Sex and sex hormone-dependent modulation of allergen-induced type 2 immunity following early-life Respiratory Syncytial Virus infection

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ABSTRACT

Respiratory syncytial virus (RSV) is one of the leading causes of acute lower respiratory tract infection (LRTI) in children. Most children are infected at least once with RSV by the age of two, and many are re-infected before the age of five. While a large proportion of infected children experience mild symptoms, others need to be hospitalised due to the severity of the infection. Roughly 200 000 deaths occur worldwide yearly due to RSV, nearly all of which are in developing countries that cannot provide adequate supportive care. Following severe RSV infection, some children develop recurrent childhood wheeze and asthma. Several studies indicate that a causal relationship may exist between severe LRTI with RSV during early-life and the development of asthma. The pathogenesis of both diseases involves innate immunity, including the innate cytokine interleukin (IL)-33 as well as innate cells such as eosinophils and group 2 innate lymphoid cells (ILC2s). The connection between these two diseases remains poorly understood. Importantly, a well-established sex difference exists in the prevalence of asthma. Before puberty, asthma is more prevalent in young males, yet post-puberty asthma is more prevalent in females. Sex differences in RSV infection are less clear; however, being a young male has been identified as a risk factor for severe disease. Prior to sexual maturity, sex hormones have been largely overlooked as factors in pathology, though they can also shape immunological responses before puberty.

We have established a murine model to examine early events to allergen exposure after early-life infection with RSV. Postnatal day (PND) 10 mice were infected with RSV and 10 days later were exposed to house dust mite (HDM) on 2 consecutive days. Inflammatory responses were examined 72h later. Eosinophil responses were increased only in female mice exposed to RSV→HDM. While ILC2 and IL-13^{+} T cell responses were enhanced in both sexes following RSV→HDM, females had significantly greater numbers of IL-13-producing ILC2s and T cells. Levels of IL-33 released by HDM exposure were greater in all mice previously infected with RSV, though a sex difference was not apparent. When the time between RSV infection and HDM exposure was extended from 10 days to one month, eosinophil responses were still preferentially increased in RSV→HDM exposed female mice. In addition, all RSV-infected females had increased ILC2 numbers, yet only RSV→HDM exposed females had airway hyperresponsiveness (AHR).

In order to better understand the importance of sex hormones in these responses, estrogen and testosterone were manipulated in pregnant females and/or neonatal mice, *prior to* RSV infection.

In males, blocking or providing exogenous androgens had no effect on responses to sequential RSV→HDM exposure. In female RSV→HDM exposed mice, the effects of manipulating androgens were mixed: blocking androgens reduced eosinophil numbers but providing exogenous androgens reduced IL-13^{+} T cells. The effects of estrogen manipulations were more consistent on the other hand: blocking estrogen signalling led to a decrease in both ILC2s and T cells producing IL-13, in female RSV→HDM mice while providing exogenous estrogen increased ILC2s and IL-13^{+} T cells in male RSV→HDM mice. In summary, our findings demonstrate that innate events, including estrogens present in late fetal development and in neonates, promote a type 2 primed environment following subsequent RSV infection. These results contribute to our understanding of the long-lasting effects of early-life viral infection in both sexes.

RÉSUMÉ

Le virus respiratoire syncytial (VRS) est l'une des principales causes d'infection aiguë des voies respiratoires inférieures (IVRI) chez les enfants. La plupart des enfants sont infectés au moins une fois par le VRS avant l'âge de deux ans, et beaucoup sont réinfectés avant l'âge de 5 ans. Alors que beaucoup d'enfants infectés présentent des symptômes légers, d'autres sont hospitalisés. Environ 200 000 décès surviennent chaque année dans le monde à cause du VRS. Presque tous les décès surviennent dans des pays en développement qui ne peuvent pas fournir des soins de soutien adéquats. À la suite d'une infection grave par le VRS, certains enfants développent la respiration sifflante récurrents et l'asthme. Plusieurs études indiquent qu'une relation causale peut exister entre une IVRI sévère avec VRS au début de la vie et le développement de l'asthme. La pathogenèse des deux maladies implique l'immunité innée, notamment la cytokine innée interleukine (IL)-33 ainsi que des cellules innées comme les éosinophiles et les cellules lymphoïdes innées du groupe 2 (ILC2). Le lien entre ces deux maladies reste mal compris. Il existe une différence bien établie entre les sexes dans la prévalence de l'asthme. Avant la puberté, l'asthme est plus répandu chez les jeunes mâles, mais l'asthme post-puberté est plus répandu chez les femelles adultes. Les différences entre les sexes en matière d'infection par le VRS sont moins claires ; cependant, le fait d'être un jeune mâle a été identifié comme un facteur de risque de maladie grave. Les hormones sexuelles sont souvent négligées en tant que facteurs participant à la pathologie jusqu'à l'âge adulte, même si elles peuvent également façonner les réponses immunologiques.

Nous avons établi un modèle murin pour reproduire une infection précoce par le VRS suivie d'une exposition aiguë à un allergène. Au jour postnatal (JPN) 10, les souris ont été infectées par le VRS par voie intranasale et 10 jours plus tard ont été exposées par voie intranasale à des acariens (AC) pendant 2 jours consécutifs. Les réponses des éosinophiles n'ont augmenté que chez les souris femelles exposées au VRS→AC. Les réponses des lymphocytes T IL-13^{+} et ILC2 étaient augmentées chez les deux sexes après VRS→AC, mais les femelles présentaient un nombre significativement plus élevé de lymphocytes T et ILC2 produisant d'IL-13. Les niveaux d'IL-33 libérés par l'exposition au AC étaient plus élevés chez toutes les souris précédemment infectées par le VRS, mais aucune différence entre les sexes ne soit apparente. Lorsque le délai entre l'infection par le VRS et l'exposition au AC était prolongé de 10 jours à un mois, les réponses des éosinophiles étaient encore préférentiellement augmentées chez les souris femelles exposées au VRS→AC. De plus, toutes les femelles infectées par le VRS présentaient une augmentation du

nombre d'ILC2, mais seules les femelles exposées au VRS→AC présentaient l'hyperréactivité des voies respiratoires (HVR). Afin de déterminer l'importance des hormones périnatales et néonatales, les œstrogènes et la testostérone ont été manipulés. Chez les mâles, le blocage ou l'administration d'androgènes exogènes n'a eu aucun effet sur les réponses au VRS→AC. Chez les souris femelles exposées au VRS→AC, le blocage des androgènes a réduit le nombre d'éosinophiles et l'apport d'androgènes exogènes a réduit les cellules T IL-13^{+}. Le blocage de la signalisation des œstrogènes a entraîné une diminution du nombre de cellules IL-13^{+}, à la fois des cellules ILC2 et T, chez les souris femelles VRS→AC, alors que des œstrogènes exogènes ont augmentés des cellules T IL-13^{+} et ILC2 chez les souris mâles VRS→AC. En résumé, nos résultats démontrent que les événements innés, y compris les œstrogènes prépubères, favorisent un environnement amorcé de type 2 après une infection par le VRS au début de la vie. Ces résultats contribuent à notre compréhension des effets à long terme de l'infection virale chez les deux sexes.

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LIST OF ABBREVIATIONS

AEC	Airway epithelial cell
AHR	Airway hyperresponsiveness
AM	Alveolar macrophage
ANOVA	Analysis of variance
AR	Androgen receptor
ATAC-seq	Assay for transposase-accessible chromatin using sequencing analysis
BAFF	B-cell activating factor
BAL	Bronchoalveolar lavage
BMDC	Bone marrow-derived dendritic cell
cDC	Conventional dendritic cell
CLR	C-type lectin receptor
COPD	Chronic obstructive pulmonary diseas
CRA	Cockroach antigen
CRSwNP	Chronic rhinosinusitis with nasal polyps
CTL	Cytotoxic lymphocyte
˚C	Degree Celsius
DC	Dendritic cell
DEHP	Di(2-ethylhexyl) phthalate
DHEA	Dehydroepiandrosterone
DTR	Diphtheria toxin receptor
DTx	Diphtheria toxin
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
EOS	Eosinophil
EPO	Eosinophil peroxidase
EU	European Union
F	Fusion protein
FACS	Fluorescence-activated cell sorting

