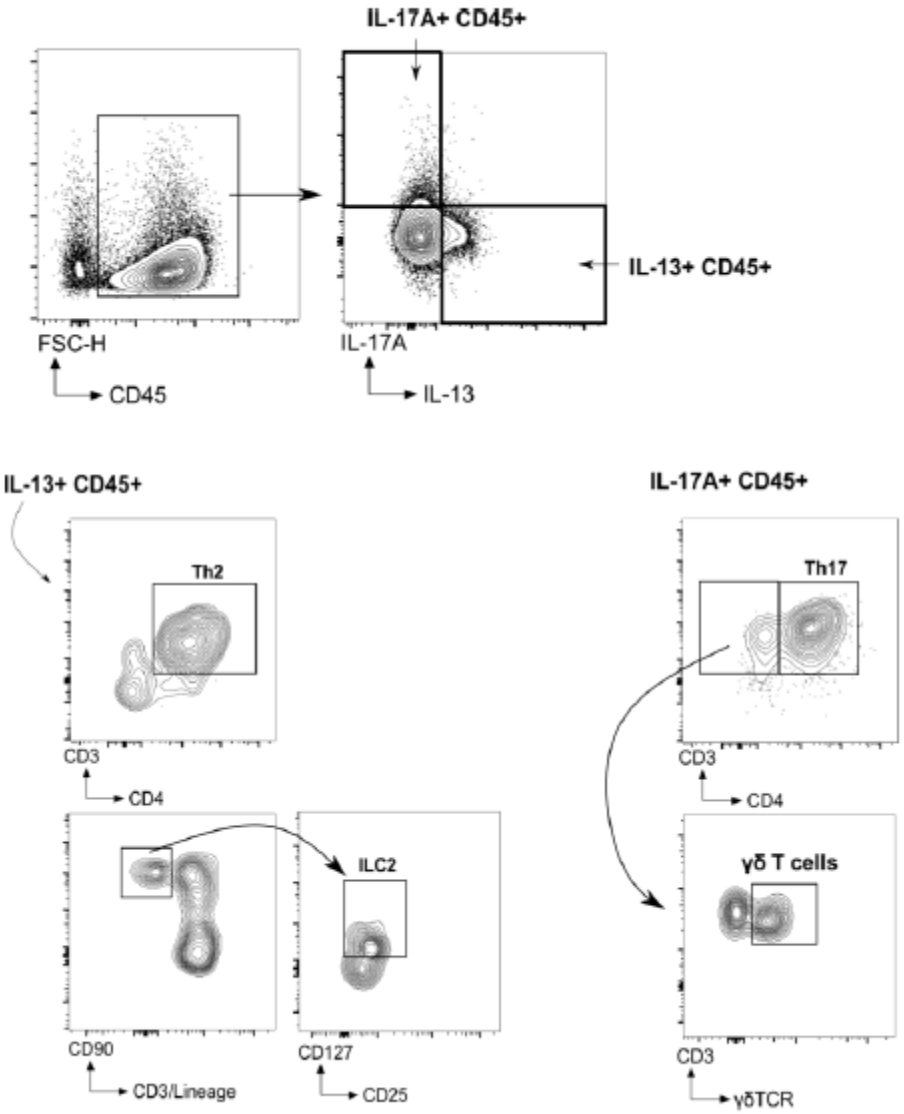

Figure 3-4: Gating Strategy for Th2, Th17, γδ T Cells and ILC2. Data. are representative of the gating strategies used to measure IL-13 and/or IL-17A+ Th2, Th17, ILC2 and γδ T cells. Samples were pre-gated for viable singlets with low to intermediate FSC-A and SSC-A properties associated with leukocytes and lymphocyte populations.

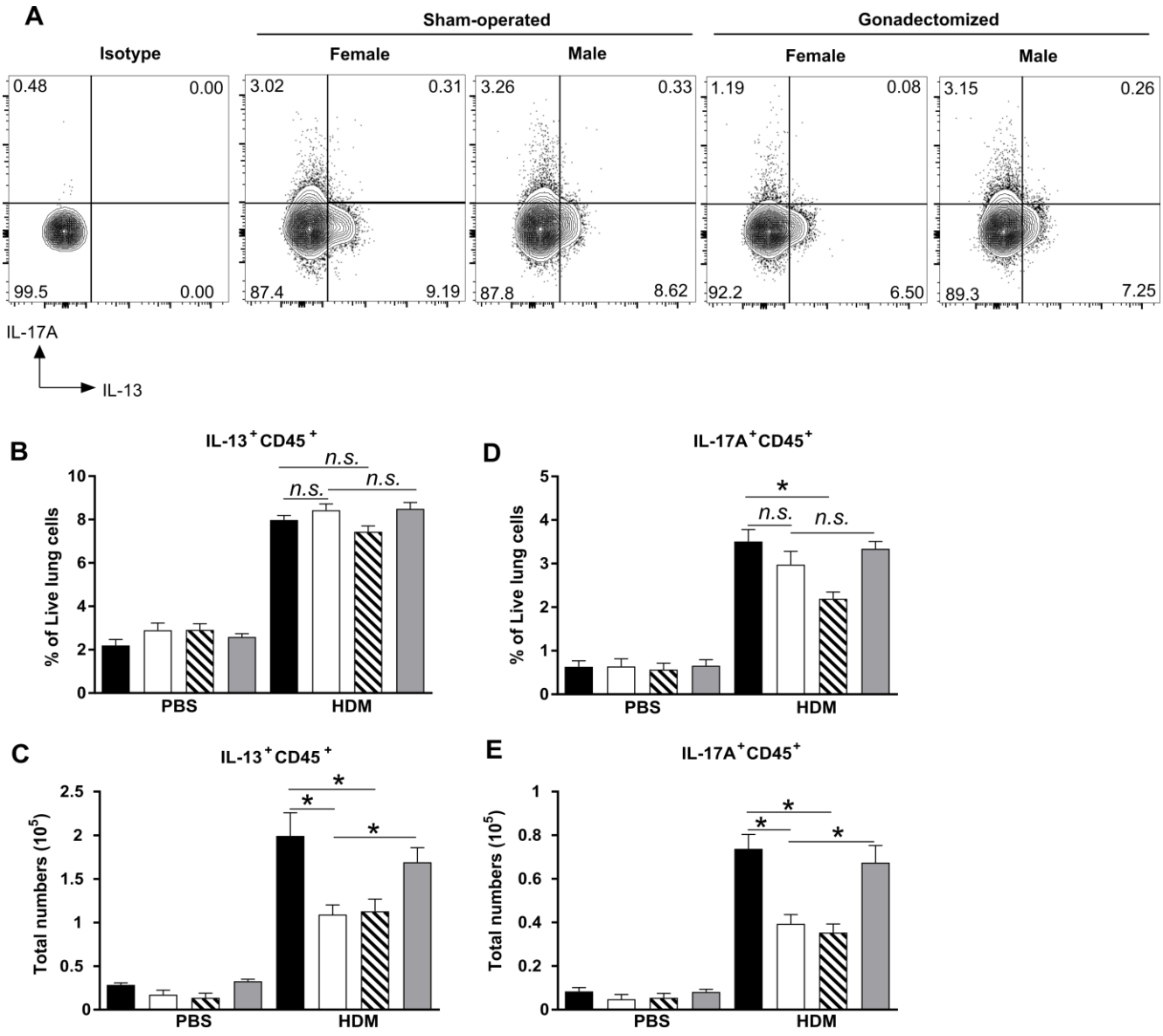

Figure 3-5: Testosterone decreased and Ovarian Hormones increased HDM-induced Total Numbers IL-13 and IL-17A Producing CD45+ cells. WT gonadectomized and sham-operated male and female BALB/c mice were challenged with HDM or PBS. Lungs were harvested 24 hours after the last challenge, and lung cells were restimulated with PMA, ionomycin and Golgi stop. **(A)** Representative dot plots of viable, CD45+ IL-13+ or IL-17A+ cells. **(B-C)** Percent and total numbers of IL-13+ CD45+ cells. **(D-E)** Percent and total numbers of IL-17A+ CD45+ cells.

Next, we determined the cell sources of CD45+ IL-13+ or IL-17A+ cells, and we found that the total number, but not the percentage, of IL-13+ Th2 cells was increased in HDM-challenged sham-operated female and gonadectomized male mice compared with sham-operated male and gonadectomized female mice (Figure 3-6 and Figure 3-7A-B). We found a significant increase in the percentage of IL-17A+ Th17 cells in the sham-operated female mice compared with the gonadectomized female mice (Figure 3-7D). Furthermore, the total number of IL-17A+ Th17 cells was increased in HDM-challenged sham-operated female mice and gonadectomized male mice compared with sham-operated male mice and sham-operated female mice (Figure 3-7E). Although the CD4 T cells accounted for ~50–60% of the IL-13 and IL-17A produced in the lung, IL-13+ ILC2 and IL-17A+ γδ T cells were also increased in HDM-challenged sham-operated female mice and HDM-challenged gonadectomized male mice compared with HDM-challenged gonadectomized female and HDM-challenged sham-operated male mice (Figure 3-7C, 3-7F). Combined, these studies showed that HDM-induced IL-13+ and IL-17A+ producing cells, predominantly Th2 and Th17 cells, were decreased by testosterone and increased by ovarian hormones and that the frequency of HDM-induced IL-17A+ Th17 cells was increased by ovarian hormones.

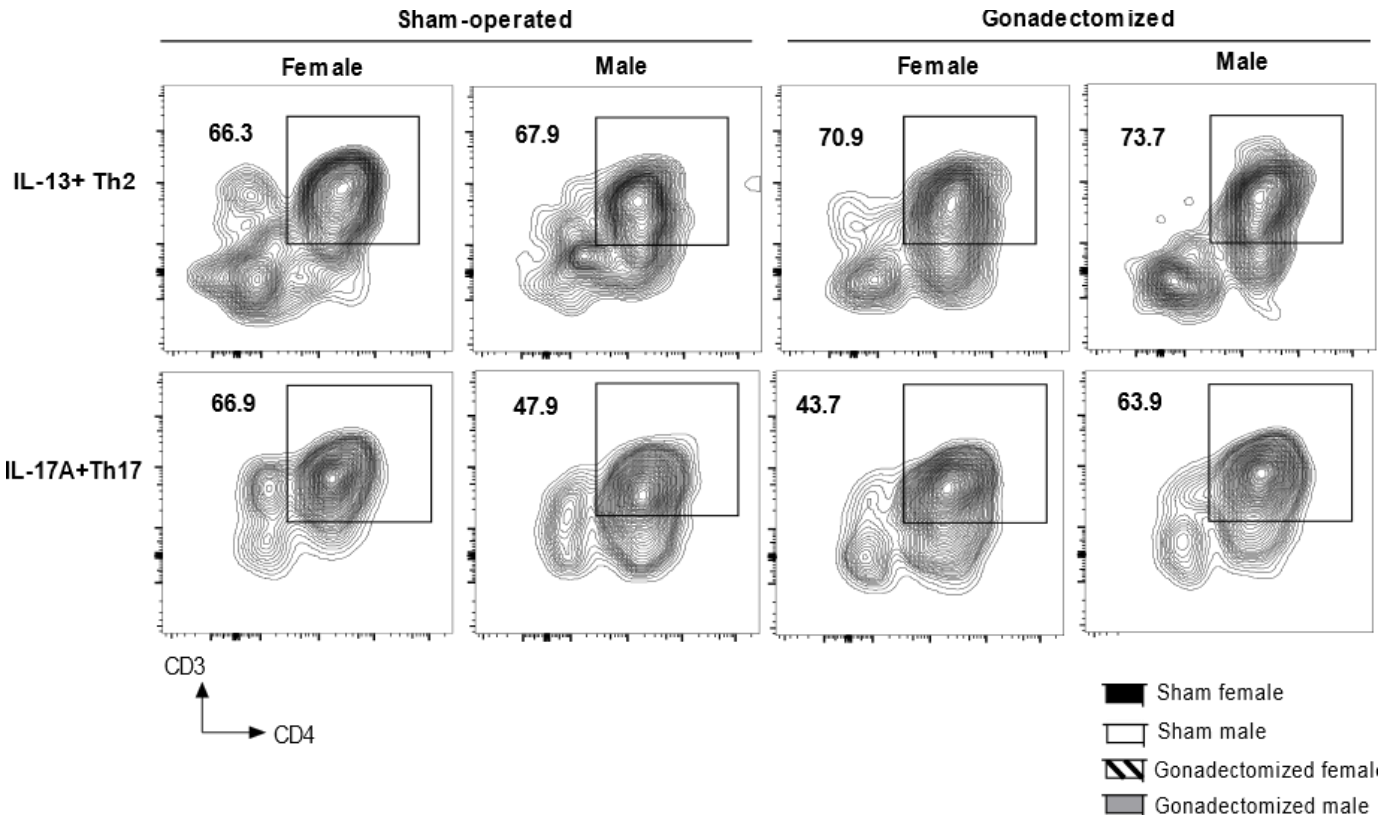

Figure 3-6: Testosterone decreased and Ovarian Hormones increased HDM-induced Total Numbers of IL-13+ Th2 and IL-17A+ Th17 cells - Gating Strategy. WT gonadectomized and sham-operated male and female BALB/c mice were challenged with HDM or PBS. Lungs were harvested 24 hours after the last challenge, and lung cells were restimulated with PMA, ionomycin and Golgi stop. **(A)** Representative dot plots of viable, CD45+ CD3+ CD4+IL-13+ or IL-17A+ cells.

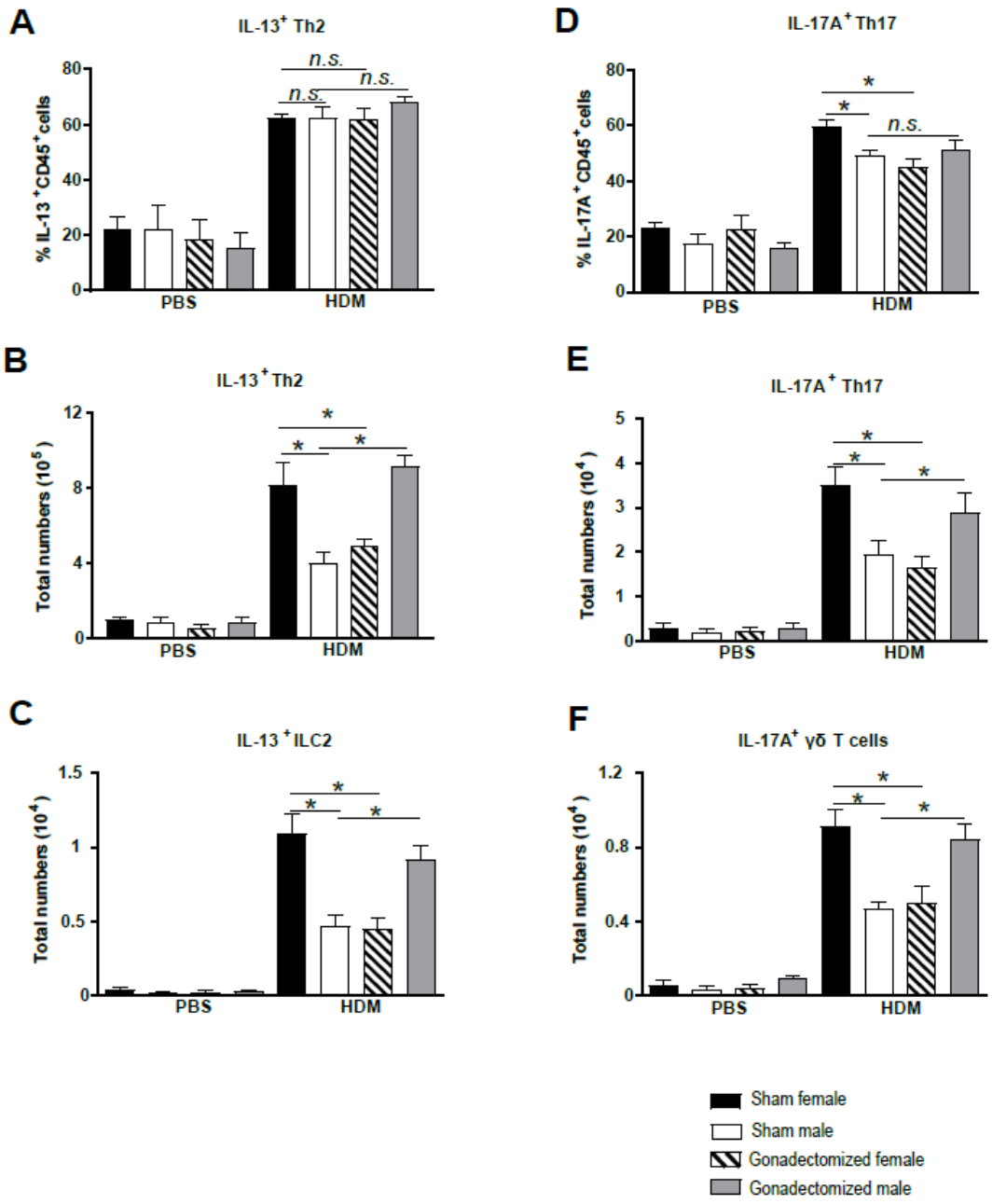

Figure 3-7: Testosterone decreased and Ovarian Hormones increased HDM-induced Total Numbers of IL-13+ Th2 and IL-17A+ Th17 cells. (B-D) Percent and total numbers of IL-13+ Th2 cells (defined as CD45+, CD3+ CD4+ IL-13+ cells) and IL-13+ ILC2 cells (defined as Lin- CD45+ CD25+ CD90+ IL-13+ cells). **(E-G)** Percent and total numbers of IL-17A+ Th17 cells and IL-17A+ γδ T cells (defined as CD45+ CD3+ CD4-γδTCR+) cells. *p<0.05 1-way ANOVA with Tukey post hoc analysis n=6-10 mice/group. Data pooled from two independent experiments. *n.s.* means not statistically significant.

3.4 Discussion

Sex hormones modulate immune responses in many autoimmune and allergic diseases, including asthma (4, 269). Clinical and epidemiological studies showed that boys have a higher prevalence of asthma than girls before puberty. However, after puberty, women become twice as likely as men to have asthma (4, 179). The sex differences in asthma prevalence coincide with changes in sex hormones levels, suggesting that sex hormones are important in regulating asthma pathogenesis. However, the underlying molecular mechanisms by which sex hormones regulate airway inflammation associated with asthma remain unclear.