FBS	Fetal bovine serum
FI-RSV	Formalin inactivated-RSV
FMO	Florescence minus one
FOT	Forced oscillation technique
FSC	Forward Scatter
G	Attachment protein
GWAS	Genome-wide association studies
HCP	Health care professional
HDM	House dust mite
HRT	Hormone replacement therapy
ICS	Inhaled corticosteroids
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
ILC2	Group 2 innate lymphoid cell
IM	Interstitial macrophage
IN	Intranasal
IP	Intraperitoneal
KLRG1	Killer cell lectin-like receptor subfamily G member 1
Lin	Lineage
LN	Lymph node
LRT	Lower respiratory tract
LRTD	Lower respiratory tract disease
LRTI	Lower respiratory tract infection
MATISSE	MATernal Immunization Study for Safety and Efficacy
MFI	Mean fluorescence intensity
mG	Membrane bound G protein
mg	Milligram
MHC-II	Major histocompatibility complex class II
mM	Milimolar

mL	Milliliter
ng	Nanogram
NOD	Nucleotide-binding oligomerization domain
NLR	NOD like receptor
nt	Nucleotides
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
pDC	Plasmacytoid dendritic cell
PND	Post natal day
PRR	Pattern recognition receptor
RV	Rhinovirus
RIG	Retinoic acid-inducible gene
RLR	RIG-like receptor
RORα	Retinoid-related orphan receptor alpha
RSV	Respiratory syncytial virus
RSVPreF3 OA	AS01E-adjuvanted RSV prefusion F protein–based vaccine
RT	Room temperature
sG	Soluble G protein
SH	Small hydrophobic
SEM	Standard error of the mean
Siglec-F	Sialic acid-binding Ig-like lectin F
SNP	Single nucleotide polymorphism
SSC	Side scatter
Tc2	Type 2 cytotoxic T cell
Tfh	T follicular helper
Th	T helper
TNF	Tumor necrosis factor
TLR	Toll-like receptor
TSLP	Thymic stromal lymphopoietin

μg	Microgram
μl	Microliter
URT	Upper respiratory tract
XCI	X chromosome inactivation
Xi	Inactive X chromosome

CONTRIBUTIONS OF AUTHORS

The candidate has chosen to present a manuscript-based thesis. This thesis contains two original manuscripts and is in accordance with the guidelines for thesis preparation provided by the Faculty of Graduate and Postdoctoral Studies of McGill University. Lydia Labrie (LL) is recognized as the principal author and to have performed the majority of the work in the manuscripts presented. The specific contributions of authors are as follows:

CHAPTER 1

The literature review was written by LL and revised by EDF and BJW.

CHAPTER 2

LL and EDF were responsible for designing the experiments as well as writing the manuscript. Experiments were performed primarily by LL with assistance from HA, RK, and VG. BJW participated in experimental design.

This manuscript was submitted to PLOS Pathogens.

CHAPTER 3

LL and EDF were responsible for designing the experiments as well as writing the manuscript. Experiments were principally performed by LL with assistance from RK who also provided edits to the manuscript. BJW participated in experimental design.

This manuscript is being finalised for submission.

CHAPTER 4

The general discussion was written by LL and revised by EDF and BJW.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The work presented in this thesis contributes original knowledge to the fields of type 2 inflammation in the lung linked to viral infection and murine sex differences. The specific contributions are as follows:

To date, various murine models have been developed to understand how RSV infection can induce enhanced responses to allergens. However, these models are generally in adult mice and mainly focus on the impact of RSV infection on adaptive immune responses after allergen sensitization and challenge. There are few models using young mice to explore how RSV alters immune events induced within days of allergen exposure. In chapter 2, we demonstrate that early-life RSV infection leads to increased numbers of innate cells, eosinophils and ILC2s, in addition to T cells following an acute exposure to HDM, a common allergen. These responses were present whether HDM was delivered within days or after several weeks after RSV infection. Many responses, such as innate cell numbers and AHR, were enhanced specifically in female mice, but sex differences were not linked to the innate cytokine, IL-33. This chapter sheds light on possible mechanisms underlying the subsequent predisposition to enhanced allergen responses after viral clearance and highlights potential sex-specific effects of early-life viral infection on the developing neonatal lung.

Although sex differences are well established in many diseases and can often be linked to sex hormones, such as estrogen and testosterone, in adults, various diseases begin in childhood and display sex differences even before the onset of puberty. The impact of early-life hormones on establishing immunological sex differences in disease is poorly understood. In chapter 3, we demonstrate the important role of sex hormones present before puberty in shaping responses in RSV→HDM mice. Blocking neonatal estrogens reduced ILC2s and T cells with the ability to produce IL-13 selectively in female mice. In contrast, treatment with exogenous estrogens increased ILC2s and T cells in male mice. Overall, removing estrogens masculinized female-pattern responses in RSV→HDM females, while providing exogenous estrogens feminised responses to RSV→HDM in males. On the other hand, when androgen signaling was modified, no changes in inflammatory responses in males were present and only minor effects in female mice

were noted, results that were unexpected based on data from adults showing that androgens inhibit ILC2s in adult males.

CHAPTER 1

Literature Review and Research Objectives

1.1 Respiratory Syncytial Virus (RSV)

1.1.1 Virology

Respiratory syncytial virus is a non-segmented, negative-sense, single-stranded, enveloped RNA virus. It belongs to the family of Paramyxoviridae, genus Pneumovirus, subfamily Pneumovirinae. RSV virions can be spherical, asymmetrical or filamentous (1). The genome is roughly 15 200 nucleotides (nt) in length (2), comprised of 10 genes that encode 11 proteins. Starting from the 3' end, these genes are non-structural proteins NS1 and NS2; nucleocapsid proteins N and P; inner envelope membrane protein M1; surface proteins small hydrophobic (SH), attachment protein (G), and fusion protein (F); M2 mRNA contains overlapping open reading frames (ORF), which encode three proteins, M2.1, M2.2 and large (L) protein (3). Figure 1.1 shows the typical morphology of the RSV virion and the viral genome.

G and F are important antigenic proteins: G functions in host cell attachment and the F functions in fusion and cell entry (4). G can be membrane bound (mG) as well as soluble (sG) (5). *In vitro* and *in vivo* murine data show that sG is detectable early post-infection and may act as an antigen decoy thereby inducing maladaptive immune responses (5). G contains a conserved CX3C chemokine motif, which binds CX3CR1 on human respiratory epithelial cells (ECs) as well as certain immune cells, including macrophages, monocytes, and dendritic cells (DC)(6). Following mG attachment to the targeted cell, F mediates fusion to allow viral entry (4). Fusion is initialed by the pre-fusion protein binding host cell receptor leading to a conformational change that fuses the virion to the host cell and forms a fusion pore (7) through which viral RNA enters the host cytoplasm and transcription of the genes encoding N, P, and L is initiated (8). New virions are assembled in the cytoplasm and either bud from the cell surface or fuse with the cell membranes of neighbouring cells to form syncytia (ie: giant cells) (9).

RSV is transmitted via respiratory droplets or fomites and infects the upper respiratory tract (URT) through the nasopharyngeal or conjunctive mucosa (10). RSV can then spread to the lower respiratory tract (LRT) in some cases where it infects polarized ciliated human airway epithelial

cells (AECs), which causes lower respiratory tract infection (LRTI), bronchiolitis and pneumonia (11-13). Human studies have also reported the ability of RSV to infect dendritic cells (14), T cells (15), alveolar macrophages (16), as well as other neighboring immune cells.

1.1.2 Epidemiology

The only natural hosts for human RSV are humans and chimpanzees, although various small animal species can also be infected experimentally (17, 18). There are two major antigenic subgroups of RSV, A and B. Co-circulation of strains of both subtypes is common, although usually one predominates (19). Molecular analyses show that in any given season and region, several genotypes can be present, yet the strains circulating in nearby regions may be different (20). There is a seasonality with RSV outbreaks in temperate regions. Onset in North America typically occurs in late fall or early winter with a peak incidence between December and February and a gradual decline in cases in late spring (21). In northern Europe, yearly outbreaks alternate between those that are early and large and those that are late and small. In contrast, in tropical regions, the RSV outbreaks are much less predictable (21). The overall incidence and severity of RSV disease in any given year varies widely and some years are considered to be 'epidemics'. Why RSV disappears between epidemics is still unclear, however this periodicity has been linked to the level of maternally-derived RSV neutralizing antibodies that decline in newborns and infants with time since the last epidemic (22).

In most healthy adults, RSV infections are asymptomatic or cause mild cold-like symptoms, such as runny nose, cough, low-grade fever and nasal congestion (23). Disease burden caused by RSV infection is highest in infants, immunocompromised individuals, and the elderly (24, 25). Nearly all children are infected with RSV at least once by the age of two (26). Worldwide, an estimated 55 000 to 200 000 deaths in children under 5 years of age are caused by RSV annually, the majority of which occur in low-income countries unable to provide adequate supportive care (27, 28). RSV is also one of the leading causes of infant hospitalization. For example, in the European Union (EU), RSV infection is responsible for 10 out of every 1000 children hospitalised annually (29). RSV is the leading cause of acute lower respiratory tract infection in infants and is characterised by bronchiolitis, interstitial pneumonitis, alveolitis, and severe apnea (30). In the elderly population, RSV infection is characterised by pneumonia, bronchiolitis, congestive heart failure, worsening of asthma or chronic obstructive pulmonary disease (COPD) and is a major

factor requiring medical intervention (31, 32). In addition, in elderly and high-risk adults (those with chronic heart or lung conditions), the disease burden of RSV is similar to that of non-pandemic influenza (32).

During the COVID-19 pandemic, the incidence of RSV infection was very low, likely in connection to hygiene practices put in place, social distancing, and school closures (33, 34). In Australia where lockdown measures were the strictest, RSV cases were reduced by 68.8% to 100%, depending on the region (33).

Reinfection rates with RSV are high, particularly in children under 2 years of age. It has been estimated that 30-75% who experience RSV infection during their first year of life will experience reinfection the following season (26, 35). Secondary infections are typically symptomatic in young children, however severity decreases with each exposure and fewer children develop bronchiolitis (35). Reinfection is common throughout older childhood and adulthood as well; however, symptoms are typically absent or restricted to the upper respiratory tract (36).

1.1.3 Immune response

Disease pathogenesis is often linked to viral replication and cytotoxicity. However, as for other viral infection, it is equally important to consider that immunological mechanisms may also be an important factor in the severity of disease and disease progression.

1.1.1.1 Innate Immunity

The innate immune response is the first-line of defense against pathogens. This early and rapid response relies on germline-encoded receptors, pattern recognition receptors (PRR) that recognise and respond to pathogen-associated molecular patterns (PAMP) conserved amongst pathogens (37). PRR-PAMP interactions following RSV infection trigger a signalling cascade that induces transcription factors to upregulate antiviral cytokines, such as type I (IFNα, IFNβ) and type III (IFNλ) interferons (IFNs), as well as pro-inflammatory cytokines, such as IL-6, IL-1 and tumor necrosis factor alpha (TNFα), which act on innate immune cells (38, 39).

1.1.1.1.1 Neutrophils

Neutrophils represent the most prominent leukocyte in the airways during RSV bronchiolitis (40). In RSV infected infants, neutrophils represented approximately 93% of cells in the URT and 76% in the LRT (40). During *in vitro* RSV infection, human respiratory epithelial

cells and macrophages produce IL-8 which drives neutrophil chemotaxis (41). Human studies show that genetic polymorphisms near the IL-8 gene are associated with disease severity (42); that nasopharyngeal IL-8 concentrations correlate with disease severity (43); and that both serum and bronchoalveolar lavage (BAL) samples have elevated IL-8 levels in infants with RSV bronchiolitis (44). Through binding of TLR4, RSV F can initiate NETosis in human neutrophils, a form of cell death where neutrophils release decondensed chromatin and granular contents leading to inflammation (45). Murine studies suggest that RSV G may also increase pulmonary neutrophils through interaction with TLR2, leading to increased expression of the CCL2 neutrophil chemokine (46).

1.1.1.1.2 Eosinophils

Several groups have shown that eosinophil cationic protein (ECP) is elevated in the serum and lower airway secretions of RSV infected patients (47, 48). During human RSV infection, eosinophil degranulation has been reported in the nasopharynx and lung parenchyma, and increased levels of ECP in the circulation have been associated with wheeze (49). Following RSV exposure in mice, increased IL-5 promotes lung eosinophil recruitment, which then leads to AHR (50). Most murine models of RSV suggest that the increase in eosinophils is linked to RSV G (51, 52). G peptides that contain a CX3C motif can induce IFNγ and IL-5 production in primed murine splenocytes and peripheral blood mononuclear cells (PBMCs) (53). While some murine models have shown that sG may link enhanced pulmonary eosinophilia with reduced RSV clearance (52), others have shown that eosinophils are protective during RSV infection by promoting RSV clearance (54). The role of eosinophils in RSV disease may be multifactorial and much remains to be understood in the contribution of eosinophils to pathogenesis.

1.1.1.1.3 Macrophages

Human alveolar macrophages, one of the first immune cells to encounter RSV during infection, respond by secreting cytokines including IL-1β, IL-6, IL-8, IL-10, IL-12 and TNFα3, which increase vascular permeability and activation/recruitment of lymphocytes as well as other innate immune cells (55, 56). In a study of ventilated children following LRT RSV infection, a modest monocyte-derived IL-12 response was inversely related to ventilation duration indicating that this antiviral cytokine may reduce disease severity (57).

1.1.1.1.4 Natural Killer Cells

In human infants, severe RSV disease correlates with single-nucleotide polymorphisms (SNPs) that increase a subset of NK cells expressing leukocyte immunoglobulin-like receptor B1 (LILRB1)(58). During the first few days following RSV infection in adult female mice, NK cells producing IFNγ accumulate in the lung (59). The exact role of these innate cells in RSV pathogenesis has yet to be fully defined; however, data from murine models suggest that NK cells are detrimental. For example, NK cell depletion reduces disease severity (60) and enhancing NK cell activation through IL-18 results in enhanced disease (61).

1.1.1.1.5 ILC2s

Group 2 innate lymphoid cells (ILC2s) produce type 2 cytokines and are activated by innate cytokines, such as IL-33, IL-25 and TSLP, as well as lipids and other mediators, such as prostaglandin D2 (62). Infants hospitalised for severe RSV infection have increased levels of ILC2s in nasal aspirates as well as increased IL-33 and IL-25 (63). Following RSV infection, ILC2 numbers in the lungs of neonatal mice are much higher than in adult mice (30). Additionally, the induction of these murine ILC2s was found to be dependent, directly or indirectly, on IL-33 levels (30). These findings suggest one possible mechanism by which disease severity may be enhanced in (human) infants compared to adults.

1.1.1.1.6 Dendritic cells

Typically, in an effective anti-viral response, dendritic cells (DCs) direct $CD4^+$ T cells toward an antiviral Th1 response, which leads to IFNγ production and viral clearance. However, murine and *in vitro* human cell data show that RSV dampens Th1 responses, at least in part, through inhibition of type 1 IFNs (via structural proteins NS1 and NS2) and antigen decoys (sG). Without type-1 immunity, DCs express co-stimulatory molecules, such as OX40L, and produce cytokines and chemokines that skew the immune response towards Th2 (2, 64, 65). Although the number of DCs increases in the lungs of both humans and mice upon RSV infection (66, 67), different DC subsets appear to have varying impacts on disease progression. The plasmacytoid DC (pDC) population demonstrates a protective role in both animal models and human studies through IFNα production, which promotes viral clearance, and by reducing Th2 responses, including AHR and mucus production (68, 69). Conversely, infants hospitalised with RSV infection have increased levels of conventional DCs (cDCs), which correlate with disease severity (69).

1.1.1.2 Adaptive Immunity

The adaptive immune response follows the innate immune response and is characterised by immunological memory, which allows rapid and robust secondary responses to previously encountered pathogens.

1.1.1.2.1 Humoral

Humoral immunity largely encompasses B-cell responses and antibody production. F and G both induce neutralizing antibodies in mice (70). In murine RSV infection, RSV-specific IgM is produced first followed by IgG in the serum and RSV-specific IgA detectable in BAL fluid (71). In humans, IgM is present within a few days, while IgG appears by week 2 (72). In very young infants, IgA production is unexpectedly variable and may not be present in respiratory secretions at all (72). In children, both IgG and IgA provide protection against infection (73). Infants infected with RSV exhibit increased circulating B cells as well as increased B cell-activating factor (BAFF) (74). Similar findings have also been reported in mice showing increased BAFF and CXCL13, a B cell chemoattractant, in the lung (75). Younger infants have immature somatic hypermutation (SHM) capability, which limits the antibody repertoire. However, with age, SHM and immunoglobulin class switching increase (76). Without the humoral response during RSV infection, animal models demonstrate that lung inflammation and reinfection are more severe, but viral clearance is not impacted (77).

Due to the transfer of maternal immunoglobulin through the placenta, most newborns have at least some RSV-specific antibodies at birth. However, the most severe cases of RSV occur between 2-6 months of age, when maternal antibodies should still be present, raising questions about the effectiveness of these maternal antibodies (72). There is some evidence that RSV IgE is present in nasal secretions of infants with acute bronchiolitis (78), though this observation hasn't been consistent between studies (79).

1.1.1.2.2 Cell-mediated

Cell-mediated immunity depends on both $CD8^+$ cytotoxic T lymphocytes as well as T helper cells. The importance of cell-mediated immunity in RSV infection is evident from studies of children lacking cellular immunity who shed virus for months and are at increased risk for severe disease, compared to immunocompetent individuals who can clear the virus within weeks even with severe infection (72).

Cytotoxic lymphocytes (CTL) directly kill virally-infected cells, though they can contribute to pathogenesis, as shown in murine models (80). In addition to their role as CTLs, $CD8^+$ T cells can produce type 2 cytokines, such as IL-4, and in infants, IL-4^+ $CD8^+$ T cells are associated with increased RSV disease severity (81).

T helper cells assist in B cell maturation and activate CTLs and macrophages. Th1 responses are associated with IFNγ, which stimulates macrophage activation and IL-2 production, while Th2 responses are associated with IL-4, which promotes IgE class switching; IL-5, which promotes eosinophil maturation; and IL-13, which promotes mucus production and AHR (82-84). Th17 responses are associated with IL-17A, which contributes to airway damage, AHR, and airway inflammation (85). T helper cell responses are dependent on which RSV antigen is encountered. For example, mice primed with vaccinia virus expressing RSV F induce Th1 responses with robust CTL activity while mice primed with vaccinia virus expressing RSV G induced a Th2 response accompanied by eosinophil recruitment to the lung upon RSV challenge (86).