Dual type 2 and IL-17A–mediated airway inflammation increases eosinophil and neutrophil infiltration into the lung and is associated with more severe phenotypes of asthma (34, 20). Our study determined that testosterone decreased, and ovarian hormones increased HDM-induced total numbers of IL-13+ Th2 cells and IL-17A+ Th17 cells, infiltration of eosinophils and neutrophils into the BAL fluid, total serum IgE, and AHR. Our 3 wk HDM protocol was sufficient to establish increased eosinophils and neutrophils but was not long enough to observe airway remodeling or smooth muscle hypertrophy that is associated with severe asthma [data not shown) (270). Therefore, our HDM challenge mouse model does not reflect all the features of severe asthma in humans.

Increased AHR and mucus production are regulated by IL-13 and IL-17A, and previous studies showed female mice had increased allergen-induced AHR and/or mucus production compared with male mice (14, 17, 225). Additional studies showed that ovarian hormones increased OVA-induced AHR (17, 19) testosterone decreased contractility of

guinea pig tracheal myocytes by negatively regulating IP3 signaling (252), estrogen increased mucus production compared with vehicle in the nasal mucosa of guinea pigs (251), and estrogen increased the production of the mucin proteins Muc5AC and Muc5B in human airway or nasal epithelial cells (253). We expanded upon these findings and showed that testosterone decreased, and ovarian hormones increased HDM-induced lung IL-13 and IL-17A production, AHR, and total serum IgE production in mice. However, to our surprise, no significant differences in mucus production were observed between HDM-challenged sham-operated and gonadectomized female and male mice. We think these discordant findings may be due to the length of our HDM protocol. Since induction of type 2 and IL-17A-mediated inflammatory responses occur prior to induction of mucus cell metaplasia, it is likely that a longer HDM challenge protocol would allow for assessment of the differences in mucus production in gonadectomized female and male mice. Taken together, these findings suggest that sex hormones differentially regulate allergen-induced AHR and mucus production through multiple pathways. CD4+ Th2 and Th17 cells were the primary sources of HDM-induced IL-13 and IL-17A, respectively.

CHAPTER 4

Androgen Receptor Signaling Attenuates CD4 Th2 and Th17 Cell-Mediated Airway Inflammation and Cytokine Expression

4.1 Introduction

Epidemiological and clinical studies have reported that androgens, including testosterone, (DHT), and dehydroepiandrosterone (DHEA) were associated with increased FEV1 and FVC scores and decreased reporting of asthma exacerbations (173, 174, 271). These studies suggest that efforts to understand the underlying mechanisms as to this protective effect of androgens asthma may be helped by understanding the foundational mechanisms by which androgen signaling effects type 2 and non-type 2 mediated airway inflammatory pathways associated with asthma.

Androgens regulate gene expression in target cells via the intracellular, DNA binding androgen receptor (AR) (210, 272, 211). Recent studies show that AR signaling negatively regulates immune cell function. In structural cells, testosterone decreased airway smooth muscle cell and airway fibroblast proliferation in humans (273, 274). Further, in guinea pig tracheal monocytes, testosterone decreased airway smooth muscle contractility potentially by negatively regulating IP_3 signaling (252). In innate cells, our group and others demonstrated that AR signaling decreased IL-5+ and IL-13+ ILC2 numbers in the lungs following allergen-induced ILC2-mediated airway inflammation via ILC2 intrinsic and extrinsic mechanisms (233, 234). AR signaling was reported to regulate alveolar macrophage differentiation, where DHT reconstitution of castrated male mice followed by OVA challenge increased M2 alveolar macrophage total numbers in the lung (244).

AR signaling is known to regulate CD4 T cell development and function and recent studies showed that AR signaling attenuated interferon-γ (IFNγ) production and decreased CD4 Th1 cell differentiation by inhibiting mRNA messenger expression of the phosphate *Ptpn1*, leading to decreased STAT4 phosphorylation (275). Combined, these studies showed AR signaling inhibited innate and adaptive type 2 immune responses by suppressing cytokine expression in airway structural cells, alveolar macrophages, ILC2s and CD4 T cells. However, the effect of androgen receptor signaling on Th2 and Th17 cell-mediated airway inflammation and cell differentiation remained unclear. In this chapter, we investigated the role of androgen receptor signaling on a dual Th2 and Th17 cell-mediated airway inflammatory response. We hypothesized that testosterone signaling, via the androgen receptor, would inhibit dual CD4 Th2 and Th17 cell-mediated airway inflammation and inhibit Th2 and Th17 cytokine production *in vitro*.

4.2 Methods

4.2.1 Mice

WT female, WT male, and AR testicular feminized (AR^{tfm}) male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME), and breeding colonies were established at Vanderbilt University Medical Center. CD90.1/CD90.2 mice were bred in-house using homozygous CD90.1 and CD90.2 C57BL/6J mice from Jackson Laboratory. Gonadectomy or sham surgeries were conducted at 3–4 wk of age by Charles River laboratories veterinary staff, and experiments were started when gonadectomized or sham-operated mice were 6–8 wk old. All animal experiments were conducted in adherence to the rules and regulations of the Association for Assessment and Accreditation of Laboratory Animal Care and

were approved by the International Animal Care and Use Committee at Vanderbilt University Medical Center.

4.2.2 BAL and Analysis of Inflammatory cell Infiltration in the Airway

BAL was performed by instilling 800 μl of saline solution through a tracheostomy tube and then withdrawing the fluid with gentle suction through a syringe, as previously described (18). The total cell count in the BAL fluid was counted using a hemocytometer and 0.04% trypan blue exclusion dye (Sigma-Aldrich). The cells from the BAL were then fixed to a slide and stained using the Three-Step Stain system (Richard-Allan Scientific, Waltham, MA). Two hundred BAL cells were classified as eosinophils, neutrophils, lymphocytes, or macrophages using standard morphologic criteria, and percentages of these inflammatory cells were determined. Total numbers of inflammatory cells were determined by multiplying the percentage of the inflammatory cells by the total numbers of viable cells in the BAL fluid.

4.2.3 Cytokine Measurements

Cytokine levels were measured from BAL fluid, lung homogenates, and/or Th0, Th2, and Th17 cell culture supernatants by ELISA using Quantikine and Duoset kits (R&D Systems). Total serum IgE levels were determined using an ELISA (BioLegend). All experiments were performed according to the manufacturer's instructions. Any OD450 value less than the lower limit of detection was assigned half the value of the lowest detectable standard.

4.2.4 Flow Cytometry of Lung IL-4, IL-13 and IL-17A Production

Lungs were harvested and digested, and lung single-cell suspensions were restimulated with 50 ng/ml of PMA (Sigma-Aldrich), 1 μg/ml of ionomcyin (Sigma-Aldrich), and 0.07% Golgi Stop (BD Biosciences) for 5 h at 37°C in RPMI 1640 plus 10% FBS. Following restimulation, cells were stained with viability dye (Ghost Dye UV 450; Tonbo Biosciences), blocked with an anti-FcR Ab (clone 2.4G2), and surface stained with biotin-labeled anti-CD3 (clone 17A2), PE-Cy5 anti-CD4 (clone 129.19), BV786 anti-CD90.2 (clone 53-2.1), Alexa Fluor 700 anti-CD45 (clone 30-F11), Alexa Fluor 488 anti-CD25 (clone OC61), PE-Cy7 anti-CD127 (clone A7R34), and FITC anti-γδTCR (clone GL3) Abs, followed by allophycocyanin-Cy7 streptavidin staining (1:250). Cells were then fixed, permeabilized using the Foxp3/transcription factor staining kit (Tonbo Biosciences), and intracellularly stained with PE–IL-13 (clone eBio13A), PE-Cy7 anti–IL-17A (clone eBio17B7), and/or PE–IL-4 (clone 11B11). Flow cytometry analysis was conducted on LSR II flow cytometer, and all data were processed using FlowJo software version.

4.2.5 Bone Marrow Chimeras

C57BL/6J heterozygous CD90.1+ CD90.2+ male recipient mice (6–10 wk old) were lethally irradiated (11 Gy) using a cesium irradiator. Three hours after irradiation, a 1:1 mixture of bone marrow (BM) from age-matched 6- to 10-wk-old WT (CD90.1+) and AR^{tfm} male mice (CD90.2+) was transplanted in the male recipient CD90.1+ CD90.2+ mice via retro-orbital injection. Six weeks later, recipient mice were challenged intranasally with HDM or PBS. Twenty-four hours after the last challenge, lungs were harvested, digested, and restimulated as described in the above section. Following restimulation, cells were stained with viability dye

(Ghost Dye UV 450; Tonbo Biosciences), blocked with an anti-FcR Ab (clone 2.4G2), and surface stained with biotin-labeled anti-CD3 (clone 17A2), PE-Cy5 anti-CD4 (clone 129.19), FITC anti-CD90.1 (clone HIS51), BV786 anti-CD90.2 (clone 53-2.1), Alexa Fluor 700 anti-CD45 (clone 30-F11), and Alexa Fluor 488 anti-CD25 (clone OC61) Abs, followed by allophycocyanin-Cy7 streptavidin staining (1:250). Cells were then fixed, permeabilized, and intracellularly stained with PE–IL-13 (clone eBio13A) and PE-Cy7 anti–IL-17A (clone eBio17B7). Flow cytometry analysis was conducted on LSR II flow cytometer, and AR^{tfm} (CD90.2) and WT (CD90.1) cytokine-producing Th2 and Th17 cells were gated as CD3+CD4+ IL-13+ or CD3+ CD4+ IL-17A+ cells. All data were processed using FlowJo software version 10. Residual cells remaining from the heterozygous recipient mice (CD90.1+/CD90.2+) were excluded from the analysis.

4.2.6 CD4 Th2 and Th17 Cell Differentiation

Naive CD4 T cells were isolated from the spleens of WT C57BL/6J female, male, and AR^{tfm} male mice using a commercially available naive CD4+ T cell isolation kit (Miltenyi Biotec). Following isolation, naive CD4+ T cells were activated with anti-CD3 (1 μg/ml; BD Biosciences) and anti-CD28 (0.5 μg/ml; BD Biosciences) and differentiated into Th2 cells by adding recombinant mouse (rm) IL-4 (10 ng/ml) and anti–IFNγ (10 μg/ml). Naive CD4+ T cells were differentiated into Th17 cells by adding rmIL-23 (10 ng/ml), recombinant human TGF-β (1 ng/ml), rmIL-6 (20 ng/ml), anti–IFNγ (10 μg/ml) and anti–IL-4 (10 μg/ml). Recombinant IL-23 protein, anti–IFNγ, and anti–IL-4 Abs were purchased from R&D Systems. All other recombinant proteins were purchased from PeproTech (Rocky Hill, NJ).

4.2.7 RNA Isolation and Quantitative Real Time PCR Analysis

Total RNA was isolated using a RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was generated by using 100 ng of total RNA, and TaqMan quantitative PCR analysis of *AR*, *gata3*, *rorc*, *il4Rα*, *il23r*, and *gapdh* mRNA expression was conducted using commercially available primers and FAM/MGB probes (Applied Biosystems). Data were reported as relative expression normalized to the housekeeping gene *Gapdh*.

4.2.8 Statistical Analysis

Unless otherwise indicated, data are represented as mean ± SEM where groups were compared by one-way ANOVA with Tukey post hoc analysis. For all analysis, $p < 0.05$ was considered significant.

4.3 Results

4.3.1 AR Signaling attenuates HDM-induced Eosinophilic and Neutrophilic Airway Inflammation

Previous studies showed that testosterone and AR signaling negatively regulated allergen-induced ILC2-mediated airway inflammation (233, 234). Our in vivo results from the HDM-challenged gonadectomized and sham-operated male and female mice showed that total numbers of IL-13+ Th2 and IL-17A+ Th17 cells, IL-13+ ILC2, and IL-17A+ γδ T are decreased by testosterone and increased by ovarian hormones. Therefore, we hypothesized that AR signaling attenuates cell numbers of HDM-induced IL-13+ Th2 cells and IL-17A+ Th17 cells. To test this hypothesis, we intranasally challenged WT female, WT male, and AR^{tfm} C57BL/6J with HDM or PBS as previously described in Figure 3-1A. AR^{tfm} male mice have a mutation in

the AR mRNA transcript, and AR^{tfm} male mice are unresponsive to androgens, including testosterone and testosterone derivatives (276). WT female and AR^{tfm} male mice had increased airway eosinophils and neutrophils compared with WT male mice (Figure 4-1A-B). Lung cells were restimulated with PMA, ionomycin, and Golgi stop and stained for IL-13– and IL-17A–producing Th2 and Th17 cells, respectively. Similar to Fig. 4, the percentage of IL-13+ Th2 cells and IL-17A+ Th17 were not different between HDM-treated groups, but IL-13+ Th2 and IL-17A+ Th17 cells were increased in WT female and AR^{tfm} male mice compared with WT male mice (Figure 4-1C–E). These results suggested that AR signaling attenuating type 2 and IL-17A–mediated airway inflammation was independent of the mouse genetic background.

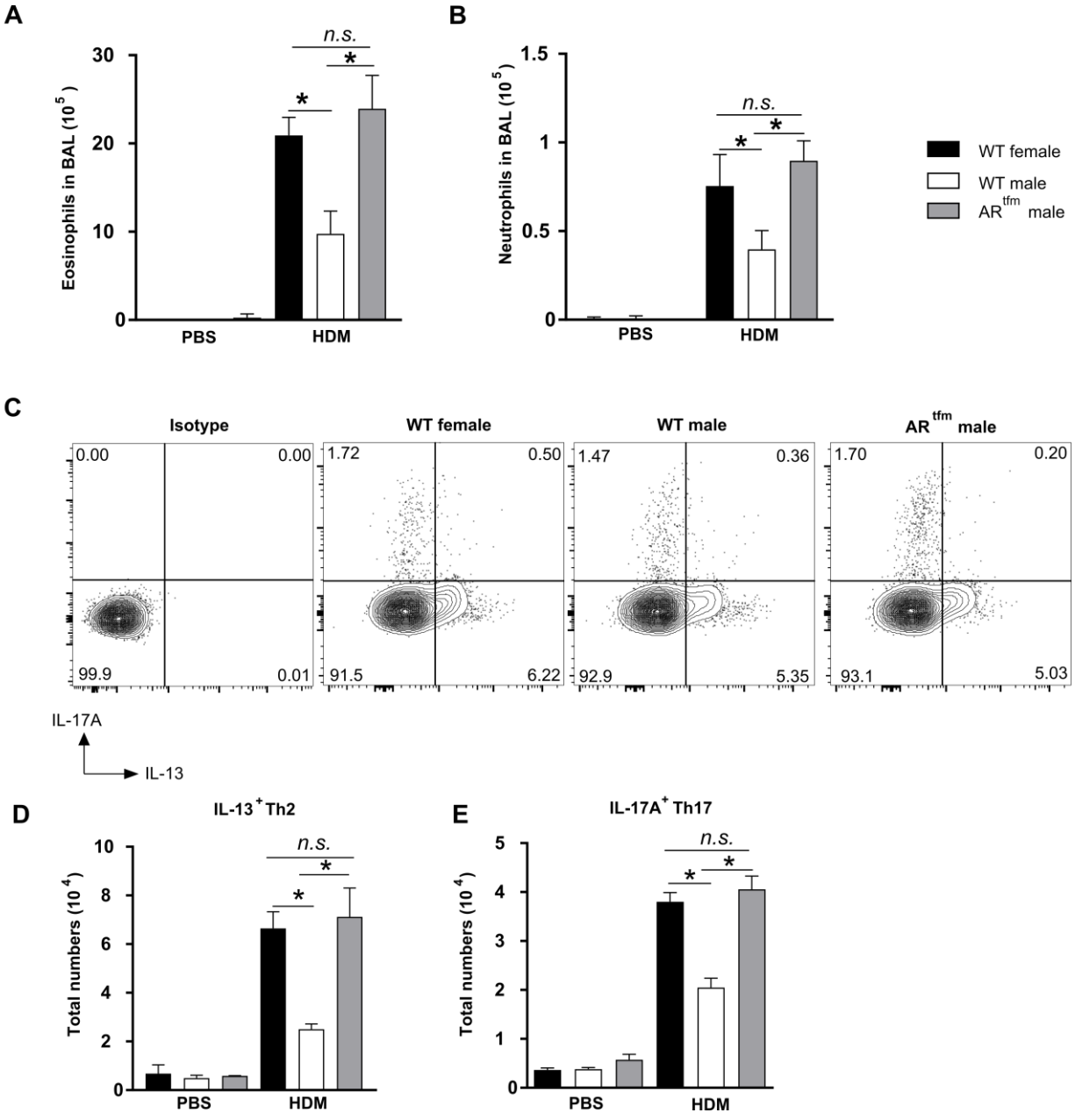

Figure 4-1: AR Signaling attenuated HDM-induced Eosinophilic and Neutrophilic Airway Inflammation. WT female, WT male, and androgen receptor testicular feminized mice (AR^{tfm}) male C57BL/6 mice were challenged with HDM or PBS. **(A-B)** BAL fluid eosinophils and neutrophils. **(C)** Representative flow staining of IL-13 and IL-17A expressing T cells. **(D-E)** Total numbers of IL-13+ Th2 cells and IL-17A+ Th17 cells. * $p<0.05$, ANOVA with Tukey post hoc test. n=3-5 mice/group. Data shown is from one representative experiment of three independent experiments. *n.s.* means not statistically significantly.

4.3.2 AR Signaling intrinsically decreased HDM-induced Total Numbers of IL-17A$^+$ Th17 cells and indirectly decreased Total Numbers of IL-13$^+$ Th2 cells

To determine whether AR signaling decreased IL-13+ Th2 and IL-17A+ Th17 cells in the lung intrinsically, we used a mixed BM chimera model. Lethally irradiated WT CD90.1+/CD90.2+ male mice were reconstituted with a 1:1 mixture of BM cells from CD90.1+ WT male and CD90.2+ AR^{tfm} male mice. Six weeks after reconstitution, the recipient mice were challenged with HDM intranasally four times a week for 3 weeks. Twenty-four hours after the last challenge, we harvested the lungs and measured the total numbers of IL-13– and IL-17A–producing CD3+ CD4+ T cells from WT CD90.1+ or AR^{tfm} CD90.2+ cells (Figure 4-2A). Based on previous findings in ILC2-mediated airway inflammation (233, 234), we hypothesized that AR signaling directly attenuates IL-13– and IL-17A–producing Th2 and Th17 cells, respectively. Surprisingly, we found that the CD90.2+ IL-13+ Th2 cells from AR^{tfm} mice were decreased compared with CD90.1+ IL-13+ Th2 cells from the WT mice (Figure 4-2B-C). These findings were different from our results in Figures 3-7 and 4-1 in which testosterone and AR signaling attenuated HDM-induced IL-13+ Th2 cells in the lung. However, it was possible that AR signaling was attenuating IL-13+ Th2 cells via an indirect, extrinsic mechanism. In contrast, CD90.2+ IL-17A+ Th17 cells from AR^{tfm} mice were increased compared with CD90.1+ IL-17A+ Th17 cells from WT mice (Figure 4-2D), suggesting that AR signaling decreased cell numbers of IL-17A+ Th17 cells present in the lung via a Th17-intrinisic mechanism.

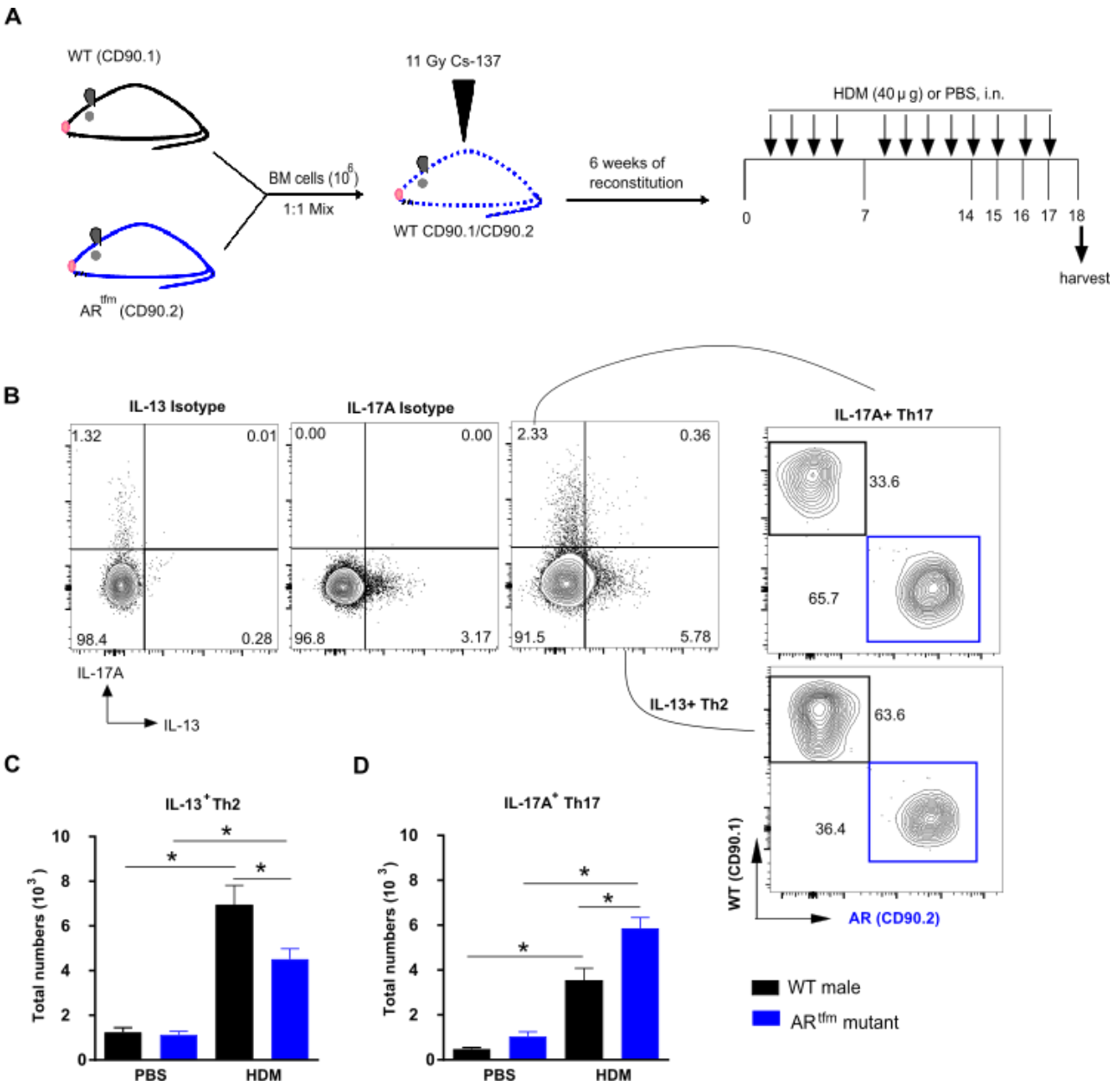

Figure 4-2: AR Signaling intrinsically decreased IL-17A+ Th17 cells and indirectly decreased IL-13+ Th2 cells. (A) Experimental design of mixed bone marrow chimera experiments with 1:1 bone marrow mixture from WT (CD90.1) or AR^{tfm} (CD90.2) male C57BL/6J mice transferred into lethally irradiated heterozygous CD90.1+CD90.2+ recipient C57BL/6J mice. After 6 weeks of reconstitution, recipient mice underwent HDM or PBS challenge and IL-17A+ and IL-13+ CD4+ T cells were determined for WT and AR^{tfm} mice. **(B)** Representative dot plots showing gating strategy. **(C-D)** Total numbers of IL-13+ Th2 and IL-17A+ Th17 cells derived from WT CD90.1 or AR^{tfm} CD90.2 lineages.* $p<0.05$ ANOVA, with Tukey post hoc analysis, n=5-10 mice/group. Data pooled from 2 combined experiments.

4.3.3 AR Signaling attenuated Th17 but not Th2 Cytokine Expression *in vitro*

Our BM chimera experiment determined that AR signaling was differentially regulating cytokine production from Th2 and Th17 cells. Therefore, to determine how AR signaling regulated Th2 and Th17 differentiation, we conducted in vitro Th2 and Th17 cell differentiation experiments. Naive CD4+ T cells isolated from the spleens of WT female, WT male, and AR^{tfm} male C57BL/6J mice were activated with anti-CD3 and anti-CD28 and differentiated into Th2 or Th17 cells for 4 days as previously described (18). After 4 days, IL-13 and IL-17A protein expression was measured in the cell culture supernatants. We also measured mRNA relative expression of *Ar*, as well as transcription factors and receptors known to regulate Th2 and or Th17 differentiation, including *Gata3*, *Il4rα*, *Rorc*, and *Il23r*, by quantitative RT-PCR analysis. *Ar* mRNA relative expression was significantly decreased in differentiated Th2 andTh17 cells from AR^{tfm} male mice compared with WT female and male mice (Figure 4-3A, 4-3E). Furthermore, *Ar* relative mRNA expression in differentiated Th17 cells was significantly decreased in WT female mice compared with male mice (Figure 4-3E), but no difference in *Ar* mRNA expression was detected in Th2 differentiated cells between male and female mice (Figure 4-3A).

No significant differences in *Gata3 or Il4rα* mRNA relative expression, or IL-13 protein expression were found between Th2 cells from our experimental groups (Figure 4-3B-D). *Il23r* mRNA relative expression and IL-17A protein expression were increased in Th17 cells from WT female mice and AR^{tfm} male mice compared with WT male mice, but no differences in *Rorc* expression were determined in Th17 cells from our experimental groups (Figure 4-3F-H). Combined, AR signaling attenuated IL-17A and *Il23r* expression in Th17 cells. However, AR signaling did not regulate IL-13 levels and/or transcription factors associated with Th2 cells.

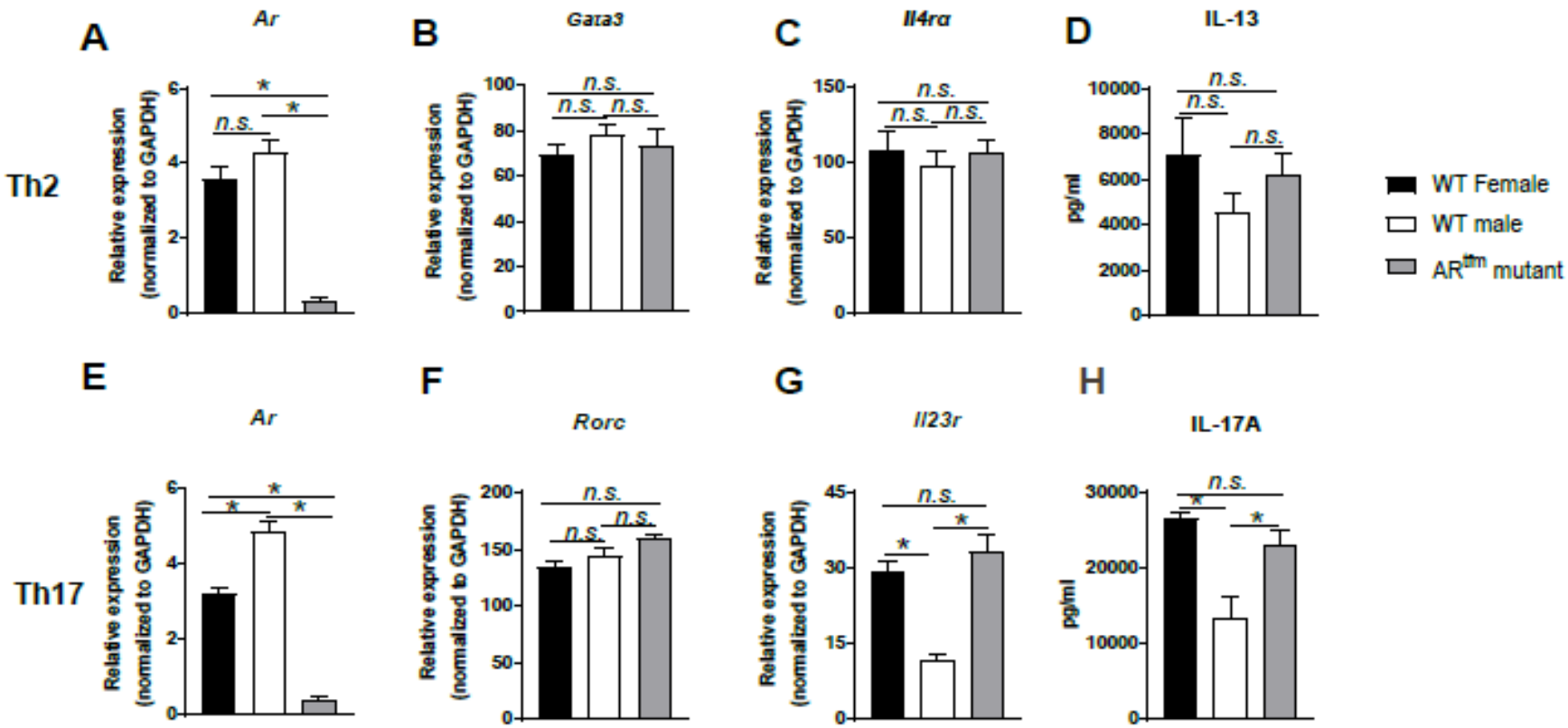

Figure 4-3: AR Signaling attenuated Th17 but not Th2 Cytokine Expression *in vitro*. Naïve CD4+ T cells from WT female, WT male, and AR^{tfm} male mice were activated and differentiated into Th2 or Th17 cells. Cell culture supernatants and RNA were collected 4 days after differentiation. **(A)** and **(E)** *AR* mRNA expression in Th2 cells (top) and Th17 cells (bottom). **(B-D)** *Gata3 and Il4rα* mRNA relative expression from in differentiated Th2 cells and IL-13 protein expression from Th2 cell culture supernatants. **(F-H)** *Rorc and Il23r* mRNA relative expression in differentiated Th17 cells and IL-17A protein expression in Th17 cell culture supernatants. IL-13 and IL-17A protein expression were measured by ELISA and mRNA relative expression measured by qRT-PCR with relative expression in Th2 or Th17 cells were normalized to *Gapdh*. * $p<0.05$, ANOVA with Tukey post hoc analysis. n=9-12 mice/group. Data pooled from 2 independent experiments. *n. s.* means not statistically significant.

4.3.4 AR Signaling decreased HDM-induced Total Numbers of IL-4 Producing cells

Our results from the HDM studies in AR^{tfm} mice, the mixed BM chimeras, and in vitro Th2 differentiation experiments suggested that AR signaling decreased Th2 differentiation and cytokine production in the lung by attenuating cytokines and/or chemokines critical for Th2 cells. Therefore, we measured IL-4, IL-10, IL-25, CCL11, CCL24, CCL4, and TARC, cytokines and chemokines that are known to regulate Th2 differentiation and migration, 24 h after the last challenge in lung homogenates of WT female, WT male, and AR^{tfm} male C57BL/6J mice (Figure 4-4A-G). Only IL-4 protein expression was increased in the lung homogenates of WT female and AR^{tfm} mice compared with WT male mice Figure 4-4A. IL-4 production is important for Th2 differentiation, and decreased levels of IL-4 in the cytokine milieu of the lung would decrease Th2 differentiation and cytokine production (96, 277). To determine the role of AR signaling on IL-4 production in the lung, we harvested the lungs of WT female, WT male, or AR^{tfm} male mice and measured IL-4 production by flow cytometric analysis. IL-4+ Th2 cells, mast cells, and basophils were determined based on the gating strategy described in Figure 4-5 and Figure 4-6. IL-4+ Th2 cells, mast cells, and basophils were significantly increased in the lungs of WT female and AR^{tfm} mice compared with WT male (Figure 4-6B-F). No IL-4+ ILC2 were detected in PBS- or HDM-challenged WT or AR^{tfm} mice (data not shown). These findings show that AR signaling decreased HDM-induced IL-4 production in vivo and provided a mechanism by which AR signaling decreased Th2 cell–mediated inflammation.

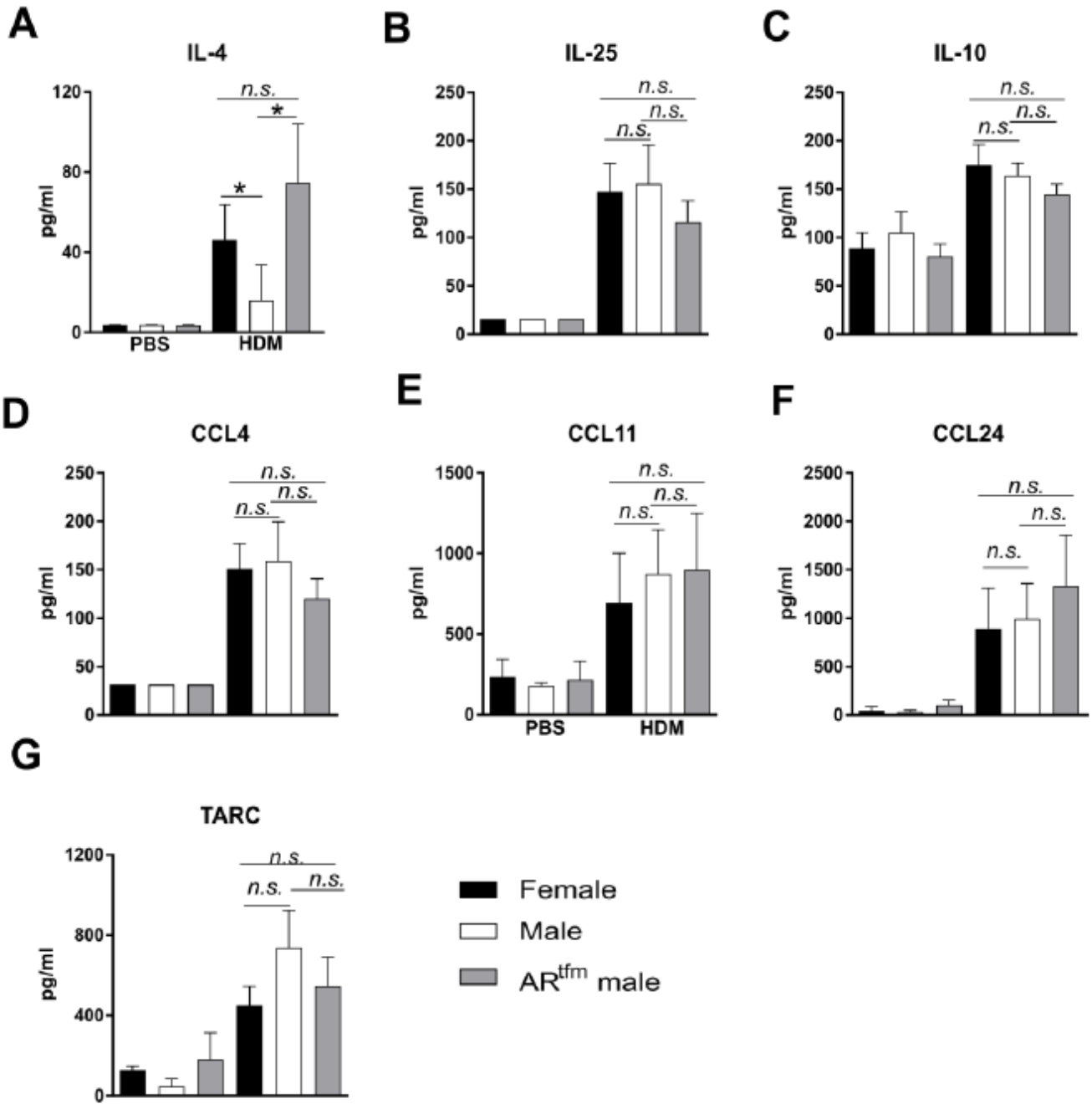

Figure 4-4: (A-G) HDM-induced Cytokines and Chemokines in Whole Lung Homogenates. WT female, WT male and AR^{tfm} male mice were challenged with HDM or PBS. The left lung was harvested, homogenized and cytokine or chemokine expression was measured by ELISA. n=3-9 mice per group. Data pooled from 2 combined experiments. *n.s.* means not statistically significant.