In summary, following RSV recognition through PRR-PAMP interactions, an immune response is triggered. In humans, this is characterised by an initial strong neutrophil response. Other innate cells such as macrophages, eosinophils, NK cells, and ILC2s contribute to the early inflammatory response through the release of cytokines and chemokines. DCs then migrate to the lymph nodes to activate RSV-specific T cells, upon which $CD8^+$ T cells mediate viral clearance. Antibodies contribute to protection, although these responses can be weak and transient, leading to frequent reinfection. Th1 responses leading to IFNγ are generally protective, while Th2 responses can be deleterious (87).

1.1.4 Treatments

In most cases of acute bronchiolitis in infants or children severe enough to require hospitalisation, high-quality supportive care is important to minimize morbidity and mortality. Nasal suction, nasogastric or intravenous fluids, supplemental oxygen and nasogastric feeding are the standard of care (88). Bronchodilators are also commonly administered although the positive impact of such treatments has not been conclusive (89). In adult or elderly populations, treatments are also largely supportive in nature and include intravenous hydration, oxygen and corticosteroids to reduce symptoms and patient discomfort (90).

1.1.1.3 Palivizumab

Palivizumab is a monoclonal antibody directed against the RSV F that acts to block fusion of the virus with host cells (91). In 1998, it was approved by the FDA for high-risk infants and children to prevent serious LRTI (91). It is administered via intramuscular infection once per month starting one month before the regional RSV season (92). Palivizumab reduces hospitalization by approximately 50%, reduces hospital length of stay by 42%, and reduces days on oxygen by 40%, but the American Academy of Pediatrics does not recommend its use in treatment of wheeze or asthma (93). In Canada, the Canadian Risk-Scoring Tool is used for premature infants to determine if palivizumab should be administered and this system has been shown to be cost-effective and convenient (94, 95).

1.1.1.4 Ribavirin

For high-risk infants, ribavirin is an FDA approved broad-spectrum antiviral agent that can be used in nebulized form in cases of severe bronchiolitis (89). Ribavirin inhibits the replication of DNA and RNA viruses. While the results of meta-analysis indicate that ribavirin may reduce the duration of ventilation and length of hospitalisation, firm conclusions on its effectiveness have not been reached (96). Ribavirin is expensive and can only be used with a specific device, the small particle aerosol generator model-2 (SPAG-2) or via oxyhood or tent (97). These delivery methods have been linked to adverse effects in healthcare workers administering it as well as in nearby family members (97).

1.1.5 Vaccines

After a disastrous vaccine trial in the 1960s, vaccine research has progressed very cautiously for RSV and, until May of this year, there were no RSV-approved vaccines. To this day, no RSV vaccine is approved for use in infants or children.

1.1.1.5 FI-RSV

In the 1960s, an unsuccessful and devastating trial of formalin-inactivated RSV (FI-RSV) took place. Following the FI-RSV vaccination, 80% of children required hospitalisation following acute RSV disease (versus ~5% of the children who received placebo) and 2 vaccinated children died with RSV infection (98). Due to the young age of the infants that experienced the most significant disease, it is hypothesised that the immaturity of the infant immune system or a lack of experience of infection contributed to vaccine-enhanced illness. Studies in mice suggest that the

poor antibody responses may have been linked to the modification of viral proteins by the formalin treatment or failing to have a robust toll-like receptor (TLR) stimulus in FI-RSV (99, 100). Given that these trials were many decades ago; it is difficult to understand precisely the mechanisms that drove the enhanced disease. Tissues collected post-mortem from these infants were initially reported to show excess eosinophils, which is recapitulated in murine models (101), however secondary analysis suggested that neutrophils were predominant in the bronchiolar epithelium (102, 103).

1.1.1.6 Arexvy

In May 2023, Arexvy was approved by the FDA to prevent lower respiratory tract disease (LRTD) caused by RSV in people 60 years of age and older. This vaccine is a $AS01_E$-adjuvanted RSV prefusion F–based vaccine (RSVPreF3 OA) (104). In participants who received Arexvy, risk of developing RSV-associated LRTD decreased by 82.6% and risk of developing severe RSV-associated LRTD decreased by 94.1% (104).

1.1.1.7 Abrysvo

Abrysvo is composed of two preFs to optimize protection against both RSV A and B strains and is unadjuvanted (105). In May 2023, Abrysvo was approved for use in older adults, 60 years of age and older, to prevent LRTD caused by RSV (106). In clinical trials, Abrysvo showed an overall efficacy of 67% in reducing risk of developing RSV-associated LRTD in adults with 2 or more symptoms and 86% efficacy in those with 3 or more symptoms (106). In August 2023, Abrysvo was approved for use in pregnant individuals 32-36 weeks of gestational age. The goal of the vaccine is to protect infants at birth through 6 months of life from LRTD and severe LRTD caused by RSV(105). A phase 3 trial, the MATISSE (MATernal Immunization Study for Safety and Efficacy) reported a vaccine efficacy of 81.8% against severe medically-attended LRTD due to RSV in infants from birth through the first 90 days of life as well as 69.4% efficacy through the first six months of life (105).

1.1.6 Mouse Models of RSV Infection

Evidence of the Th2-biasing effect of RSV in the lung is often obtained using adult mice models (107, 108). However, adult mice are not susceptible to RSV. Therefore, these models require high titers of virus delivery to the LRT (109). In addition, adult mice mount potent Th1 responses to RSV infection leading to rapid viral clearance (110).

Due to the limitations of adult murine models, models of RSV infection in neonates have also been developed. Reinfection models where young mice are infected with RSV during early-life (within 10 days of birth) and reinfected during adulthood, demonstrate that neonatal priming leads to severe weight loss, increased inflammatory cell recruitment (eosinophils and Th2 cells) and enhanced AHR upon reinfection compared to delayed priming (111, 112). Models of neonatal RSV infection followed by exposure to allergens demonstrate that prior RSV infection exacerbates allergic airway disease, leads to long term pulmonary inflammation and induces alternative activation of macrophages (113, 114).

1.2 Asthma

Asthma is a heterogeneous clinical disease that affects all age groups (115). Worldwide, over 300 million people are affected by this chronic inflammatory disease (116). Common symptoms include cough, wheeze, shortness of breath, chest tightness and intermittent attacks of breathlessness (116). These symptoms are caused by lung inflammation, which in turn leads to mucus production, remodeling of the airways and AHR (117).

1.2.1 Epidemiology

Across the globe, the prevalence, severity, and mortality of asthma vary. In low to middle income countries, the prevalence of asthma continues to rise, while it has mostly plateaued in certain developed countries (118). Often asthma begins in childhood, however some individuals also develop asthma later in life. Typically, late-onset asthma is more severe and the association with allergy is lower compared to childhood-onset asthma (117). Traditionally, asthma is divided into two main forms: allergic and non-allergic. Allergic asthma is induced by environmental allergens such as HDM, pollen, and animal dander and often begins in childhood, a crucial period in immune system and lung structural development, but may also begin later in life upon exposure to new allergens (117). Allergic asthma is mainly associated with type 2 immunity. In childhood, asthma is typically controlled through the use of inhaled medications, such as corticosteroids and long-acting beta agonists, but may require more intense therapy if it persists (116). Non-allergic asthma, which typically begins later in life and can be type 2 or non-type 2, is linked to obesity, aging and smoking (119). Despite mortality being infrequent, asthma patients place a heavy burden on the health care system. In the United States, asthma patients account for approximately 10

million office visits, 400 000 hospitalizations and 200 000 emergency room visits leading to an annual economic cost of nearly $20 billion (116). Risks factor for asthma include: deficiency in vitamin D, gastrointestinal and respiratory microbiome, tobacco smoke exposure, air pollution, upper respiratory tract viral infections, as well as genetic factors (120).

1.2.2 Immune response during allergic asthma

The overall immune response during allergic asthma involves first innate immunity through the recognition of the allergen and second adaptive immunity through the activation of allergen-specific Th2 which produce, amongst others, IL-4, IL-5, IL-9, and IL-13 leading to downstream effects such as eosinophil recruitment to the lung, excess mucus production, and IgE synthesis by allergen-specific B cells (117). Airway obstruction is a hallmark feature of asthma and is caused by a decrease in the diameter of the airways. Chronic inflammation leads to the narrowing of the airways and is mediated by infiltration and activation of both innate and adaptive immune cells including DCs, eosinophils, neutrophils, ILCs, lymphocytes and mast cells (117).

1.2.2.1 Innate Immunity

Asthma is initiated at mucosal surfaces where epithelial cells are the first point of contact. The epithelial cell layer serves as both a physical barrier to environmental allergens as well as a collection of immunologically active cells that can initiate inflammatory responses. Many allergens, including HDM and cockroach proteins, possess protease activity with the ability to cleave intercellular junctions, leading to a loss in tight junction proteins and increased permeability (121). Epithelial cells in the lung respond to a plethora of external stimuli through the expression of a variety of PRR such as TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and protease-activated receptors (117). Upon binding of danger-signalling molecules to these PRRs, epithelial cells produce a wide range of chemokines and cytokines. In the context of asthma, arguably the most important cytokines released are “alarmins”, IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) (122).