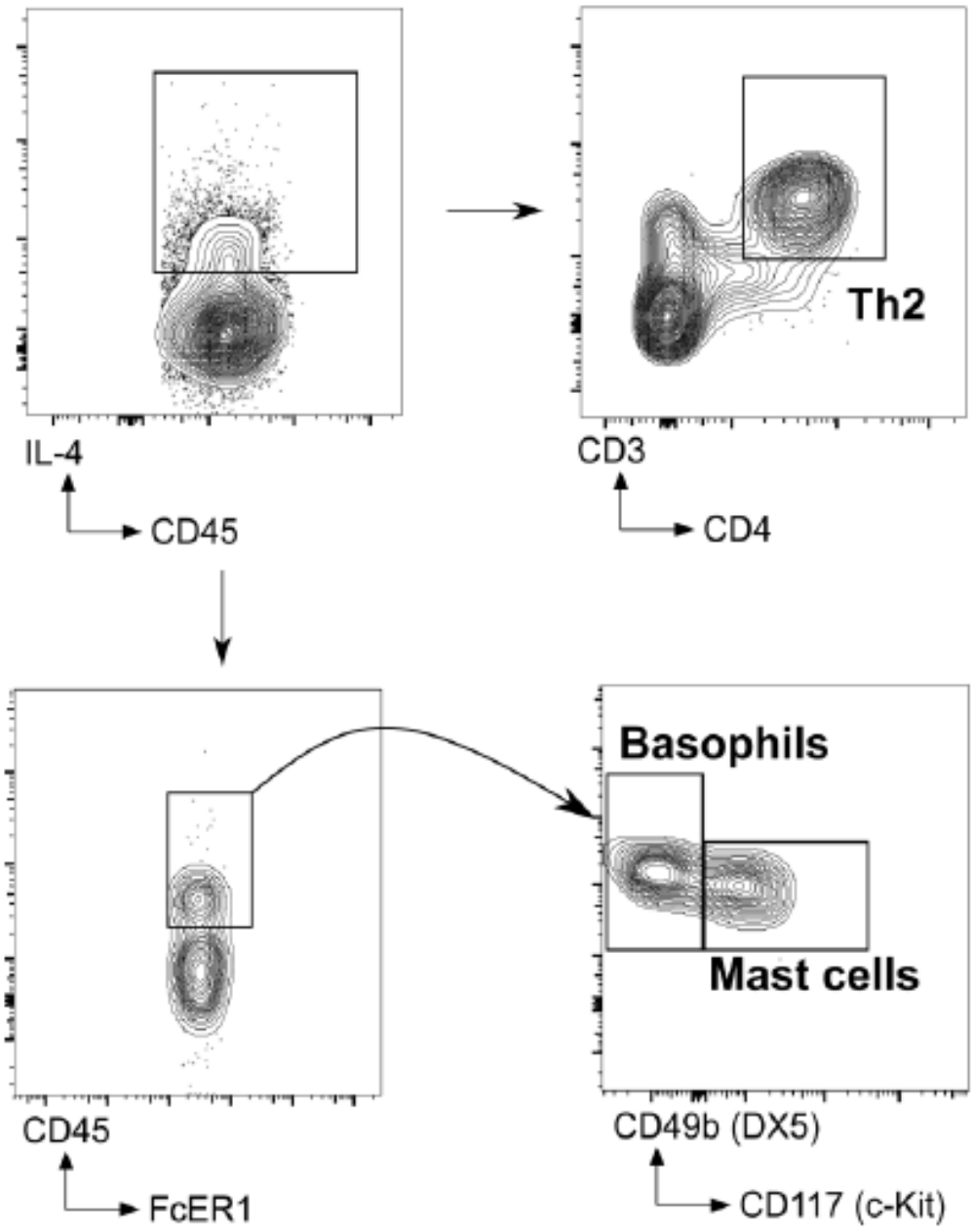

Figure 4-5: Gating Strategies for IL-4 Producing Th2, Mast cells and Basophils. Data are representative of the gating strategies used for data in Figure 4-6. Samples were pre-gated with viable singlets with low to intermediate FSC-A and SSC-A properties associated with leukocytes and lymphocyte p

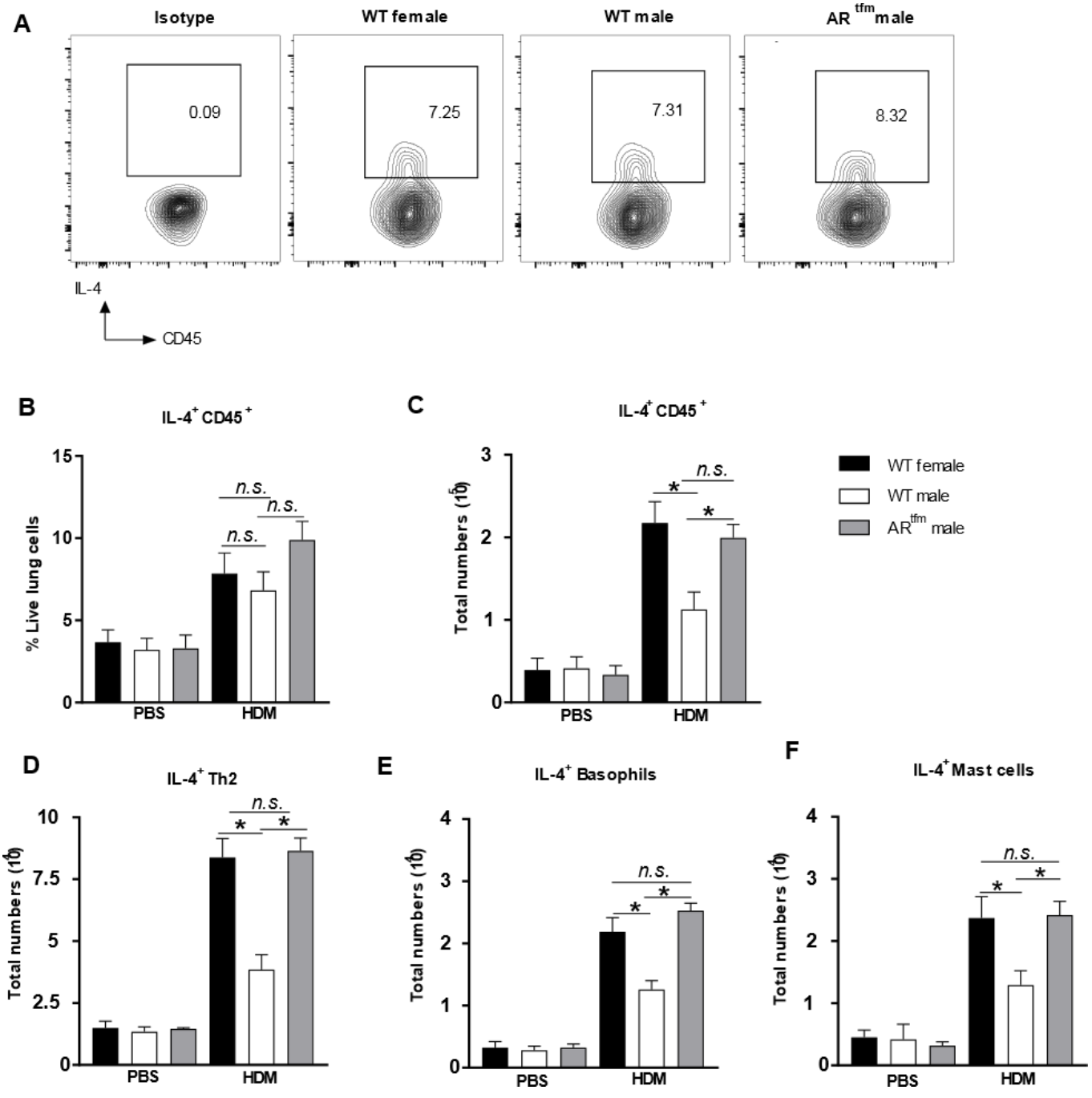

Figure 4-6: AR Signaling decreased Total Numbers of IL-4 Producing Cells. WT female, WT male and AR^{tfm} male mice were challenged with HDM or PBS. **(A)** Representative dot plots of IL-4 producing CD45+ cells. **(B-C)** Percentage and total numbers of viable IL-4+ CD45+ cells. **(D-F)** Number of IL-4 producing Th2 cells, basophils and mast cells. Th2 cells were defined as CD45+ CD3+ CD4+ cells, mast cells were defined as CD45+ FcεRI+ CD117+ DX5- cells, and basophils were defined as CD45+ FcεRI+ CD117- DX5+ cells. * $p<0.05$, ANOVA with Tukey post hoc test. n=3-9 mice/group with data pooled from 2 independent experiments. *n.s.* means not statistically significant.

4.4 Discussion

AR signaling decreased IL-17A+ Th17 cells in the lung via a Th17 cell intrinsic mechanisms and decreased IL-17A protein expression and *Il23r* mRNA expression in differentiated Th17 cells. Other studies have also reported a critical role for IL-23R signaling in Th17 cytokine production, in which inhibition of IL-23R expression decreases optimal IL-17A production (264, 136, 18, 278). Surprisingly, AR signaling indirectly attenuated Th2 cells in the lung by suppressing lung IL-4 production. IL-4 signals through the IL-4R and is important for Th2 cell differentiation by increasing STAT6 activation and Gata3 expression (101). Our *in vitro* Th2 differentiation studies showed that Th2 cells from WT male and AR^{tfm} male mice had similar levels of IL-13 production, as well as *Gata3* and *Il4ra* mRNA relative expression. Although Th2 cells produce IL-4, our Th2 culture conditions included a large concentration of rmIL-4 (10 ng/ml). These data provide additional evidence to suggest that AR signaling attenuation of IL-4 cytokine expression in the lung limited the numbers of Th2 cells that differentiate or proliferate, leading to decreased type 2–mediated allergic inflammation in males compared with females.

Recent studies from our group and others reported that AR signaling intrinsically decreased ILC2-mediated allergic inflammation cell proliferation and cytokine production (233, 234). These findings differ from our study in that we determined that AR signaling negatively decreased Th2 cell–mediated inflammation by a Th2 cell indirect extrinsic mechanism. Previous reports have also shown that AR signaling extrinsically regulates T cell development and T cell function in the periphery (279). BM chimera models in which AR expression was attenuated in thymocytes or thymic epithelial cells revealed that

suppression of AR expression on thymic epithelial cells inhibited the ability of the thymus to respond to androgens via indirect mechanisms (280-282). However, addition of DHT, a derivative of testosterone, to splenic, activated CD4+ T cells decreased IL-4, IL-6, IL-12, and IFNγ levels (283). Therefore, AR signaling may intrinsically or extrinsically regulate immune responses depending on the cell type and/or tissue.

Understanding the foundational mechanisms underlying airway inflammation associated with type 2 and IL-17A inflammatory pathways is imperative for the development of novel asthma therapeutics that are not only effective but personalized specifically for women and men with asthma. Our studies showed that testosterone attenuated total numbers of IL-13– and IL-17A–producing CD4 T cells and suggest that increasing AR signaling may be important for decreasing airway inflammation associated with asthma. Recently, a phase IIa clinical trial has demonstrated that nebulized dehydroepiandrosterone-3-sulfate (DHEAS), a non-virilizing hormone derivative of testosterone that does not have unwanted side effects on females, improved asthma control in patients with moderate to severe asthma (271). These findings suggest a potential role for testosterone derivatives, including nebulized DHEA, in attenuating asthma symptoms and warrant additional investigation.

CHAPTER 5

Estrogen Signaling through ERα increased IL-23R Surface Expression and IL-17A Production in Th17 cells

5.1 Introduction

Gender differences exist in baseline immune responses between male and females and studies demonstrate that in asthma and autoimmune diseases such as multiple sclerosis and lupus, females have increased CD4 Th17-mediated inflammatory responses compared to males (284, 166, 258, 49). Estrogen signaling is well known for regulating reproductive processes, but studies further demonstrate that estrogen signaling also regulates immune cell function through estrogen receptor α (ERα) and estrogen receptor β (ERβ) signaling by directly binding DNA to regulate gene expression in target cells (285, 269). CD4 T cells express both ERα and ERβ and studies demonstrate that estrogen signaling upregulates IFNγ production in Th1 cells by directly binding to estrogen response elements (EREs) in the promoter region of the *Ifnγ* gene (286). Additionally estrogen signaling in Th1 cells was shown to upregulate expression of the Th1 transcription factor T-bet (286). In inducible Tregs (iTregs), estrogen (E2) treatment of naïve CD4 T during *in vitro* iTreg differentiation increased the frequency of Foxp3+ iTregs in E2 treated cells compared to placebo treated cells (287).

The effects of estrogen signaling on Th17 cells has been reported, but with discordant findings. For instance, in an T cell adoptive transfer colitis model, ERα deficiency within effector T cells inhibited T cell accumulation as well as inhibited both Th17 and Th1 mediated inflammation in the mesenteric lymphoid nodes of recipient mice (287). In contrast, E2-induced signaling specifically via ERα provided a protective effect against experimental autoimmune encephalomyelitis (EAE), and ERα deletion in IL-17A+ CD4+ T cells abrogates this E2 induced

protection against EAE (288). Additionally, treatment of WT mice with a ERβ-specific ligand called PHTPP after onset of EAE symptoms decreased Th17 and Th1 mediated spinal cord inflammation (289). Combined, these studies show that ERβ has a immunosuppressive effect on Th17 cell function, but E2 signaling via the ERα in Th17 cells can be both immunostimulatory or immunosuppressive depending on the on the immunological and disease context.

Women have an increased prevalence of asthma, particularity severe asthma, compared to men (258). Our group have previously published that the frequency and total numbers of IL-17A+ Th17 cells were increased in the peripheral blood of women, compared to men, with severe asthma (18) . Furthermore, IL-17A protein expression was increased in Th17 cells from ovariectomized female mice implanted with 17β-estradiol (17β-E2) and progesterone (P4) hormone pellets compared to Th17 cells from ovariectomized female mice implanted with vehicle pellets. Our group determined that estrogen and progesterone inhibited *Let-7f* microRNA expression, leading to increased IL-23R expression and increased IL-17A protein expression in Th17 cells (18). These findings demonstrated a role for estrogen and progesterone in regulating IL-23R mRNA expression to increase IL-17A protein expression in Th17 cells, but it was unclear whether ERα or ERβ signaling was responsible for upregulating IL-23R expression in Th17 cell differentiation. In this chapter, we hypothesized that E2 signaling through ERα increased IL-23R expression and IL-17A protein expression in Th17 cells.

5.2 Methods

5.2.1 Mice

WT female, WT male, estrogen receptor α (ERα) and β (ERβ) female knockout C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME), and breeding colonies were established at Vanderbilt University Medical Center. Gonadectomy and sham surgeries were conducted at 3-4 weeks of age at Jackson Laboratory veterinary staff, and, experiments were started when gonadectomized or sham-operated mice 6-8 weeks old. All animal experiments were conducted in adherence to the rules and regulations of the Association for Assessment and Accreditation of Laboratory Animal Care and were approved by the International Animal Care and Use Committee at Vanderbilt University Medical Center.

5.2.2 Flow Cytometry Sorting of Naïve CD4 T Cells

Naïve CD4 T cells were isolated from the spleens of WT female, male, as well as knockout ERα and/or β ERβ female mice using a commercially available kit. Naïve CD4 T cells isolation kit (Miltenyi Biotec). Following isolation, naïve CD4 T cells were FACS sorted for naïve $CD4^{+}CD62L^{+}$ T cells (> 96% purity).

5.2.3 CD4 Th1, Th2 and Th17 Cell Differentiation

After sorted, naïve CD4 T cells were activated with anti-CD3 (1 μg/ml; BD Biosciences) and anti-CD28 (0.5 μg/ml; BD Biosciences) and differentiated into Th1 cells by adding recombinant mouse (rm) IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml) or into Th2 cells by adding recombinant mouse (rm) IL-4 (10 ng/ml) and anti–IFNγ (10 μg/ml). Sorted naive CD4+ T cells were differentiated into Th17 cells by adding rmIL-23 (10 ng/ml), recombinant

human TGF-β (1 ng/ml), rmIL-6 (20 ng/ml), anti–IFNγ (10 μg/ml) and anti–IL-4 (10 μg/ml). Recombinant IL-23 protein, anti–IFNγ, and anti–IL-4 Abs were purchased from R&D Systems. All other recombinant proteins were purchased from PeproTech. In some experiments Th17 cells were treated with increasing doses of rmIL-23 (0, 10, 20, 30 ng/ml).

5.2.4 Cytokine Measurements

Cytokine levels were measured from Th0, Th1, Th2, and Th17 cell culture supernatants by ELISA using Duoset kits (R&D Systems). All experiments were performed according to the manufacturer's instructions. Any OD450 value less than the lower limit of detection was assigned half the value of the lowest detectable standard.

5.2.5 Flow Cytometric Analysis of IL-17A Production in Th17 cells

On day three of Th17 cell differentiation, 0.07% of Golgi Stop (BD Biosciences) was added to the cell culture medium for 4 hours. After 4 hours, cells were stained with viability dye (Ghost Dye UV 450; Tonbo Biosciences), blocked with an anti-FcR Ab (clone 2.4G2), and surface stained with APC-Cy7 anti-CD3 (clone 145-2C11), FITC anti-CD4 (clone GK1.5), BV786 anti-CD90.2 (clone 53-2.1) and APC anti-IL-23R (clone 12B2B64). Cells were then fixed, permeabilized using the Foxp3/transcription factor staining kit (Tonbo Biosciences), and intracellularly stained PE-Cy7 anti–IL-17A (clone eBio17B7). Flow cytometry analysis was conducted on LSR II flow cytometer, and all data were processed using FlowJo software version 10.

5.2.6 RNA Isolation and Real Time Quantitative PCR Analysis

Total RNA was isolated using a RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was generated by using 100 ng of total RNA, and TaqMan quantitative PCR analysis of *Rorc*, *Il23r*, and *Gapdh* mRNA expression was conducted using commercially available primers and FAM/MGB probes (Applied Biosystems). Data were reported as relative expression normalized to the housekeeping gene Gapdh. For the detection and quantification of *Let-7f* miRNA, qRT-PCR was conducted with SYBR green mix and a provided universal qPCR reverse primer (200 nM). Forward primer sequences were used as follows: *Let-7f* forward primer, TGAGGTAGTAGATTGTATAGTT; *U6b* forward primer, TGACACGCAAATTCGTGAAG (Integrated DNA Technologies). Data was reported as relative expression normalized to the housekeeping gene, *U6b*.

5.2.8 Statistical Analysis

Unless otherwise indicated, data are represented as mean ± SEM where groups were compared by one-way ANOVA with Tukey post hoc analysis. For all analysis, $p < 0.05$ was considered significant.

5.3 Results

5.3.1 ERα Signaling increases IL-17A Protein Expression in Th17 Cells

Our previous studies showed that estrogen and progesterone were important in increasing Th17 cell differentiation and IL-17A production (18). However, it remained unknown if estrogen signaling through ERα and/or ERβ was important for driving Th17 cell differentiation and IL-17A expression *in vitro*. Naïve CD4 T cells were sorted from WT female, WT male, ERα knockout (KO) female, and ERβ KO female C57BL/6J mice were differentiated into Th1, Th2 or Th17 cells for 3 days as previously described (18). IL-17A protein expression in Th17 cells was significantly decreased in WT male mice and ERα KO female mice compared to WT female and ERβ female mice (Figure 5-1A). IFNγ protein expression from Th1 cells was also significantly decreased in WT male and ERα KO female mice compared to WT female and ERβ KO female mice (Figure 5-1B). However, no change in IL-13 protein expression was found in Th2 cells from group (Figure 5-1C). Th17 cells are plastic cells and may shift towards other Th1 or Th2 subsets that produce IFNγ and IL-13, respectively. Therefore, we measured IFNγ and IL-13 in Th17 cells and found values were below the limit of detection for all groups. (Figure 5-1D-E). These findings suggest that ERα, but not ERβ signaling increases IL-17A production in Th17 cells and IFNγ production in Th1 cells. Additionally, our results show that neither ERα nor ERβ effects IL-13 protein expression in Th2 cells.