1.2.2.1.1 IL-33

SNPs in the loci of IL-33 and its cognate receptor IL1RL1 or ST2, have been strongly associated with asthma in numerous genome wide associations studies (GWAS) and candidate-gene based studies, particularly in childhood onset asthma (123, 124). IL-33 is elevated in the

lungs of asthmatics. Bronchial biopsies from asthmatics contain more IL-33^{+} cells compared to healthy controls (125). In addition, the bronchoalveolar lavage fluid (BALF) from asthmatics has increased concentrations of IL-33 compared to non-asthmatic controls and IL-33 levels are inversely correlated with lung function (126). Major targets for IL-33 include mast cells, ILC2s, Th2 cells and eosinophils (127). IL-33 promotes recruitment and activation of these cells, all of which contribute to type 2 inflammatory responses. Current biologics targeting IL-33 are addressed below.

1.2.2.1.2 IL-25

In bronchial biopsies of individuals with chronic asthma, IL-25 mRNA levels are elevated (128). In addition, compared to asthmatics with low-IL-25, those with high-IL-25 mRNA also have greater AHR, increased airway eosinophils, higher IgE levels, and increased expression of Th2 signature genes (129). Murine models of allergic asthma also show that neutralizing antibodies against IL-25, delivered at the time of challenge, reduce Th2 cytokine production and decrease the number of airway eosinophils, airway inflammation and AHR (130-132). Cellular targets for IL-25 include T cells, endothelial cells, mast cells, and eosinophils (133). To date no IL-25 targeting antibodies have proceeded to clinical trials in humans.

1.2.2.1.3 TSLP

TSLP protein is elevated in the epithelial layer of airway biopsies, serum, sputum, exhaled breath condensates, and BALF of asthmatics compared to healthy individuals (134-138). In addition, the level of TSLP protein in asthmatic patients correlates with disease severity and airway obstruction (138). Major cellular targets for TSLP include ILC2s, T cells, mast cells, and eosinophils (139). Interestingly, TSLP, which induces phosphorylation and activation of STAT5, promotes resistance to corticosteroids in a subset of ILC2s (140). Current biologics that target TSLP are discussed below.

1.2.2.1.4 ILC2s

The number of ILC2s, which are activated by IL-33, IL-25 and TSLP, is elevated in the blood, sputum, and BALF of asthmatic patients (141, 142). Through ex-vivo co-cultures of ILC2s with human bronchial epithelial cells it was shown that IL-13 produced by ILC2s could disrupt epithelial cell barrier function (143). In addition, when asthma treatment is successful, blood ILC2

numbers are reduced (144). However, rechallenging with an allergen increases the number of ILC2s expressing type 2 cytokines in the sputum (145).

In murine models, ILC2 accumulation in the allergic lung, especially in early-life, has been linked to the ability of certain allergens, like HDM, to induce IL-33 production by epithelial cells (146, 147). Upon IL-33 stimulation, murine ILC2s produce type 2 cytokines IL-13 and IL-5. The release of these cytokines is associated with lung eosinophilia, AHR as well as increased migrations of DCs to the draining lymph nodes (LNs) leading to induction of Th2 differentiation (148, 149). Murine ILC2s also have the ability to present antigen as they express MHCII and co-stimulatory molecules (150). However, this has largely been shown in parasitic models and this function has yet to be fully elucidated in the lung after allergen exposure. Based on abundant data from murine models, it is considered that human ILC2s have similar functions. On a per cell basis, ILC2s have been shown to produce large quantities of type 2 cytokines, even greater than those produced by T cells (148, 151).

1.2.2.1.5 Eosinophils

In the pathogenesis of type 2 asthma, eosinophils are considered key pro-inflammatory granulocytes. Disease severity is associated with eosinophilic inflammation. A direct correlation exists between tissue/blood eosinophil count and the occurrence of irreversible airway obstruction as well as the number of exacerbations (152). Eosinophils are recruited from the blood to the lung by IL-5 produced from other immune cells. Upon activation, human eosinophils release a variety of cytotoxic proteins such as major binding protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN), which when prolonged leads to damage of lung structural cells (153). Eosinophils also release a variety of other molecules that contribute to the inflammatory cascade. These include Th2 cytokines, acute pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8), chemokines, and lipid mediators, which lead to the hallmark features of asthma such as AHR and goblet cell metaplasia (154). In addition, in murine models, eosinophil-derived IL-4 and IL-13 promote ILC2 accumulation in the allergic lung (155). *In vitro* experiments have shown that eosinophil release of soluble mediators leads to ILC2 proliferation (155). IL-5 and IL-5R biologics are discussed below.

1.2.2.1.6 Dendritic cells

The epithelial layer from the conducting airways to the alveoli is patrolled by a heterogeneous group of DCs and macrophages (156). Following PRR signalling, cytokines activate DCs migration to the draining LN to promote Th2 development. In the lungs, conventional type 2 DCs (cDC2s) are an important subset of DCs that drive Th2 responses. In murine models, this particular subset is targeted by cytokines such as IL-33, TSLP, IL-1α and GM-CSF (157-159). Asthmatic patients have elevated DC numbers in their airways compared to non-asthmatics. In patients with type-2 high asthma, CD1c+ DCs in the lung express the high-affinity receptor FcεRI, which correlates with serum IgE levels in humans (160, 161).

1.2.2.2 Adaptive Immunity

When asthma patients present with high sputum and/or blood eosinophilia they are termed type 2-high patients (162). This form of asthma will be the focus of the following sections. For type 2 asthma, inflammation is driven by Th2 cells that produce IL-13, IL-5 and IL-4.

1.2.2.2.1 T cells

Type 2 asthma is considered to be Th2 driven, given that allergen-specific Th2 cells as well as IL-4, IL-5 and IL-13 are present in the BALF, blood, sputum, and lung of allergic asthmatic patients (163-166). In both humans and mice, Th2 cells demonstrate similar gene expression including specific receptors recognizing epithelial-derived signals (e.g. ST2, TSLPR, and IL-25R) and the nuclear receptor PPAR-γ, which upregulates expression of the ST2 subunit of the IL-33 receptor and also contributes to cytokine production (167, 168). IL-4 through IL-4Rα induces IgE class switching by B cells, promotes AHR, and upregulates expression of adhesion molecules on endothelial cells, to promote entry of inflammatory cells into the lungs (169). IL-13 has many similar effects to IL-4 as it also binds IL-4Rα, but in addition IL-13 promotes mucus production as well as collagen deposition (170). In humans, IL-5 drives the differentiation, recruitment, and activation of eosinophils (171). $CD4^+$ resident memory T (Trm) cells are present in the lungs of type 2-high asthmatic patients (172). Murine data show that Trm cells rapidly produce large amounts of type 2 cytokines compared to circulating Th2 following re-exposure to allergens (173).

1.2.2.2.2 B cells

Following activation, distinct IL-4 competent T cell populations in the lymph nodes will exit via the draining lymph and enter circulation where they migrate towards sites of inflammation

to become fully mature Th2 cells, while others migrate to B cells zones and become T follicular helper (Tfh) cells (174). Tfh cells induce maturation of B cells to plasma cells that produce antibodies (174). Based on human data, in response to IL-4 and/or IL-13 from Tfh cells, memory IgG-positive B cells class switch to IgE (175). Typically, IgE production occurs in the secondary lymphoid organs; however, it can also occur in the lung mucosa of asthmatic patients (176). IgE affects many immune cells as well as structural cells. Studies in human cells demonstrate that IgE can bind the high-affinity FcεRI present on basophils, mast cells, eosinophils, and DCs as well as airway smooth muscle cells, endothelial cells and epithelial cells (177). Crosslinking of IgE molecules by allergens leads to mast cell and basophil activation leading to release of preformed mediators like histamine, neutral proteases, lipid mediators, and Th2 cytokines (117). In mice, mast cells located near submucosal mucous glands release these molecules to promote mucus production by goblet cells (178). In addition, data from human studies suggest that mast cells localized to the airway smooth muscle bundles promote smooth muscle hypertrophy and hyperplasia, which contribute to AHR (179).

1.2.3 Current treatments

Currently, inhaled corticosteroid (ICS) are one of the most effective anti-inflammatory medications to treat asthma symptoms (180). ICS are recommended for children and adults with asthma as first-line maintenance therapy (180). Regular use of ICS reduces symptoms and exacerbations, while improving lung function and quality of life (181). Although they are most effective in allergic asthmatics, ICS are not sufficient to control asthma in all patients. Biologics, which have been approved for use in humans for several years, have been specifically designed to target the mediators of disease (Figure 1-2).

1.2.3.1 Blocking IL-5 and IL-5R

As mentioned previously, eosinophils are considered to participate in asthma pathogenesis, and are largely dependent on IL-5 signalling. There are currently two IL-5 monoclonal antibodies approved in Canada for adults with asthma that is not well controlled by high-dose ICS therapy and who present with elevated blood eosinophils (182). Both are given every 4 weeks (182). First, mepolizumab is an IgG1 antibody targeting IL-5 given by subcutaneous injection. In clinical trials mepolizumab was shown to reduce corticosteroid use and improve lung function (183). Second, reslizumab is an anti-IL-5 IgG4 antibody given by intravenous infusion. In clinical trials,

reslizumab significantly reduced the frequency of asthma exacerbations (184). Benralizumab, which has also been approved in Canada for use in adult patients with eosinophilic asthma, is a cytotoxic antibody that targets cells (e.g. eosinophils and basophils) expressing the IL-5 receptor (IL-5R) alpha chain (185). In clinical trials, benralizumab decreased the need for oral corticosteroids and reduced exacerbation rates (185).

1.2.3.2 Targeting IL-4Rα

As previously mentioned, both IL-4 and IL-13 bind IL-4Rα and downstream signalling from these cytokines drives IgE production, AHR, mucus production and fibrosis. Dupilumab is a humanized monoclonal antibody that binds IL-4Rα that was first used in patients with eosinophilic asthma and was shown to improve lung function, reduce exacerbations, and reduce Th2-associated inflammatory markers (186). Even patients with non-eosinophilic asthma had improved lung function and reduced exacerbations while taking dubilumab (187). Dupilumab is currently approved for use in patients of various ages with atopic dermatitis, asthma, chronic rhinosinusitis with nasal polyposis, or eosinophilic esophagitis (188).

1.2.3.3 Targeting IgE

IgE contributes to the early phase of allergic asthma as well as the chronic phase of disease. Omalizumab is a monoclonal anti-immunoglobulin E antibody (189). Omalizumab binds free IgE which reduces the ability of IgE to bind to its high-affinity receptor, thereby downregulating FcεRI expression and decreasing function of the cells expressing FcεRI (189). In clinical trials, omalizumab reduced exacerbations rates, reduced doses of corticosteroids required, and improved lung function (190, 191). Patients with high eosinophil counts showed improvements in lung function when taking omalizumab (192-194).

1.2.3.4 Targeting Innate cytokines

Epithelial derived alarmins, IL-33, IL-25 and TSLP induce type 2 responses, upstream of type 2 cytokines and IgE. Clinical trials of biologics targeting TSLP and IL-33 have begun. In a phase 3 study, Tezepelumab, a human monoclonal antibody specific for TSLP, reduced the frequency of exacerbations in patients with type 2-high asthma as well as those with type 2-low asthma, providing evidence that it may be effective in the treatment of a broad range of asthma patients (195). Itepekimab is a monoclonal antibody specific for IL-33 (196). In a clinical trial, itepekimab decreased the number of events associated with loss of asthma control, improved lung

function, and reduced mean blood eosinophil count in patients with moderate-to-severe disease (196). Astegolimab is a human monoclonal antibody that selectively inhibits the IL-33 receptor ST2 (197). In a clinical trial, Astegolimab reduced asthma exacerbation rates and was well-tolerated in a broad patient population (197).

1.3 Linking RSV and Asthma

Several studies have observed an association between RSV LRTI in early life and recurrent wheeze of early childhood (RWEC) and/or asthma indicating that a causal link may exist between the two (198-200). However, other studies, including those where monoclonal antibodies targeting RSV are used in a heterogeneous infant population report no reduction in the diagnosis of RWEC or asthma in the treatment group supporting the hypothesis that RSV LRTI is simply a marker of an underlying predisposition for RWEC and/or asthma (201-203). If a true causal link exists between RSV and subsequent asthma development, RSV treatments and vaccines for young children would be a large public health value as they would not only reduce severe acute RSV disease in infants, which poses a significant public health burden, but they would also be predicted to reduce many future cases of asthma.

1.3.1 Predisposition to RWEC and asthma

1.3.1.1 Genetics

Genetic factors may underlie the association between LRTI with RSV and asthma development. It is often speculated that children who experience RSV infection share common genetic vulnerability that predisposes them to both serious LRTI with RSV and asthma (204, 205). Candidate gene studies focused on RSV bronchiolitis found that susceptibility genes were related to immune regulation and surfactant proteins (206). Similarly, many of the risk markers for RSV were also identified as risk markers for asthma (207, 208). Therefore, shared genetics may explain the association between severe RSV infection and asthma. Twin studies support a similar conclusion. For example, one such study in Denmark demonstrated that, although RSV and asthma were positively correlated and that the genetic determinants for each overlapped, modeling the direction of causation did not indicate that RSV caused asthma, but instead indicated a genetic predisposition to asthma (209).

1.3.1.2 Microbiome

Another theory proposes that the microbiome may be the underlying cause of the association between RSV and asthma. The gut microbiome is recognised as an important risk factor for asthma and may impact susceptibility to viral infection of the airways, highlighting the importance of the gut-lung axis (210). In a murine model, mice that were supplemented with *Lactobacillus johnsonii* were protected from RSV-induced inflammatory responses and AHR (211).

In addition to the role of the gut microbiome, the nasopharyngeal microbiome may be equally important in driving severe RSV infection and subsequent RWEC. In one study, an association was found between the bacterial species, *Haemophilus influenza* and *Streptococcus*, and RSV infection and hospitalization (212). In another study, infants who did not present with symptoms but had *Streptococcus* as the dominate species in their nasopharynx had increased risk of developing RWEC (213).

1.3.1.3 Monoclonal antibody studies

One indirect indicator that RSV and asthma may not have a causal link comes from the results of studies using palivizumab, a monoclonal antibody against RSV. If a causal link exists between the two diseases, the treatment of RSV in early life should lead to a reduction in the overall prevalence of RWEC and asthma as well. Following hospitalization caused by RSV, the use of palivizumab reduced the risk of RWEC, but did not reduce risk for asthma (203). Similarly, results from prospective studies with RSV immunoprophylaxis in which pre-term infants treated with palivizumab showed decreased numbers of wheezing episodes, but the incidence of asthma by age six remained unchanged (201, 202). Second-generation monoclonal antibodies targeted to RSV have been effective at reducing the rate of severe acute RSV disease while not preventing recurrent wheeze (214).

1.3.2 Causal relationship

1.3.2.1 Epidemiologic link

Three criteria must be met to establish a causal relationship between severe LRTI with RSV and subsequent RWEC/asthma. First, a temporal relationship placing RSV infection prior to asthma must be present. Second, a dose-dependent relationship must be established to demonstrate

that more severe infection leads to increased risk for asthma. Third, a sound biological mechanism must be proposed and evaluated.

In addressing the first criterion, several longitudinal studies have demonstrated that acute RSV infection occurred prior to allergic sensitisation and asthma development (215-217). A study of approximately 95 000 infants born between 1995 and 2000 sought to determine the relationship between the first winter viral infection in infancy and the development of asthma in childhood (218). Findings from this study demonstrated that if infants were 4 months of age at the peak of winter viral season they had an increased likelihood of developing clinical bronchiolitis and childhood asthma (218). Interestingly, even though the winter viral peak shifted by 2 months during the six-year study, the risk of asthma also shifted each year by a similar extent (218). Infants born 4 months before the first winter viral peak continued to have the highest risk for asthma in childhood (218). The authors of this study speculated that the susceptibility to RSV infection may reach its peak around 4 months of age due to the decay of maternal antibodies and low IgG near this time in infants (219, 220). These results support that winter virus seasonality occurred prior to and was a causal factor in asthma development.

In addressing the second criterion, many studies have explored the relationship between severity of viral infection during infancy and subsequent asthma risk and support the hypothesis that the effect is dose-dependent (216, 221) In a cohort of 90 341 children, an infant bronchiolitis visit was reported in 18% of children. Of the 18%, 31% went on to report early childhood diagnoses of asthma. Compared to infants with no bronchiolitis visit the odds ratios were 1.86 in outpatient groups, 2.41 in emergency department groups and 2.82 in the hospitalised groups. These findings demonstrate that the more severe the disease (based on level of care required), the higher the risk of a subsequent asthma diagnosis (222).

In addressing the third criterion, several different groups, including the Fixman and Ward labs, have proposed biological mechanisms by which aberrant immune response during RSV could potentially lead to asthma development (223-225). However, the exact mechanism(s) that occurs in humans has yet to be elucidated. Although a clear conclusion has yet to be reached, there is strong evidence towards a causal relationship between severe LRTI with RSV during infancy and the subsequent development of RWEC and/or asthma in childhood.

Several studies show a strong association between severe RSV infection and RWEC and/or asthma (198-200). In addition, a history of RSV bronchiolitis is associated with more severe asthma. One study demonstrated that prior hospitalisation with RSV LRTI was associated with a 3-fold higher rate of asthma admission and medication use (198). Another reported that 42% of children with RSV bronchiolitis during infancy had further episodes of wheezing compared to only 19% in controls (226). In a population-based cohort study including approximately 90 000 children, data showed that infants with reported healthcare visits for bronchiolitis during RSV season were more likely (odds ratio of 1.89) to develop asthma by 4 years of age (227). Through a retrospective cohort of 150 infants with RSV bronchiolitis, infants who required hospitalization were more likely to have wheeze at 30-42 months (odds ratio 2.3), 69-81 months (odds ratio 3.5) and asthma diagnosed by a doctor at 91 months of age (odds ratio 2.5) (228). In a 20-year long-term follow-up study, 17-22% of 18-20-year-old patients who had RSV bronchiolitis or RSV pneumonia before the age of 2 were reported to have asthma compared to only 11% in control patients (229). A meta-analysis of infants hospitalised with severe RSV infection demonstrated a 63% prevalence of asthma under the age of 5 and a prevalence of up to 92% between 5 and 12 years of age. Given that the average background rates of asthma in children without a history of RSV hospitalisation are 1-7%, these results support a causal relationship between RSV infection early in life and subsequent asthma development later in life (230). Human studies are further supported by the results from murine studies, which demonstrate that RSV-infected mice have AHR and histologic changes similar to humans, both of which are maintained long after viral clearance (231).