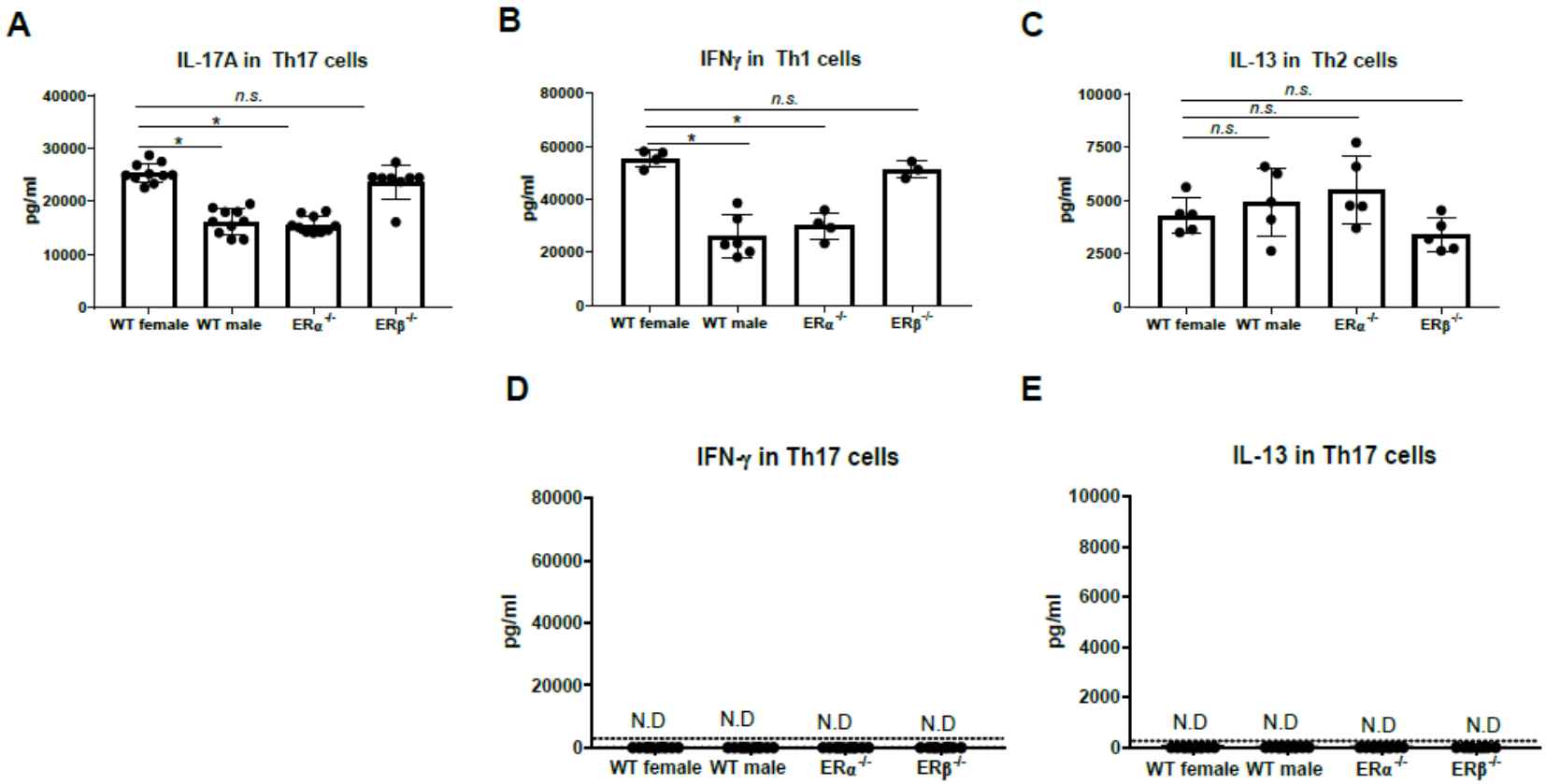

Figure 5-1: ERα Signaling increases IL-17A Protein Expression in Differentiated Th17 cells. FACS sorted naïve CD4+ CD62L+ T cells from WT female and male and ERα and ERβ KO female mice were activated and differentiated into Th17, Th1 and Th2 cells for 3 days. Cell culture supernatants were collected 3 days after differentiation. **(A)** IL-17A, **(B)** IFNγ and **(C)** IL-13 protein expression in cell culture supernatants from differentiated Th17, Th1 and Th2 cells, respectively. (D-E) IFNγ and IL-13 protein expression in Th17 cells. N.D. means not detectable and dotted line shows the limit of detection. * $p<0.05$, ANOVA with Tukey post hoc analysis. n= 8-10 mice/group. Data from Figure 5-1A, D and E pooled from 3 independent experiments. Data Figure 5-1B-C from B and C represented as one representative experiment from three independent experiments. *n.s.* means not statistically significant.

5.3.2 ERα Signaling increases the Frequency and Total numbers of IL-17A Producing CD4 T Cells

Figure 5-1A showed that ERα signaling increased IL-17A protein expression in Th17 cells. Therefore, we next wanted to determine whether ERα signaling was increasing the amount of IL-17A produced from each cell, increasing numbers of Th17 cells, or both. On day 3 of Th17 cell differentiation, golgi-stop was added to the cell culture medium for 4 hours followed by flow cytometry analysis of IL-17A producing Th17 cells, labelled as viable CD3+ CD4+ IL-17A+ cells. Despite seeding the same number of cells on Day 0, the percentage and total number of CD3+CD4+ IL-17A+ Th17 cells were significantly decreased in WT male and ERα KO female mice compared to WT female mice (Figure 5-2A-C). We also determined the amount of IL-17A produced from each cell by measuring mean fluorescent intensity (MFI), but found no difference in the MFI of IL-17A staining in Th17 cells from between groups (data not shown). ER-α deletion in CD4 T cells has been shown to inhibit cell proliferation and promote gene expression of apoptotic genes such as Bcl2 following TCR-stimulation (287), thus the decreased numbers of IL-17A+ CD4+ T cells in ERα KO female mice may have been due to decreased cell proliferation and cell viability. Combined, these data showed that ERα increases both the amount and total numbers of IL-17A+ Th17 cells.

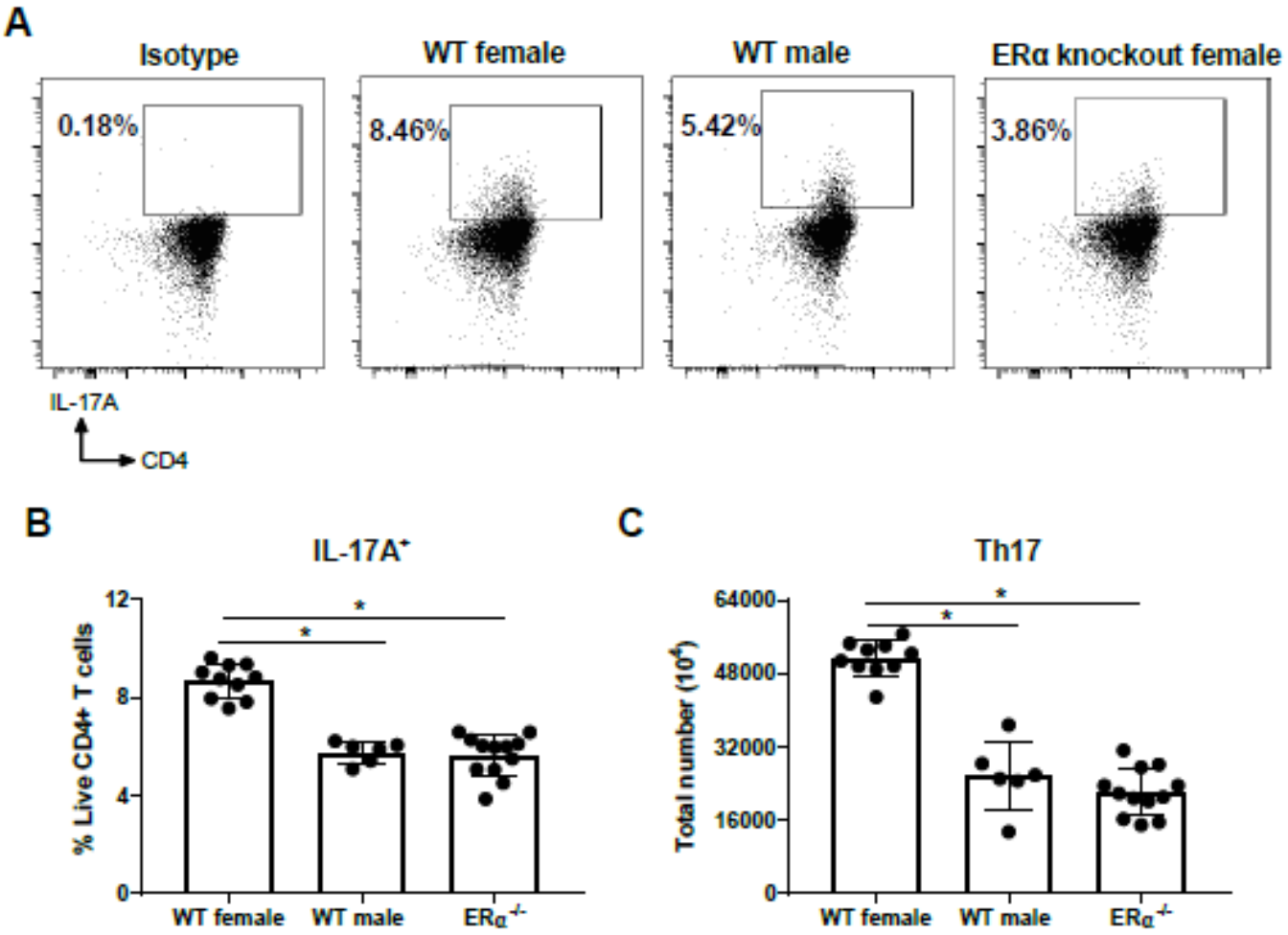

Figure 5-2: ERα Signaling increased the Frequency and Total numbers of IL-17A+ Th17 Cells. Th17 cells were differentiated from WT female, WT male and ERα female knockout for 3 days. On day 2, Golgi-stop was added to the cell culture media for 24 hours. Twenty-four hours later, cells were harvested and intracellularly stained for IL-17A+ Th17 cells. **(A)** Representative flow gating of IL-17A cytokine expression in viable CD3+CD4+ T cells (Th17 cells). **(B-C)** Frequency and total numbers Th17 cells**. (D)**. Frequency of viable CD3+CD4+ T cells. *p<0.05 1-way ANOVA with Tukey post hoc analysis n=6-10 mice/group. Data pooled from two independent experiments. *n.s.* means not statistically significant.

5.3.3 ERα Signaling increases IL-23R Surface Expression on Th17 Cells, Leading to increased IL-17A Production

Our group has previously published that ovarian hormones, including estrogen, increased IL-17A protein in Th17 cells by upregulating IL-23R expression, however it remained unclear whether ERα signaling regulated IL-17A protein expression in Th17 through IL-23R. We hypothesized that ERα promotes IL-23R surface expression in Th17 cells, leading to increased IL-17A protein expression. To determine the effect of ERα signaling on *Il23r* mRNA relative expression in Th17 cells, we conducted real-time quantitative PCR analysis from differentiated Th17 cells from WT female, WT male and ERα KO female mice. Our results showed that *Il23r* mRNA relative expression was significantly increased in Th17 cells from WT female mice compared to Th17 cells from WT male and ERα Ko female mice (Figure 5-3A). We also measured mRNA expression of *Rorc*, a master transcriptional regulator of IL-17A and IL-23R expression in Th17 cells. We did not find a statistically differences in *Rorc* mRNA relative expression between our experimental groups (Figure 5-3B).

We confirmed these findings by measuring IL-23R surface expression on differentiated Th17 cells by flow cytometry. On 3-day cultured Th17 cells, we found detectable IL-23R surface expression (Figure 5-3C) and increased IL-23R surface expression was on Th17 cells from WT female mice compared to Th17 cells from WT male and ERα KO female mice (Figure 5-3D). Furthermore, the geometric mean fluorescence intensity (MFI) of IL-23R staining was significantly increased on Th17 cells from WT female mice compared to WT male mice and ERα KO female mice (Figure 5-3E). Combined these data showed ERα signaling was important for IL-23R surface expression on Th17 cells.

IL-23 is not required for Th17 cell differentiation, rather it is important for Th17 cell phenotypic stability and increased IL-17A protein expression (133). Therefore, we hypothesized that Th17 cells from WT female mice have increased IL-17A production compared to Th17 cells from WT male and ERα KO female mice following Th17 cell differentiation in the prescence of varying amounts of rmIL-23. To test this hypothesis, we measured IL-17A production by flow cytometry after differentiation Th17 cells from our experimental groups with varying concentration of rmIL-23 (0, 10, 30 ng/ml). Our data showed that at baseline (0 ng/ml), WT female mice had significantly increased IL-17A production compared to WT male and ERα KO female mice (Figure 5-4A-B). Further, the addition of increasing concentrations of rmIL-23 significantly increased the frequency and total numbers of IL-17A+Th17 cells in WT female and male mice but not in ERα KO female mice (Figure 5-4B-C). MFI of IL-17A staining was significantly increased in WT female mice compared to WT male and ERα KO female mice at higher concentrations of rmIL-23 (30 ng/ml) but not at lower concentrations (10 ng/ml) (Figure 5-4D). These findings showed that ERα signaling promotes IL-23R independent and dependent mechanisms in Th17 cells, leading to increased IL-17A protein expression.

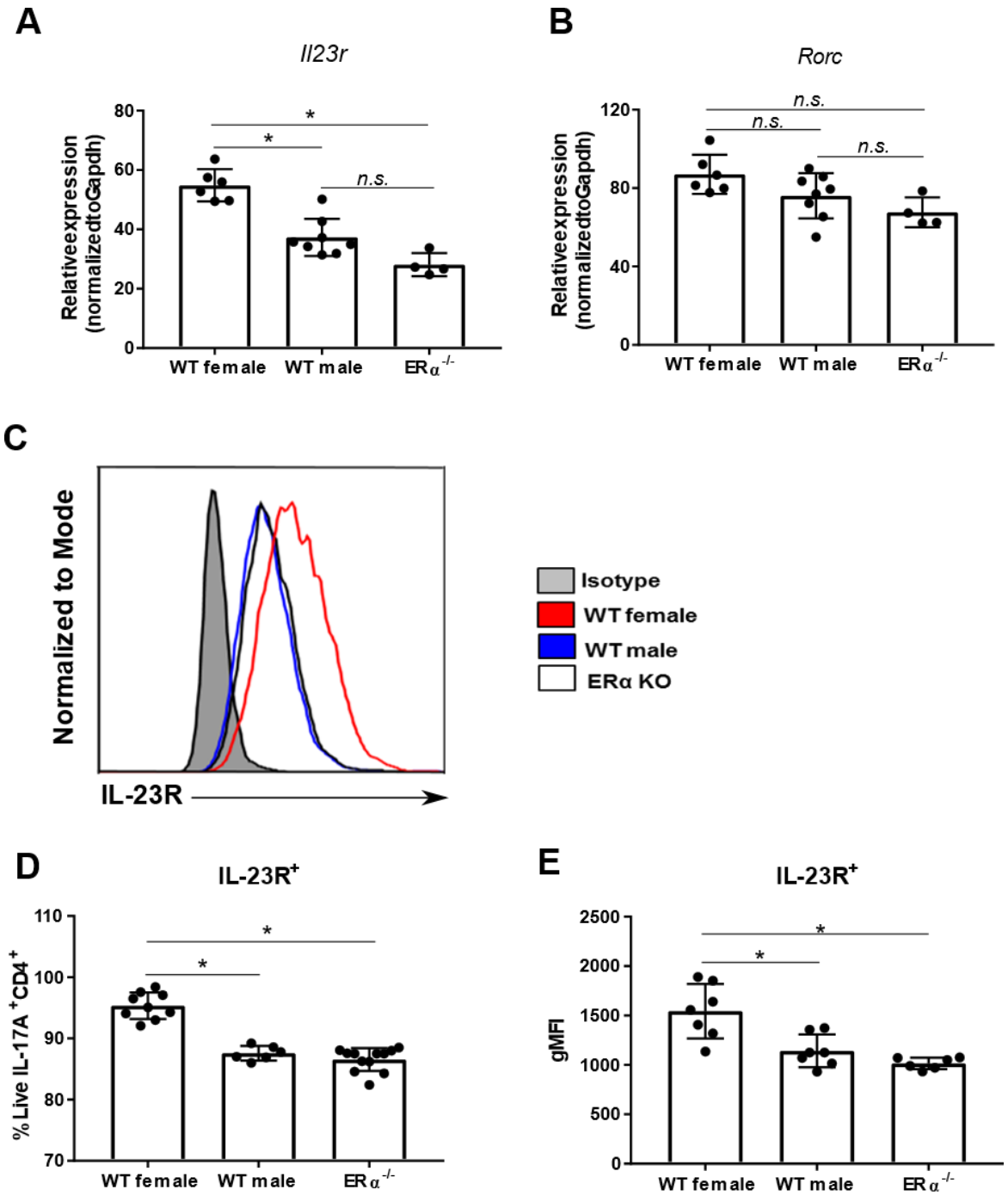

Figure 5-3: ERα signaling increased IL-23R Surface Expression on Th17 cells, Leading to increased IL-17A Production. Th17 cells were differentiated from WT female, WT male and ERα female knockout mice. (A-B) *Il23r and Rorc* mRNA relative expression were normalized to *Gapdh*. (C-E) IL-23R on IL-17A+CD3+CD4+ Th17 cells 3 days after differentiation. (C) Representative flow gating of IL-23R surface expression in CD3+CD4+ Th17 cells (D-E) Frequency and total numbers Th17 cells expressing IL-23R. *p<0.05 1-way ANOVA with Tukey post hoc analysis n=6-10 mice/group. Data pooled from two independent experiments. *n.s.* means not statistically significant.

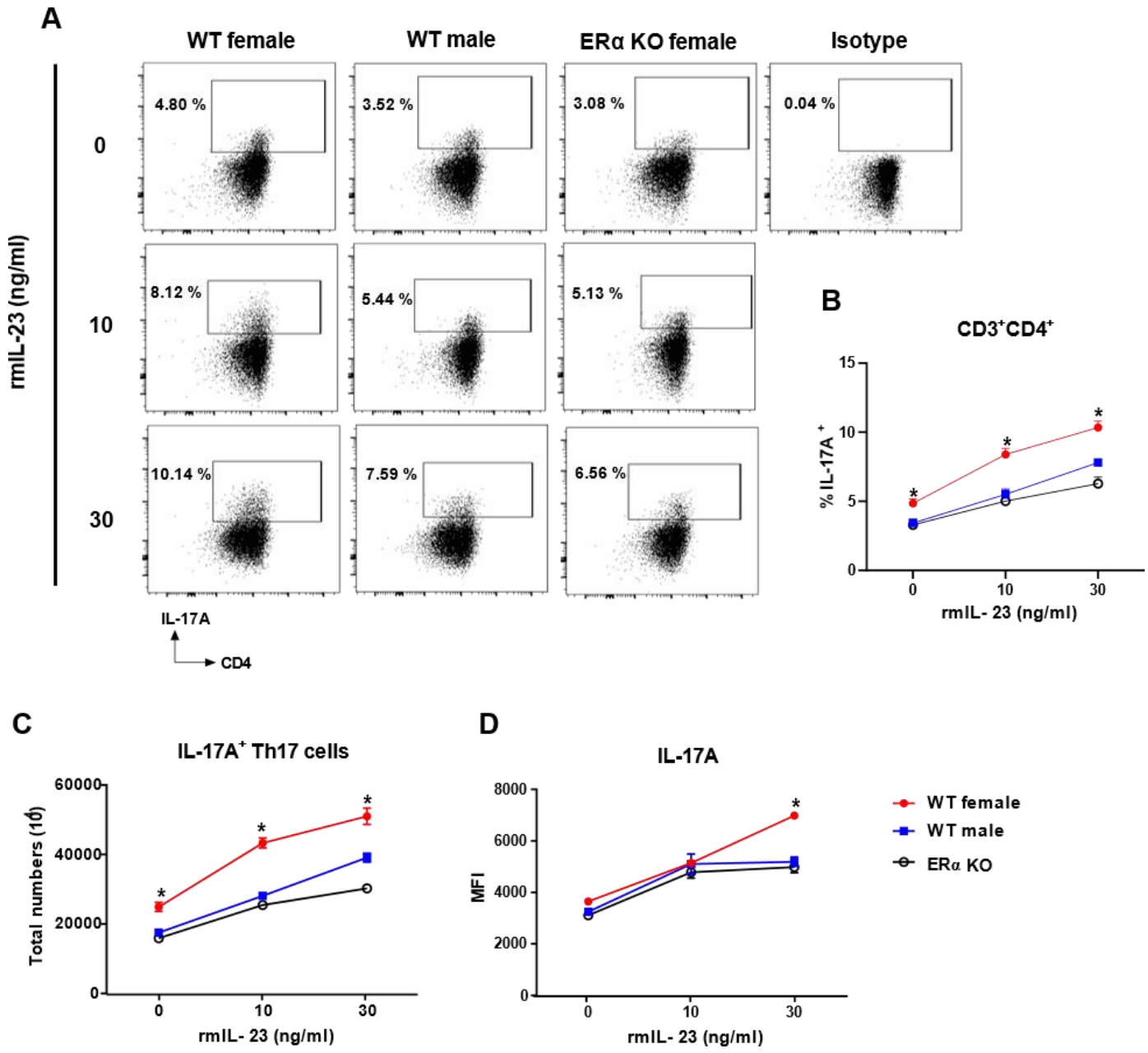

Figure 5-4: ERα Signaling increased IL-17A Protein Expression in Th17 Cells via IL-23/IL-23R Signaling. Th17 cells were differentiated from WT female, WT male and ERα female knockout for 3 days in the presence of varying concentrations of rmIL-23 (0, 10 and 30 ng/ml **(A)** Representative flow gating of IL-17A expression in viable Th17 cells. **(B)** Frequency and **(C)** Total numbers of IL-17A+ Th17 cells following rmIL-23 treatment **(D)** MFI of IL-17A staining. *p<0.05 1-way ANOVA with Tukey post hoc analysis n=3 mice/group. Significance reported as IL-17A protein expression in WT female mice compared to WT male and ERα female knockout mice. One representative experiment of 3 independent experiments. *n.s.* means not statistically significant.

5.3.4 ERα signaling deficiency increases Let-7f miRNA relative expression in Th17 cells

We have previously published that *Let-7f*, a microRNA that has been shown to regulate IL-23R expression (18), is significantly increased in Th17 cells from ovariectomized female mice implanted with 17β-E2 and P4 hormone pellets compared to Th17 cells from ovariectomized female mice implanted with vehicle pellets. Estrogen signaling is known to inhibit miRNAs, including *Let-7f*. Therefore, we hypothesized that ERα signaling inhibits *Let-7f*, thereby promoting increased IL-23R surface and IL-17A protein expression. To test this hypothesis, we first determined *Let-7f* miRNA expression in differentiated Th17 cells from WT female, WT male and ERα KO female mice. Our resulted showed that *Let-7f* was significantly decreased in WT female mice compared to WT male and ERα KO female mice (Figure 5-5A). These findings showed that ERα deficiency increased *Let-7f* mRNA expression.

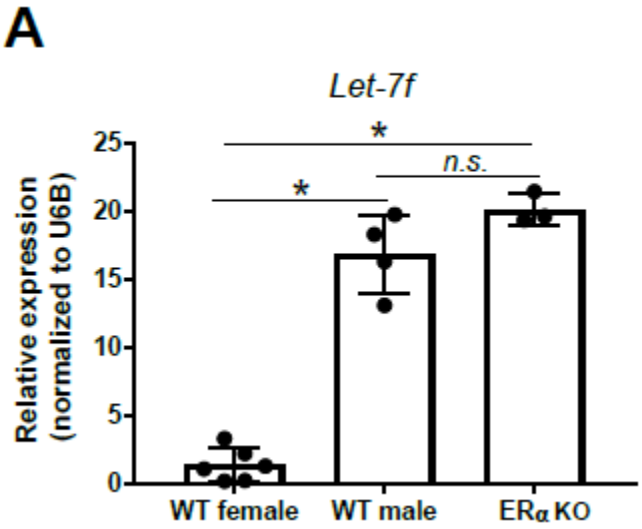

Figure 5-5: ERα deficiency increases *Let-7f* miRNA expression in Th17 cells. Naïve CD4 T cells from WT female, WT male and ERα KO female mice were differentiated into Th17 cells for 3 days and RNA was isolated for qRT-PCR analysis of *Let-7f* miRNA relative expression. Relative expression was normalized to *U6B*. **(A)** *Let-7f* miRNA relative expression in differentiated Th17 cells. *p<0.05 1-way ANOVA with Tukey post hoc analysis n=6-9 mice/group. Data pooled from two independent experiments. *n.s.* means not statistically significant.

5.3.5 Discussion

Women of reproductive age are twice more likely than men to have asthma, including severe asthma (259). Th17-mediated airway inflammation is associated with severe asthma (3) and our laboratory previously published that that women with severe asthma have increased numbers of IL-17A+ Th17 cell compared to men with severe asthma (18). Further, ovarian hormones promoted IL-17A protein expression in Th17 cells and IL-17A-cell mediated airway inflammation (249, 18). In this chapter, we showed that ERα, but not ERβ signaling, increased IL-17A in Th17 cells, as well as IFNγ in Th1 cells but not IL-13 protein expression in Th2 cells. Further, we showed that lack of ERα signaling did not promote conversion of Th17 cells to other T cell subsets, as IFNγ and IL-13 protein expression in Th17 cell culture supernatants were not

detectable. Our findings were also consistent with previous studies in a colitis model that showed ERα signaling increased T cell proliferation and IL-17A and IFNγ cytokine expression (287). Combined, these studies showed that ERα signaling increased IL-17A protein expression and Th17 cell differentiation in Th17 cells.

ERα deletion in CD4 T cells is known to decrease cell viability and promote expression of pro-apoptotic genes such as Bcl2 (287), however it remained unclear how ERα signaling in Th17 cells increases IL-17A protein expression. We assessed relative expression of *Il23r* and *Rorc*, genes known to regulate Th17 cell differentiation and IL-17A production and found that ERα signaling increased IL-23R mRNA expression and surface expression. Surprisingly, our findings showed that even in the absence of rmIL-23, IL-17A protein expression was increased in Th17 cells from WT female mice compared to WT male and ERα female knockout mice.

These findings are in alignment with previous studies where IL-23R was shown to be important for optimal IL-17A production and maintenance of the Th17 cells phenotype but not for Th17 cell differentiation (133), and suggested IL-23 independent mechanisms by which ERα signaling promotes IL17A production in Th17 cells. ERα signaling has recently been shown to regulate mitochondrial activity in CD4 T cells where TCR-mediated activation of naïve CD4 T cells from $ERα^{fl/fl}$ or CD4-$creERα^{fl/fl}$ significantly increased the oxygen consumption rate (OCR) in $ERα^{fl/fl}$ compared to CD4-$creERα^{fl/fl}$ (287). Based on these findings, it plausible that ERα signaling may promote increased mitochondrial activity following TCR-mediated activation during Th17 cell differentiation. ERα binds to DNA, thus it is also possible that ERα binding to estrogen response elements (EREs) in Th17-promoting genes, including the aryl hydrocarbon receptor (*AhR*), a

transcription factor that is essential for Th17 cell activation and differentiation. *AhR* is highly expressed in Th17 cells and is expressed at low levels in other T cell subsets, including Th1 and Th2 (290). Thus, it is possible that ERα signaling may promote expression of *AhR* to alter T cell activation and increase Th17 cell differentiation and IL-17A protein expresssion.

Our studies also showed that ERα deficiency decreases miRNA expression of *Let-7f*, a microRNA that is known to negatively regulate IL-23R expression in breast cancer cells (291). ERα signaling is known to regulate *Let-7f* mRNA in human breast cancer cells and murine uterine tissue (220), thus is likely that ERα deletion may promote IL-23R surface expression by inhibiting *Let-7f* miRNA expression in Th17 cells. In future studies, it will be important to determine if ERα signaling is sufficient to increase IL-17A production. These experiments would define if ERα signaling regulates Th17 cell differentiation by inhibiting *Let-7f* miRNA expresssion.

CHAPTER 6

Summary and Conclusion

6.1 Synopsis of Findings

Sex hormones regulate inflammatory pathways associated with airway inflammation in asthma (171, 259, 172), and our findings extended this knowledge by further defining how AR signaling attenuates and ovarian hormones promote type 2 and IL-17A mediated pathways (Figure 6-1). In chapter 3, we showed that testosterone decreased while ovarian hormones increased HDM-induced infiltration of eosinophils and neutrophils, total numbers of IL-13+ Th2 cells and IL-17A+ Th17 cells, and AHR to methacholine challenge. Furthermore, in chapter 4, we determined that testosterone signaling, via the androgen receptor, decreased total numbers of HDM-induced IL-13+ Th2 and IL-17A+Th17 cells, but through different mechanisms. AR signaling intrinsically, or directly, decreased IL-17A+Th17 cells, but indirectly suppressed IL-13+Th2 cells by decreasing IL-4 production, a cytokine required for Th2 differentiation and cytokine production. In Chapter 5, we determined that ERα, but not ERβ, increased IL-17A protein expression in Th17 cells through IL-23R dependent and independent mechanisms. Furthermore, ERα deficiency increased *Let-7f* miRNA relative expression, a negative regulator of Th17 cell differentiation, suggesting that ERα negatively regulates *Let-7f* expression in Th17 cells. Collectively, these findings showed that ovarian hormones and ERα signaling are important in increasing while testosterone and AR signaling are important in decreasing allergen-induced airway inflammation and provide a potential explanation for why women have increased asthma prevalence compared to men.

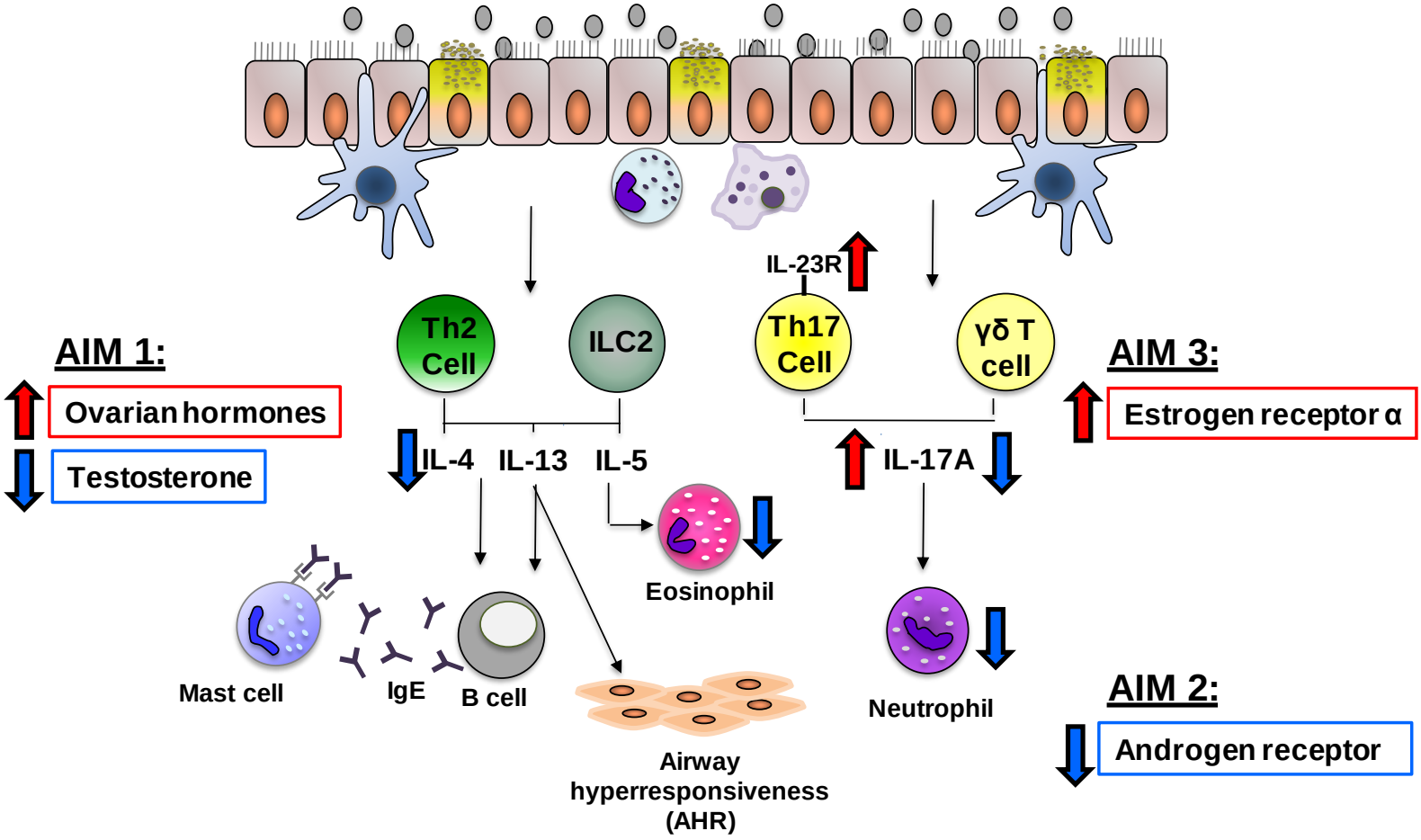

Figure 6-1: Graphical Abstract of Dissertation Results.
Summary of findings for this dissertation with arrows depicting how sex hormones regulated different inflammatory pathways associated with airway inflammation and asthma. Arrows give general directionality to findings for each aim. In Aim 1, we found that testosterone decreased and ovarian hormones increased BAL eosinophils and neutrophils following HDM challenge. Further, IL-13 and IL-17A production, predominantly from CD4 Th2 and Th17 cells, respectively, increased type 2 and IL-17A- mediated airway inflammation, leading to increased IgE production and AHR. In Aim 2, we found that AR signaling attenuated CD4 Th2 and Th17 cell-mediated airway inflammation by intrinsically suppressing IL-17A production in Th17 cells and indirectly inhibiting Th2 development by suppressing cell sources of IL-4. In Aim 3, we found that ERα signaling increased IL-17A protein expression in Th17 cells by upregulating IL-23R surface expression.

6.2 Future Directions and Implications

6.2.1 Sex Hormone Receptor Transcriptional Regulation of T cells

Our studies revealed a role for ERα and AR signaling on Th17 cell differentiation and cytokine expression, however the underlying cellular mechanisms by which ERα and/or AR-mediated signaling modulate gene expression of Th17 associated genes remain unclear. In chapter 4, our studies showed that AR signaling was intrinsically suppressing cell numbers of IL-17A+Th17 cells. In preliminary studies, we also assessed the role of AR signaling on Th17 cell proliferation, and we found that AR signaling did not decrease the Th17 cell proliferation. AR signaling is known to suppress cell proliferation but also promote apoptosis (280), thus it is likely that AR signaling decreases Th17 cell numbers of by promoting apoptosis. Future studies focused on assessing the role of AR signaling on Th17 cell apoptosis will be important for understanding the role of AR signaling on Th17 cell viability.

Apart of modulating apoptosis in Th17 cells, it is also possible that AR signaling may modulate the plasticity of Th17 cells into other Th subsets, including T regulatory cells (Tregs) or Th1 cells. Th17 cells are known to be very plastic, where permissive epigenetic marks such as histone modification have observed at the *Il10* and *Ifnγ* gene loci in differentiated TH17 cells, leading to increased production of cytokines typically attributed to other Th1 subsets, including IL-10 and IFNγ (292, 293). TGF-β is an important cytokine for Treg and Th17 cell differentiation and is known to modulate the expression of SMAD proteins that regulate Foxp3 and RORγT expression in T cells (294). Furthermore, recent studies demonstrate that steroid receptor 1 (SRC1), which is known to be regulated by sex hormones, including androgens and estrogen promotes Th17 lineage commitment by disrupting the dimerization of Foxp3 to RORγT, leading to increased dimerization of RORγT to RORα and promote of Th17 cell

differentiation (295). Based on these findings it is likely that AR and ER-mediated signaling may serve as the transcriptional and/ protein regulators of SRC1 function to regulate Th17 cell differentiation and cytokine expression.