1.3.2.2 Potential Immune mechanisms of a possible causal relationship

1.3.2.2.1 Type 2-biased neonatal lung mucosa

The precise timing of RSV infection early in life may help explain how viral infection leads to subsequent asthma development. Postnatal time periods are highly dynamic and important for lung maturation. During alveolarization (post-natal day (PND) 4-21 in mice), the lung mucosa is highly skewed toward type 2 immunity: ILC2s, mast cells, eosinophils and basophils increase (146). At this development time in mice, a large population of tissue resident alveolar macrophages express CD206, a marker of an alternatively activated phenotype, consistent with the type 2-skewed environment (232, 233).

In murine asthma models, HDM delivery in early life leads to enhanced allergic airway disease that includes increased eosinophils in the lung and AHR (146, 234). These responses are IL-33 dependent and are shaped by Th2 cells producing IL-13, IL-5 and IL-4 (146). In addition, the T cells of neonates express GATA-3 more readily and produce greater quantities of Th2 cytokines (235). The type 2 biased neonatal mucosal lung environment creates an ideal temporal window for asthma development following exposure to external factors such as a virus or an allergen. In RSV reinfection models, neonatal priming by RSV infection increases Th2 numbers and eosinophil recruitment at reinfection (113, 236). If infection is delayed to after the neonatal period, IFNγ responses are enhanced and disease following reinfection is less severe (237).

1.3.2.2.2 RSV effects on Th2/Th1 balance

Considerable evidence demonstrates that RSV glycoproteins (F and G) directly impact the Th1/Th2 balance in the lung. In mice vaccinated with vaccina virus expressing RSV G, Th2 cytokine production and recruitment of eosinophils into the airways are increased following RSV infection (51, 238). Based on human and murine data, by signalling through TLR4, the RSV F also contributes to the formation of a Th2-skewed lung microenvironment (239, 240). In murine models, allergic airway disease in response to allergens, such as HDM, relies on TLR4 signalling (in lung structural cells) (147). In a model using 4-week-old mice, RSV infection was shown to enhance aspects of HDM-induced allergic airway disease such as lung eosinophilia and mucus-producing epithelial cells (113).

As mentioned previously, RSV infection induces the release of innate cytokines IL-33, IL-25, and TSLP. Upon RSV infection in neonatal mice, IL-33 release in the lungs contributes to ILC2 expansion (30). These data are supported by data from humans showing IL-33 levels are elevated in nasal aspirates of infants hospitalised with severe RSV infection (30). In murine models of RSV infection or RSV-induced asthma exacerbations, neutralizing IL-25 or using mice lacking the IL-25R reduces Th2 cytokine production, mucus production and AHR (241). Release of TSLP is greater following RSV infection of airway epithelial cells from asthmatic children compared to similarly infected epithelial cells from healthy children (242). Finally, when human primary bronchial airway ECs are infected with RSV, the TSLP receptor is upregulated (243).

RSV has also been implicated in modifying DC responses to support Th2 responses. Findings from human studies show that the RSV G-protein reduces DC activation in RSV-infected

primary myeloid and plasmacytoid-derived DCs (244). RSV NS1 and NS2 proteins also reduce maturation of human DCs and reduce the activation and proliferation of human $CD8^+$ T cells and Th17 cells (245). In contrast, NS proteins enhance activation and proliferation of human $CD4^+$ Th2 cells (246). The Th2-biased neonatal lung mucosa combined with the ability of RSV to skew toward type 2 responses favors asthma development.

While RSV infection promotes type 2 immunity, it also promotes dysregulated immune responses by preventing Th1 responses. IFNγ is a key cytokine to induce and maintain robust Th1 responses. In studies using recombinant RSV expressing IFNγ, neonatal mice exhibited reduced disease upon re-infection compared to wild-type RSV-infected neonates (247). These results highlight the protective role of Th1 responses during RSV infection (247). In mice, macrophages, which are sensitive to IFNγ stimulation, make up a substantial proportion of the immune cells in the neonatal lung (233). However, there are striking differences between macrophages in neonatal mice compared to adult mice. Following RSV infection, lung macrophages in adult mice increase M1 macrophage markers, including MHC II, CD86, and CCR7, whereas upregulation of these markers is delayed upon RSV infection of lung macrophages from neonates (233). Consequently, without M1 macrophages, neonatal mice produce lower levels of inflammatory cytokines required to clear virus (233). In summary, RSV skews an already type 2-biased neonatal lung microenvironment further towards type 2 responses by simultaneously promoting Th2 responses and preventing proper Th1 responses.

1.3.3 Challenges in determining definitive causative relationship

One of the major challenges in determining if the link between RSV LRTI and asthma is causal stems from the fact that the diagnosis of asthma in children under the age of 6 is difficult. First, young children may experience viral-associated recurrent wheeze in the absence of asthma (248). Second, the use of spirometry to measure airway obstruction is difficult to perform in young children (248). Recently, forced oscillation technique (FOT) has also been used to measure respiratory system resistance and compliance successfully in children as young as six weeks of age, serving as a promising alternative to spirometry (249).

Another challenge stems from confounding variables that add a source of bias to the results obtained from observational studies. Controlling for risk factors for both RSV LRTI and

RWEC/asthma such as age, prematurity, access to health care, co-morbidities, exposure to air pollution, and exposure to second-hand smoke can be extremely difficult (250).

Last, a major limitation of human studies of RSV, including the randomised controlled trials with palivizumab, is that they are largely underpowered to find a statistically significant effect on RWEC and asthma (250). In order to accurately determine if treatment with palivizumab has an effect on RWEC and asthma, the sample size in each trial would need to be increased.

1.4 Sex Differences

Immune responses in human males and females differ significantly, which explains why sex differences are present in many immunological disorders. While it is too simplistic to label one pattern of immunity as 'good' and another as 'bad', females mount more robust immune responses to many pathogens and therefore have increased resistance to these infection (251, 252). For example, females have stronger antibody responses to many vaccines (253). Epidemiological trends for sex differences in both RSV and asthma will now be explored, followed by potential mechanisms for these differences through male and female hormones as well as X-linked factors.

1.4.1 RSV

The biological sex of an individual affects the outcome of many respiratory viral infections. The pathogenesis of virally-induced respiratory infections is different between sexes and varies with age. In humans disease severity in response to RSV, rhinovirus (RV) and influenza, is highest in the young (infants and children) and the elderly (65 years and older) (254, 255). In early life and childhood, compared to females, males are typically more vulnerable to severe outcomes from respiratory viral infection (252). In the older population, the male immune system experiences a greater deterioration in B cell-specific loci compared to females, which may explain why males are more susceptible to infections (255). In contrast, during the reproductive years, females experience worse outcomes from respiratory viral infections. During pregnancy, females experience more severe disease upon infection with viruses such as febrile human RV, which has been associated with increased hospitalization in pregnant females and low birth weight for infants born to RV-infected mothers (256, 257). RSV infection during pregnancy is associated with increased respiratory distress and hospitalization compared to infection in non-pregnant, age-matched adults (258). Non-pregnant females of reproductive age also experience greater

inflammatory immune responses and tissue damage post-infection compared to males of the same age (259).

Sex is also a risk factor for worse outcomes following RSV infection. RSV can infect individuals of any age, however young males are admitted more frequently to the hospital with asthma exacerbations due to RSV infection compared to young females (254). A review of studies performed in the last 30 years identified the risk ratio for development of RSV LRTI of young males to females was 1.425 (260-262). Male infants may be more prone to severe disease as they have shorter and narrower airways (263), which increase the risk of developing bronchial obstruction (263).

Another study examined RSV illness in children that had a confirmed diagnosis of RSV or had a history of recurrent wheeze (264) and found that females infected with RSV at any age had higher white blood cell counts compared to age-matched males (264). However, between the ages of 2 and 3, RSV-infected males had significantly higher blood eosinophil counts compared to females (264). These results demonstrate that male sex worsens inflammatory responses due to RSV disease in children with a history of wheezing.