In chapter 5, ERα signaling was important in increasing IL-17A cytokine expression and total numbers of IL-17A+ T cells, suggesting that ER signaling promotes Th17 cell differentiation by increasing the amount of IL-17A secreted the as well as the proliferative capacity as well of the Th17 cell. Our data showed that ERα signaling modulated IL-17A protein expression in Th17 cells by promoting IL-23R surface expression, but it remains unclear if ERα directly modulates *Il23r* mRNA expression directly by binding at the *Il23r* locus or by an indirectly affect, potentially via modulate of *Let-7f*. Estrogen and progesterone have been shown to decrease *Let-7f* miRNA expression leading to increased IL-23R and IL-17A expression (18). Furthermore, in chapter 5, ERα deficiency increased *Let-7f* miRNA expression in Th17 cells, suggesting that ERα signaling may modulate IL-17A protein expression by negatively regulating Let7f miRNA expression in Th17 cells. In these prior studies, *Let7f* miRNA expression was significantly increased and *Il23r* mRNA in Th17 cells from ovariectomized female mice administered dihydrotestosterone (DHT) hormone pellets compare to Th17 cells from ovariectomized female mice administered vehicle pellets. Further, suggesting that AR signaling induced by DHT, may also regulate *Il23r* and *Let-7f* miRNA expression in Th17 cells.

The underlying mechanisms by which AR and ERα transcriptional regulate gene expression in Th17 cells remains unclear primarily due to lack of knowledge about the specific hormone binding sites of AR and ER receptors in target cells and low expression of these hormone receptors in T cells. Efforts to identify AR and ER binding sites in Th17 cells and other T cell subsets may be helped by utilizing technologies such as Chromatin-immunoprecipitation

followed by DNA sequencing (ChIP-Seq). ChIP-Seq is a method of identifying genome-wide DNA bind sites of DNA binding proteins. Following cell lysis, sonication or enzymatic digestion is used to fragment chromatin and then DNA-bound proteins can be immunoprecipitated using AR and ER specific antibodies. The bound DNA fragment is then coprecipitated, purified and sequenced (296). Assay for Transposase-Accessible Chromatin using sequencing (ATAC-Seq) is a powerful technique that can be used to identify open chromatin regions genome-wide. This method determines DNA accessibility by probing open chromatin with a mutant Tn5 transposase that insert sequencing adapters. Sequencing reads can then to use to infer regions of accessible DNA and also map regions of transcription factor binding (297). In Chapter 4 and 5, AR decreased while ERα increased *Il23r* mRNA relative expression in Th17 cells, suggesting that AR and ERα binding to hormone binding elements at the *Il23r* locus may regulate IL-17A protein expression in Th17 cells. In future studies, AR and ERα interactions with regions of Th17 cells-specific genes should be assessed, including at the *Il23r* and *Let-7f* gene loci using ChIP-Seq and/or ATAC-Seq. Furthermore, future studies should assess the role of AR and ER signaling on gene promoting and repressive histone modifications at gene loci in Th17 cells, including H3K4me3 and H3K27me3 marks via CHIP-assays.

6.2.2 Sex Hormone Receptor Regulation of T cell Metabolic Reprogramming

Activated T cells can be skewed towards the Th17 cell phenotype in the presence of IL-6 and TFG-β, leading to the generation of "non-pathogenic" RORγT+IL-17+Th17 cells, while IL-6, TFG-β and IL-23 promote the generation of "pathogenic" RORγT+IL-17+Th17 cells (298). IL-23 has plays a critical role in generating pathogenic Th17 cells, specifically by stabilizing the Th17 phenotype endowing Th17 cells with pathogenic effector functions (133). Transcriptional

profiling of Th17 cells differentiated with TGF-β and IL-6 in the presence or absence of IL-23 further showed that IL-23 promoted the expression of serum glucocorticoid kinase 1 (SGK1), a serine/threonine kinase found that is highly expressed in IL-23R+IL-17A+ Th17 cells and γδ T cells, leading to increased memory Th17 effector responses (299). In chapter 4, AR signaling decreased IL-23R mRNA expression in Th17 cells. Future more, in chapter 5 ERα signaling increased IL-23R mRNA expression in Th17 cells. These findings suggested that AR-mediated inhibition of IL-23R expression would promote the differentiation of non-pathogenic Th17 cells, while ERα-mediated upregulation of IL-23R expression would promote the differentiation of pathogenic Th17 cells. Future studies should determine the role of AR and ERα signaling on Th17 cell pathogenicity during allergic airway inflammation by adoptively transferring "pathogenic and "non-pathogenic" derived OVA-specific Th17 cells from AR and ERα OT II mice into WT recipient mice.

In chapter 5, IL-17A protein expression was increased in Th17 cells from WT mice differentiated in the absence of rmIL-23, compared to Th17 cells from ERα KO female mice differentiated in the absence of rmIL-23. These findings suggest that ERα signaling can promote IL-17A protein expression in Th17 cells via IL-23R independent mechanisms. Transcriptomics analyses have demonstrated that ER signaling in CD4 T cells increased expression of genes associated with TCR signaling, including *Cd3e*, *Cd4*, *Zap40* and *Cd69* (287). In control or CD4-creER$\alpha^{fl/fl}$ C57BL/6J mice, activation of naïve T cells from these mice with varying concentrations of anti-CD3 and/or anti-CD28 for 24 hours showed that deletion of ERα in CD4 T cells decreased frequency of CD69+ T cells in the presence of high doses of anti-CD3 (5 µg/ml) alone or a low-dose of anti-CD3 (1 µg/ml) in combination with increasing amounts of anti-CD28. Surprisingly, a high-dose of anti-CD3 with increasing concentrations of anti-CD28

restored the frequency of CD69+ T cells from CD4-creER$\alpha^{fl/fl}$ mice to similar a frequency as CD69+ T cells from control mice (287). These findings suggest a T cell-intrinsic role for ERα signaling in regulating expression of genes associated with TCR activation.

Previous studies demonstrated that the strength of TCR stimulation is important for T cell differentiation, where intermediate doses of antigen stimulation of naïve T cells promotes the generation of Th1 effector responses, while low or high doses of antigen bias activated T cells towards Th2 responses (300). Furthermore, low doses of antigen presentation by dendritic cells induced weak TCR activation, decreased the mechanistic target of rapamycin (mTOR) pathway, which decreased cell proliferation and generation of Foxp3+ T regs as opposed to effector T cells (301). The Akt-mTOR pathway is heavily implicated in T cell metabolic activity (302), thus it is possible that ERα signaling may regulate metabolic programming in activated T cells to promote IL-17A production and Th17 cell differentiation.

Resting lymphocytes, including naïve T cells, rely primarily on catabolic and oxidative metabolic programming including oxidative phosphorylation to meet their energy consumption requirements (303). However, TCR activation induces expression of the glucose transporters, including glucose transporter 1 (Glut1) on activated T cells, leading to increased glucose uptake, increased glycolytic activity and increased lactate production. In addition to glucose, glutamine uptake and glutaminolysis further promotes T cell differentiation and activated cells that fail to meet these energy requirements subsequently undergo apoptosis (304). Metabolic reprogramming in activated T cells is regulated by various transcriptional factors and signaling pathways, including the AMP-sensitive signaling kinase (AMPK) and mTOR signaling pathways. The AMPK pathway is induced during low nutrient and/or decreased ATP conditions, leading to inhibition of glycolysis and glutaminolysis and increased reliance of cells on catabolic

metabolic processes like oxidative phosphorylation (303). The mTOR signaling pathway upregulated during when nutrients are abundant, leading to promotion of glycolysis and glutaminolysis. The functions of mTOR are mediated via two different complexes, mTORC1 and mTORC2, leading to the induction of distinct downstream signaling event (305). ERα signaling increased the total numbers of IL-17A+ Th17 cells, therefore, it likely that ERα signaling promotes increased mTOR signaling upon TCR activation, leading to increased glycolysis and glutaminolysis and increased IL-17A protein expression during Th17 cell differentiation.

Activated T cells can different in various T helper subsets and previous studies showed that T helper subsets have distinct metabolic signatures (306). Th1, Th2, Th17 and T follicular helper (Tfh) cells rely heavily upon glycolysis for ATP generation, while Tregs utilize fatty acid oxidation and/or glycolysis (306). The addition of exogenous fatty acids to naïve T cells during T cell differentiation, was shown to inhibit the formation of Th1, Th2 and Th17 cell differentiation but had only a minor inhibitory effect on Treg differentiation (304). Further studies showed that in cases where glycolysis is inhibited, Th17 cells are also known to depend heavily on glutaminolysis (307). Recent studies demonstrated that the Let-7 family of miRNAs, including Let-7f, decreased B cell metabolic activity by decreasing glycolysis and glutaminolysis (308). Let-7adf miRNAs directly decreased the expression of *hexokinase 2* (*Hk2*), an enzyme involved in glycolysis, leading to decreased IgM secretion and decreased lactate production. Furthermore, Let-7fadf cluster was shown to decrease expression of a glutamine transporter called *Slc1a5* and decreased expression of a glutaminolysis specific enzyme called *Gls*, leading to decreased glucose update and decreased glutaminolysis. Previous studies showed that ERα signaling regulates mitochondrial respiration, leading to increased Th1 and Th17 responses in a mouse model of colitis. Based on the previous findings and data, it is likely that ERα may rincrease

mitochondrial activity, glycolysis and glutaminolysis in Th17 cells to promote increased Th17 cell differentiation following TCR activation. Mitochondrial stress test experiments as well as glycolysis and glutaminolysis assays are further needed to determine the role of ERα signaling regulates on these metabolic pathways in Th17 cells via Let-7f dependent manner. Understanding the metabolic activity of Th17 cells may lead to the identification of metabolic targets that can be used to specifically inhibit Th17 metabolic activity and function in women and men with Th17-mediated airway inflammation.

6.2.3 Sex Hormone-mediated Epigenetic Regulation in T cells

Although master transcriptional regulators are highly implicated in T cell lineage commitment and stability, epigenetic mechanisms have recently been implicated in T cell differentiation as well as plasticity (309). Eukaryotic chromatin is structured into octamers of four major histones, including H2A, H2B, H3 and H4 and post-translational epigenetic modifications of histone residues have been shown to regulate gene expression in T cells, by modulating chromatin remodeling and DNA accessibility(293).

Various epigenetic mechanisms are associated with T cell differentiation and plasticity, including methylation, acetylation and sumoylation of DNA and histones. Histone modifications are well implicated in regulating T cell differentiation (310). Histone H3 lysine 4 trimethylation (H3K4me3) is associated with active promoters while Histone H3 lysine 4 mono-, di- and tri-methylenation are typically found near gene enhancer sites (311). Histone H3 lysine 36 trimethylation is associated with actively transcribed coding regions (310). Histone modifications are associated decreased gene expression, where histone H3 lysine 27 trimethylation (H3K27me3) and histone H3 lysine 9 trimethylation (H3K9me3) was found at

promoter sites of repressed genes (310). Previous studies in effector T cells from mice showed that H3K4me3 marks were significantly increased at the gene promoters for T effector with respect to their subset-specific genes, including *Ifnγ* and *Tbx21* in Th1, *Il4* and *Gata3* in Th2, *Rorc* and *Il17a* in Th17 and *Foxp3 in* Tregs with respective to their transcription (293) In contrast, H3K27me3 marks were significantly increased at the promoter genes of *Ifnγ* and *Tbx21* in Th2 cells and increased at the *Il4* and *Gata3* gene promoters in Th1 cells. Since our data showed that AR decreased and ERα increased Il23r expression, it is likely that AR and ER may modulate histone modifications in T cells regulate IL-23R expression on Th17 cells. Future studies using ChIP-assays may determine AR and ERα-induced histone modifications in Th17 cells.

Histone modifications are regulated by enzymes complexes that catalyze epigenetic changes of histone residues, including the Polycomb and Trithorax complexes. While both complexes possess methyltransferase activity, the Polycomb complex is implicated in gene repressive histone modifications while Trithorax complexes promote gene activating histone marks (310). The Polycomb complex is composed of two major proteins, PCR1 and PCR2, where the PCR2 encompasses a enhancer of zeste (Ezh) methyltransferase that generates H3K27 methylation marks(312). Human T cells express two Ezh protein, where Ezh2 is highly expressed in T cells and Ezh recruitment at the *Gata3* locus in Th2 cells has been shown decrease Th2 cell differentiation (313). Furthermore, Ezh2-induced H3K27me3 marks at the *Il4* and *Il13* gene loci in Th1 cells was shown to decrease gene expression of these genes (310).

The Trithorax complex has a protein called the Mixed lineage leukemia (MLL) methyltransferase, which has been shown to promote Th2 and Th17 cell differentiation mediated by a recruiting protein called Menin (314, 315). Menin is highly specific recruiter of the MLL

that is required for the ability of MLL to bind to DNA (316). The interaction of menin to MLL is necessarily for the maintenance of Gata3 expression in human Th2 cells and in mice, lack of MLL leads to memory Th2 responses (315). Menin is also important in Th17 cell differentiation, where Th17 cells that lack Menin were shown to have increased H3K27me3 marks at the Rorc locus, decreased IL-17A protein expression (314). AR and ER binding to hormone response elements can promote gene expression by recruiting DNA and histone modifying enzymes to the specific regions of targets genes, leading to hormone-induced DNA and histone epigenetic changes that promote or repress gene express (317).

AR signaling has been shown to promote the recruitment of the Polycomb complex to androgen response elements in prostate cancer, leading to increased addition of H3K27me3 marks. MLL are H3K4 methyltransferases and studies show that induction of ERα signaling is associated with increased MLL activity. Previous studies showed that ERα recruits MLLs to the homebox-containing gene, *HOXC13* gene, which is involved in human leukemia and hair development. Inhibition of MLL via RNAi silencing decreased ERα-induced gene activation of *HOXC13* (318). Based on these previous findings, it is likely that AR and ERα signaling may regulate the recruitment of histone modifying enyzmes such as MLL and Ezh2 to the site of Th17-specific gene loci, including *Il23r and Il17a.* ChIP-Seq analyses of the interaction of these methyltransferases with DNA binding regions at the *Il23r* gene locus in AR and ERα treated Th17 cells would determine the role of AR and ERα signaling on regulation of histone modifications in Th17 cells.

6.2.4 Sex Hormone Regulation of IFNγ in Asthma

Non-type 2 cytokines have been implicated in asthma pathogenesis, including IFNγ. In BAL fluid samples of asthmatics patients, *IFNG* mRNA expression percentages of IFNγ+Th1 cells are increased in severe asthma patients compared to those with milder asthma phenotypes (3). Characterization of immune cell phenotypes in the BAL fluid of asthmatics further showed 50% of patients with severe asthma had increased percentages of IFNγ+Th1 cells are increased compared to those with milder asthma phenotypes (3). The presence of increased IFN-γ levels in the airways of patients with asthma has raised the question of what triggers IFNγ production. Viruses and bacteria are well known to regulate the production of interferons, thus it has been suggested that chronic viral and bacterial may increase the risk of atopic allergic asthma (83).

Sex hormones are known to regulate IFNγ and type 1 immunity. In vivo studies, gonadectomized and sham-operated male and female mice infected intratracheally with a high dose of *Mycobacterium tuberculosis* strain H37Rv to assess the role of sex hormone signaling on pulmonary tuberculosis (319). These studies demonstrated that intact sham-operated female mice and gonadectomized male mice had increased Mycobacterium-induced airway inflammation with increased granuloma formation and increased pneumonia, compared to intact sham-operated male mice and gonadectomized female mice. Furthermore, intact female mice and gonadectomized male mice had increased had increased IFNγ. IL-12 induced nitric oxide synthase (iNOS) and IL-17A in whole lung homogenates compared to intact male mice and gonadectomized female mice (319). Treatment of mouse splenic T cells with 17β-estradiol (17-βE2) significantly increased IFNγ mRNA and protein levels compared to vehicle treated splenic T cells (320). In subsequent experiments, it was shown that ER signaling increased *Ifnγ* mRNA expression by directly binding to estrogen response elements in the promoter region of the Ifnγ

gene locus (286). In Chapter 5, IFNγ protein expression was increased in differentiated Th1 cells from WT female mice compared to WT male and ERα KO female mice, suggesting that ERα signaling promotes Th17 cells differentiation and Th1 responses in vivo. Since IFNγ-mediated airway inflammation is known to promote neutrophilic-mediated airway inflammation (321), utilizing the house-dust model described in Chapter 3 would be important for determining the role of ovarian hormones and testosterone on IFNγ-mediated allergic airway inflammation is important for patients with neutrophil-dominant asthma, especially obese women with asthma. Subsequent experiments should assess the specific mechanisms by which ERα signaling promotes IFNγ expression in Th17 cells. Since estrogen signaling is known to increase *Ifnγ* messenger expression (286), CHIP assays could potentially be used to assess the role of ERα in regulated *Ifnγ* messenger expression via epigenetic mechanisms, including gene permissive histone modifications at the *Ifnγ* gene locus.

6.2.5 Role of Sex Hormone Signaling on Non-T cell Subsets in Asthma

Sex hormones known to regulate other immune cells during asthma pathogenesis, including ILCs, macrophages, dendritic cells and airway epithelial cells (171). DHT treatment of ILCs decreased IL-5 and IL-13 protein expression in ILC2 and decreased IL-2-dependent ILC2 proliferation, leading to decreased MFI expression of CD25 (233). Mixed bone-marrow chimera studies in Alternaria challenge mice showed that AR signaling intrinsically decreased cell numbers of Alterneria-induced lung ILC2 cells (233). These findings showed that AR signaling attenuated ILC2-mediated airway inflammation by directly suppressing cell proliferation. Estrogen receptor signaling is also known to regulate ILC2 function (322) . In uterine tissue, ST2+ILC2 expressed both ERα and ERβ, and implantation of estrogen hormone pellets to

ovariectomized mice increased ILC2 numbers following ex vivo stimulation of ILCs with IL-33 (235). ILC2 numbers are increased in women with severe asthma compared to men with severe asthma (233). Further, ILC2 produce 40-fold more IL-5 and 10-fold more IL-13 than Th2 cells, and ILC2 are critical for the induction of Th2 cell responses in asthma (118, 116). Therefore, understanding the role of sex hormone signaling on ILC2-mediated airway inflammation is important for the design of future clinical trials that account for the sex-bias in asthma.

Alveolar macrophage (AMφ) and dendritic cells (DCs) are major mediators of allergic airway inflammation and studies show a role for androgens and estrogen on the inflammatory responses of AMφ and DCs (243, 241). In castrated mice, administration of DHT was shown to reduce OVA-induced airway inflammation and enhance the polarization of bone marrow derived macrophages towards the M2 phenotype following IL-4 stimulation *in vitro* (244). Furthermore, mice that lack AR in the macrophage/monocyte compartment showed decreased BAL eosinophil cell numbers and decreased airway inflammation following OVA sensitization and challenge, potentially due to impaired M2 polarization (244). Although their numbers are correlated with increased asthma severity in asthma, M2 macrophages are associated with wound healing and tissue repair, thus the increased numbers of these cells in chronic airway inflammation may be correlated with attempts to repair tissue damage to the airway (323) . Therefore, AR-mediated expansion of M2 macrophages supports the anti-inflammatory mechanisms by which androgens are known to modulate airway inflammation. Since AR is implicated promoting the development of M2 macrophages in vivo, future studies should focus on how understanding the cell-intrinsic mechanisms by which AR signaling regulates macrophage polarization towards the M2 phenotype. These studies may reveal specific M2 genes that can be targeted using AR therapeutics to promote conversion of M1 macrophages towards the M2 phenotype to inhibit

airway inflammation.

In contrast to the studies, estrogen has been implicated in promoting M2 polarization (242). Ovariectomized mice who were previously sensitized to OVA, were administered placebo or estrogen (E2) hormone pellets and then challenged with aerosolized OVA (242). Macrophages from ovariectomized implanted with E2 pellets had increased MFI expression of a M2 macrophage marker called YM1 and increased BAL eosinophils compared to ovariectomize mice administered placebo pellets, however these effects were not mediated by ERα, as IL-4 treatment of bone marrow derived macrophages (BMMs) did not change ERα expression in BMMs in female and male mice (242). Estrogen treatment is known to upregulate both ERα and ERβ signaling. However, while ERα is known to promote inflammation, ERβ is known to negatively regulate inflammation (324). Based on these findings, it is likely that ERβ-mediated signaling events may regulate the increased cell numbers M2 macrophages following OVA-induced allergic airway inflammation. Like the effects of AR signaling on M2 macrophage differentiation (244), ERβ-mediated regulation of M2 macrophage development may potentially promote tissue repair in the lung following chronic airway inflammation (242). Combined these studies show while estrogen signaling can be mediated by different receptors, leading to induction of pro-inflammatory events if mediated by ERα or immunosuppressive/homeostatic events if mediated by ERβ. Understanding the role of estrogen receptor specifically though ERα and/or ERβ is important for determining which specific estrogen receptor to target in modulating immune functions of various immune cells.

DCs play a critical role in allergic airway inflammation and sex hormones are known to regulate DCs cytokine expression and antigen presentation (239). In OVA sensitized and challenged female and male mice, females had increased numbers of myeloid and plasmacytoid

DCs migrating to the lung draining lymph nodes compared to male mice (239). In vitro, estrogen treatment of LPS-stimulated DCs increased IL-6 cytokine expression and increased MHC-mediated T-cell activation compared to vehicle-treated LPS-stimulated DCs (240). Furthermore, estrogen treatment of GM-CSF stimulated bone marrow mice cells increased CD11b+, CD11c+ DCs compared to vehicle treated GM-CSF stimulated cells (241). However, deletion of ERα significantly decreased cell numbers of CD11b+, CD11c+ DCs in estrogen treated GM-CSF treated bone marrow cells compared to WT GM-CSF stimulated cells (241). Ovarian hormones increased while testosterone decreased cell numbers of IL-13+ and IL-17A+Th17 cells in the lungs of HDM-challenged mice, therefore, it likely that AR and ERα may regulate the DC trafficking to the lung drain lymph nodes as well as DC-mediated activation of T cells. We determined that AR signaling decreased Th2 cell numbers in the lung, thus it likely that AR signaling may modulate DC-mediated activation and Th2 differentiation to attenuate Th2-cell mediated airway inflammation.

Sex hormones have been shown to regulate mast cell function, but the underlying mechanisms by which sex hormones regulate mast cells remain unclear. Studies in isolated peritoneal mast cells (PMCs) from Sprague-Dawley rats showed that stimulation of PMCs with substance P decreased histamine release in PMCs from female rats administered estradiol, progesterone, testosterone or DHT (238). Upon stimulation of female PMCs with substance P and IgE, estradiol treatment increased histamine release, while progesterone, testosterone and DHT treatment significantly decreased histamine release. In contrast, stimulation of male PMCs with substance P and IgE did not increase histamine release with estradiol treatment (238). Our data from chapter 3 showed that IgE levels are increased in intact female mice compared to intact male mice and ovarian hormone increased while testosterone decreased IgE levels in HDM-

challenged mice. Thus, it is likely that female mice have increased mast cell activation as result of increased IgE, however further studies are needed to determine the estrogen signaling on mast cell activation and effector functions.

6.2.6 Implications for Women with Asthma at Various Reproductive Phases

Our findings from this dissertation work has implications for women with asthma throughout various reproductive ages, including during menstruation. Approximately 30-40% of women with asthma report worsening of asthma symptoms during the pre or perimenstrual phase of their menstrual cycle (187), however the underlying mechanism regulating the cyclic changes and worsening of asthma symptoms remains unclear. Studies in healthy female subjects, ages 18-25 with normal regular menstrual cycles, showed a significant increase the percentage of peripheral neutrophils during luteal phase (secretory phase) compared to the follicular phage (proliferative phase) of the menstrual cycle (325). Furthermore, lymphocyte count was significantly increased during the proliferative phase and secretory phases compared to the menstrual phase (325). Our studies showed that ovarian hormones, including estrogen increased BAL neutrophil numbers following HDM-challenge, thus it is likely that increased estrogen levels during the luteal phase may promote increased neutrophilic-mediated airway inflammation airway inflammation in women with perimenstrual asthma. However, further studies are needed to understand the role of sex hormone signaling on premenstrual asthma in women and this is challenging to conduct in mice since mice undergo estrus and not a menstrual cycle like women.

Discordant findings have been reported in pregnancy and changing asthma symptoms in women with asthma. Increased asthma exacerbations are reported in one-third of pregnant women but the remaining 2/3 report either no change in their asthma symptoms or a an

improvement during their pregnancy (259). A more recent study showed that women with severe asthma prior to getting pregnant are more likely to have worsening asthma symptoms during pregnancy (326). About 12.6% of the women in this study were initiated classified with mild asthma had exacerbations wild pregnant, but over 25.7% of patients classified as moderate and 51.9% of patients classified with severe asthma suffered from exacerbations (326). Progesterone levels are highly increased in pregnant women (> 140 ng/ml after 36 weeks) compared to non-pregnant women ($\leq$ 15 ng/ml at 21st day menstrual period) (327). Since ovarian hormones increased type 2 and IL-17A mediated airway inflammation as well as increase IL-17A protein expression in women compared to men (18), it is possible that during pregnancy IL-17A and/or type 2-mediated inflammation is further increased in some women with severe asthma.

The human chorionic gonadotropin (hCG) hormone serum levels are significantly increased during pregnancy and has been shown to have an immunosuppressive effect on dendritic cells and T cells (328), mediated in part through hCG's ability to increase the expression of a tryptophan-metabolizing enzyme called indoleamine 2,3-dioxygenase (IDO) (329). Treatment of bone marrow derived macrophages with hCG and IFNγ increased IDO mRNA expression and enzymatic activity, leading to increased IL-10 production in these cells (329). Furthermore, hCG has been shown trigger expansion and migration of Treg cell populations as well as regulate T reg conversion into Th17 cells, potentially via IDO expression (328). Serum hCG levels are highest during the first trimester, and are significantly reduced in the subsequent trimesters (330), thus it is likely that oscillating levels of hCG may influence asthma exacerbations in pregnancy women and studying the effect of HCG on asthma exacerbations in women with asthma may potentially elucidate the reason for these discordant findings in pregnant women with severe asthma. Oscillating levels of sex hormones in

menopausal women may also account for the discordant finding in menopause and changing asthma symptoms in women with asthma. Some studies report a worsening of asthma after menopause, while other studies showed an improvement in asthma symptoms following menopause. These discordant findings are most likely influenced by hormone replacement therapy in menopausal women.

6.2.7 Implications for Patients with Obesity-Linked Asthma

Obesity is a risk factor for asthma and at least two asthma phenotypes associated with obesity, including early allergic-asthma and late onset-asthma (180). Patients with asthma who have undergone bariatric surgery report significant improvements in asthma control and lung function compared to healthy subjects who also underwent bariatric surgery (331). However, compared to healthy subjects, BAL lymphocyte numbers and CD T cell-mediated IL-5, IL-5, IL-13, IL-17A and IFNγ production was still increased in patients with asthma who received bariatric surgery (331). Furthermore, a mouse model of obese-asthma found that following consumption of a high-fat diet for 12 weeks and becoming obese, mice spontaneously developed AHR with allergen exposure (332). In B and T cell deficient Rag knockout mice, obesity-linked AHR was induced independent of adaptive immunity and was associated with increased numbers of IL-17A+ILCs (ILC3s) (333). These studies suggest that obesity is associated with more severe asthma phenotypes.

Cluster analyses in patients with asthma determined that women are more likely to have severe asthma and are less likely to respond to corticosteroids compared to men (32, 7). Furthermore, these studies revealed another phenotype composed of mainly women with

late-onset obese, atopic asthma with increased sputum neutrophils. The Unbiased Biomarkers in Prediction of Respiratory Disease Outcomes (U-BIOPRED) enrolled a cohort of asthma patients ranging from mild to severe asthma and found a cluster of obese female patients with asthma who experience frequent exacerbations and with normal lung function (334).

Sex hormone are known to regulate metabolism and adiposity in men and women (335). Estrogens are known to promote subcutaneous fat accumulation and loss of estrogens in menopausal women is associated with increased abdominal fat accumulation (336). Ovariectomy of female rats increases adiposity and administration of estrogen pellets to ovariectomized female mice changed body fat distribution to resemble that of intact female mice (337). Progesterone and androgen receptors are also expressed in adipose tissues , where ERs and PRs expression is higher in subcutaneous fat (338) and visceral adipose tissue has higher AR expression (339). Deletion of AR expression in murine adipose tissue increased subcutaneous fat and hyperleptinemia and increased estrogen levels in adipose tissue (340). Post-menopausal women have a greater risk of developing obesity-associated health complications compared to women of reproductive age (335) , thus it likely that the emergence of this late-onset obese asthma phenotype may coincide with menopause in women. Th European Community Respiratory Health Survey (ECHRS 1) determined that asthma risk was increased in lean women taking hormone replacement therapy (HRT), but no difference in asthma risk was observed in pre-menopausal and post-menopausal women not taking HRT (202). In a second ECHRS trial, women who were in menopause transition, head lower lung function and increased asthma exacerbations compared to post-menopausal women (203). These studies demonstrate the role of fluctuating levels of sex hormones asthma exacerbations, but more studies are needed to

determine if the development of this late onset obese-asthma phenotype is related to menopause transition in women or factors and mechanisms associated with adiposity.

6.2.8 Use of Asthma Biologics in Men and Women with Asthma

Current asthma therapies, including corticosteroids, are very effective for asthmatics with mild/moderate symptoms, however patients with more severe asthma phenotypes are less responsive to corticosteroids (3). Therefore, development of novel therapeutics for individuals with uncontrolled severe asthma is urgently needed. Given the important roles of type 2 cytokines and IL-17A in mediating eosinophilic and neutrophilic mediated airway inflammatory responses, these cytokines have been heavily targeted as therapeutic targets for patients with severe asthma. Some patients with severe asthma are highly responsive to these anti-inflammatory pharmacotherapies, including monoclonal therapies like omalizumab for neutralization of IgE, dupilumab, for inhibition of both type 2 and IL-13 signaling and various IL-5/ IL-5 receptor monoclonal therapies (100).

Our studies suggest that sex hormone-mediated differences in allergic type 2 and non-type mediated airway inflammation may have implications for the design of clinical trials in patients with asthma. In chapter 3, we showed that ovarian hormone signaling in females increased type 2 cytokine expression and increased serum IgE levels compared to males. Conversely, our data showed that testosterone decreased type 2 cytokine expression and IgE levels, thus it likely that men with low testosterone levels may require the same dose of these biologics administered to women. Other factors to include in the use of these biologics for women and men with asthma is the increased prevalence of obese-asthma in women. Obesity is associated with increased neutrophilic mediated inflammation (341), and we have previously

shown that female mice have increased Th17-cell mediated airway inflammation compared to male mice (18). Therefore, women with obese-asthma may be less responsive to these biologics compared to lean women because of increased neutrophilic airway inflammation.

Patients with severe asthma may have airway inflammation mediated by both type 2 and non-type 2 cytokines, including IL-17A and IFNγ and are more resistant to corticosteroids (342). Efforts to identify potential therapeutic strategies for patients with these inflammatory signatures should focus on understanding the reciprocal crosstalk that has been identified between the type 2 and IL-17A inflammatory pathways. Our group reported that human and mouse Th17 cells express a functional IL-13 receptor (IL-13R) and that recombinant IL-13 treatment of naïve CD4 T cells at the time of Th17 cell differentiation attenuated IL-17A and IL-22 protein expression vi an IL-10 dependent manner (255, 256, 343). Furthermore, other groups have also reported that compared to primary human epithelial cells treated with IL-13 alone, those exposed to both IL-13 and IL-17A had increased IL-13-induced gene expression and increased STAT6 activation (163). Combined these studies suggests reciprocal interplay between the type 2 and IL-17A inflammatory pathways, where type 2 cytokines inhibit IL-17A expression but IL-17A boosts type 2 cytokine-mediated inflammatory responses. As anti-IL-4R therapy, dupilumab, is known to inhibit IL-4 and IL-13 signaling. Future clinical trials for patients with persistent mixed eosinophilic and neutrophilic mediated asthma should assess the efficacy of administrating dupilumab plus an anti-IL-17A and/or an anti-CXCL8 antibody. This clinical approach would assess the role of dual inhibition of type 2 cytokines and IL-17A on dual type 2 and IL-17A-mediated airway inflammation.

6.2.9 Implications for Patients with Steroid Resistant Asthma

Neutrophilic and mixed eosinophilc and neutrophilic-mediated airway inflammation is associated refractory severe asthma. Corticosteroids, including glucocorticoids are known to inhibit Th2 cells and eosinophilic-mediate airway inflammation it but Th17 cells and neutrophils are resistant to glucocorticoids treatment. (161, 73, 344). Given that Th17 cells production of IL-17A contributes to neutrophilic-mediated airway inflammation and IL-17A production is increased in women with severe asthma compared to men with severe asthma, it is possible that sex hormone signaling may modulate glucocorticoid resistance in Th17 cells. Glucocorticoids, suppress inflammation by via glucocorticoid receptor (GR)-mediated cell death (345), and a sexual dysmorphism in GR expression exists in men and women. MFI expression of GR is increased in CD3+ T cells, CD14+ monocytes, and CD16+ granulocytes from healthy men compared to healthy women. Furthermore, serum testosterone levels were significantly correlated with GR expression in CD4 T cells and NK cells (346). In chapter 4, we found that AR mRNA levels were significantly increased in differentiated Th17 cells from male mice compared to Th17 cells from female mice. Based on these findings, it is likely that GR activation, following corticosteroid treatment may promote AR expression in Th17 cells, leading to decreased IL-17A protein expression in males compared to females. Since GR and AR are increased in CDT cells from males compared to females, it is likely that glucocorticoid stimulation promotes increased AR activation in Th17 cells from male mice compared to Th17 cells from female mice, leading to increased glucocorticoid-resistance in Th17 cells from female mice.

6.2.10 Implications for the use of Androgens in Asthma

The immunosuppressive effects of testosterone and proinflammatory effects of female sex hormones potentially have therapeutics implications for patients with type 2 and IL-17A mediated airway inflammation. Androgens with anti-inflammatory effects but non-virilizing properties may potentially serve as therapeutics options for pre-pubescent boys and women with severe asthma. Adrenal androgens, including dehydroepiandrosterone (DHEA), are produced both females and males have non-virilizing properties. A phase IIa clinical trial showed that inhalation of dehydroepiandrosterone-3-sulfate (DHEAS) to females with moderate-severe asthma, improved their asthma control scores and trended for improved proportions of symptom-free days and nights (271). The utilization of non-virilizing in other Th17-associated diseases such as systemic lupus erythematosus (SLE) has been tested in small clinical trials. In a study of 50 female patients with mild-moderate SLE treated with DHEA daily, women reported decreased disease activity after 6 months-one year (347). Only 34 women completed the trial, where acneiform dermatitis was sites as the most common reason for discontinuing therapy (347). Similarly, therapeutic that aim to interfere female sex hormone actions at a level that would not be detrimental general activity elsewhere in the body are potential therapeutic targets for women. Currently the FDA approved ERα/β antagonist tamoxifen is utilized for the treatment of ER+ breast cancer, thus the development of lung tissue specific ER antagonists in asthma could be potential therapeutic option for women (348).

An alternative therapeutic approach is gender-specific asthma therapies for women with severe asthma and for men with low-testosterone levels. Clinical data shows that men with rheumatoid arthritis (RA) with low testosterone levels have increased disease severity compared to men with normal serum testosterone levels (349). Clinical studies showed that testosterone

replacement therapy in these patients increased numbers of CD8 T cells, decreased IgM rheumatoid factor, and decreased the number of affected joints and lowered usage of anti-inflammatory therapeutics (349). Studies further show that men with other autoimmune diseases including SLE, Sjogren's syndrome and Klinefelter's syndrome go into clinical remission after undergoing testosterone replacement therapy (350). More studies in patients with asthma are needed to further evaluate a potential protective role for testosterone as well as provide further understanding of the mechanisms driving the sex bias in asthma prevalence.

6.3 Closing

This dissertation work demonstrates the underlying mechanisms by which testosterone and ovarian hormones, including estrogen, regulate type 2 and IL-17A mediated airway inflammation and Th17 cell differentiation. These studies showed that testosterone signaling through AR was important for attenuating airway inflammation by decreasing cell numbers of pro-inflammatory Th2 and Th17 cells, suggesting that higher testosterone level in males potentially has a protective role in severe asthma. In contrast, estrogen signaling through ERα increased IL-17A protein expression in Th17 cells by increasing IL-23R surface expression, suggesting that high estrogen levels in females with severe asthma may promote IL-17A production in Th17 cells and potentially increase the prevalence of steroid resistant Th17-cell mediated severe asthma in females compared to males. Our findings are important in that they can inform clinical trials to personalize asthma therapeutics strategies for patients with asthma including women menstruating and menopausal women, men with low testosterone levels. Moreover, our findings will provide the foundational mechanisms required for potential future clinical trials focused on assessing the protective role of testosterone in patients with asthma.

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