Malinczak et al. designed a neonatal murine model to examine the impact of sex on early-life RSV infection (at PND 7) and subsequent allergic airways disease. At early time points following infection, male mice exhibited increased viral mRNA and decreased IFNβ compared to female mice (225), demonstrating better viral control in females through greater cytokine production. Four-weeks post infection, when virus had been cleared, male mice had increased levels of mRNA for *Muc5ac*, *Il-4, Il-5, Il-13, Il-17a, Tslp and Il-33* as well as increased numbers of ILC2s compared to female mice (225). Male mice demonstrated Th2 and Th17-skewing. Four weeks after RSV infection, mice were sensitized and challenged with cockroach antigen (CRA). Consistent with the enhanced type 2 skewing after RSV infection, AHR, mucus production, and ILC2 numbers were all greater in males compared to females (225). Sex differences were abolished in mice lacking TSLP receptor, providing evidence that following early-life RSV infection, TSLP may change the immune environment specifically in male mice, leading to enhanced responses to allergen exposure later in life.

In a follow-up study, Malinczak et al. investigated innate immune responses in male and female mice following RSV infection at PND 7, focusing on bone marrow-derived dendritic cells

(BMDCs). In both humans and mice, cDCs have been shown to accumulate in the LRT during RSV infection, although the origin of these cells remains unknown (265, 266) Four-weeks post-infection, bone marrow was collected and cultured for 6 days to differentiate BMDCs.. Early-life RSV-infected females generated BMDCs with greater levels of IL-12a mRNA and increased surface expression of IFNAR1, which enhanced T cell production of IFNγ (267). These findings are compatible with their in vivo data showing that the adoptive transfer of BMDCs generated from RSV-infected males, enhanced Th2/Th17 responses following RSV infection of recipients (267) while female recipients of similarly treated female BMDCs were largely protected upon RSV infection (267). Experiments using ATAC-seq (to examine 'open', more transcriptionally active chromatin) showed that type-1 immune response genes were more accessible in the chromatin of BMDCs from RSV-infected females (267). To further support these data, ATAC-seq analysis was performed using human monocytes isolated from the cord blood of male and female neonates. These data confirmed similar accessible chromatin near type-1 immune genes in female monocytes (267). Altogether, these data indicate that epigenetic factors impact sex-associated responses to early-life RSV infection, leading to type-1 signalling selectively in female-derived cells and activation of type-1 trained immunity.

1.4.2 Asthma

Both sex (male or female, based on biological characteristics) and gender (socially constructed roles and behaviors) influence the diagnosis and prevalence of asthma across the world.

1.4.2.1 Sex

During childhood males have a higher prevalence of asthma compared to females (268). However, following puberty the prevalence is higher in females compared to males (Figure 1-4) (268). Differences in childhood may be related to fetal and postnatal lung development. Compared to females, males are more likely to suffer from dysanaptic lung development, which is a mismatch between the size of the airway tree and lungs in relation to airway flow rates (269). The bronchial airways as well as the lung parenchyma in females grow proportionally. In males, however, growth of the airway is delayed compared to the growth of the lung, which leads to fewer alveoli for the number of airways (270). Once males fully mature, the diameter of the airways as well as lung volume, maximum expiratory flow, and diffusion surface become greater compared to adult

females, which may explain, at least in part, the “switch” in prevalence following puberty (271). Although there is no direct correlation between dysanaptic lung growth and AHR (272), it may correlate with allergen sensitisation as higher IgE levels have been reported in young males compared to young females (273).

Some females report worsening of asthma symptoms during certain phases of their menstrual cycles (274). During the luteal phase (when estrogens and progesterone rise), AHR and asthma-related healthcare utilisation has been reported (275, 276). Some studies show that roughly 20 to 40% of females with asthma experience more frequent or more severe symptoms during the pre- and peri-menstrual period (277). In addition, in some studies the premenstrual period has been associated with increased sputum eosinophils (278). However, other studies report no association with asthma and the menstrual cycle when examining factors such as airway reactivity, visits to the emergency room, and asthma-related events (279, 280).

Pregnancy has been reported to have effects on asthma control, though outcomes are highly variable and often unpredictable (281). During pregnancy, some females report worse symptoms, some report improvements and some report no changes (282). One study found a link between worse symptoms and increased exacerbations, especially in women with more severe disease compared to those with mild or moderate asthma (283). Despite recommendations to maintain asthma treatment during pregnancy, asthma symptoms may increase if asthma medication is stopped due to unfounded safety concerns (284).

A small number of studies have linked menopause to more severe asthma and heightened risk for new-onset asthma (285, 286). In addition, hormone replacement therapies (HRT) for menopausal women have been associated with distinct asthma outcomes depending on the HRT used, yet conclusions have not been consistent between studies. One case-control study reported that estrogen monotherapy or combined estrogen with progesterone HRT were associated with an increased risk for asthma while progesterone monotherapy decreased asthma risk (287). A different study found that both estrogen monotherapy and combined estrogen and progesterone HRT reduced risk for asthma development (288).

While the effects of menstruation, pregnancy and menopause are quite variable, several studies support the use of hormonal contraceptives to improve asthma outcomes and reduce incidence. Hormone contraceptives reduce asthma exacerbations, reduce the risk of new-onset

asthma, reduce asthma incidence, decrease asthma related healthcare visits, and decrease wheezing in asthmatics (289-292). Mechanism to explain these results have yet to be elucidated, but they may be related to hormonal contraceptives reducing levels of female hormones, estrogen and progesterone, during the menstrual cycle (293).

Sex-specific SNPs in gene or epigenetic variabilities are implicated in the pathogenesis of pediatric and adult asthma. SNPs in *TSLP* are associated with either decreased asthma risk in males or decreased asthma risk in females, depending on the variant (294). DNA methylation, which decreases in males, but increases in females from pre- to post-adolescence, has been linked to asthma susceptibility and could theoretically modulate the "switch" in asthma prevalence at during puberty (295).

1.4.2.2 Gender

Different behaviors or exposures associated with gender also have considerable impact on asthma risk and asthma outcomes. Males and females are exposed to different triggers due to gender roles and occupations. For example, exposure to flour or flour products leading to baker's asthma, is disproportionality higher in males compared to females (296). Females have a higher likelihood of being exposed to chemicals related to cleaning and hairdressing while males are more likely to be exposed to wood dust and cooling lubricants (297). Lifestyle choices are also highly gendered. Males tend to be more physically active and are less likely to be obese compared to females (298, 299). Both low levels of physical activity and obesity are risk factors for asthma as well as increased asthma severity (300).

Patient perception of symptoms also varies based on gender. Females tend to perceive asthma symptoms as more bothersome, frequent and severe compared to males even if asthma severity is similar (301). Compared to males, females are more likely report asthma symptoms to a healthcare professional (HCPs) (301). These behaviors may be linked to societal expectations for males to appear stronger and more resilient to pain (302). In addition, gender bias exists in behaviors by HCPs, which has an impact on diagnosis and management of asthma. Surveys demonstrate that females feel less well understood by HCPs, which may be linked to the finding that the amount of time between symptom onset and diagnosis is longer in females compared to males (303, 304).

1.4.3 Roles for Male Hormones in Immunity

Androgens, such as testosterone or dehydroepiandrosterone (DHEA), are associated with decreased incidence of asthma as well as reductions of asthma symptoms. In one pediatric cohort study, males with higher levels of DHEA had a decreased risk of asthma in adolescence, while females with higher estradiol had increased risk (305). In adult males over the age of 45, lower testosterone levels are associated with increased asthma prevalence and in adults, both male and female, increased testosterone levels correlate with decreased asthma prevalence, decreased symptoms, and decreased hospitalisations due to asthma (306, 307). In older male populations, decreasing levels of testosterone are associated with increased asthma prevalence (308). Given the benefits observed following DHEA treatment in COPD and pulmonary hypertension patients, similar therapies were considered for asthma patients. In pilot clinical trials of exogenous androgen administration in females with asthma and low DHEA levels, both nebulised and oral DHEA reduced asthma burden (309, 310).

The ability of androgens to modulate some immune responses (252, 253) has been confirmed in mice where testosterone upregulates IL-10 and downregulates IFNγ (311). Activation of murine macrophages is downregulated by testosterone by decreasing TLR4 expression (312). Androgens acting directly on T cells decrease cytokine production by Th2 cells and reduce allergic airway inflammation (313). ILC2s are also negatively regulated by androgens. In mice, androgens lower the functional capacity of ILCs and decrease the number of ILC2s that lack expression of the killer-cell lectin like receptor G1 (KLRG1) (314). Furthermore, upon activation of the androgen receptor (AR) on murine ILC2 progenitors, maturation is reduced (315).

1.4.4 Roles for female hormones in Immunity

In both humans and mice, many cells of the immune system such as T cells, B cells, NK cells, macrophages and DCs express estrogen receptors (ER-α and ER-β) and the presence of these receptors on immune cells indicates that they have the potential to be controlled by estrogens (252, 253, 316, 317). In many instances of human disease, such as autoimmune disease, estrogens appear to act as immune activators (318). Murine macrophages show increased activation in the presence of estradiol (319). In mice, estrogen upregulates IFNγ, TNFα and IL-12, and downregulates IL-10 (320). Finally, estrogens enhance allergen-induced type 2 inflammation by increasing IL-33 levels in the murine lung (321).

1.4.5 X-linked factors in Immunity

Females are more prone to autoimmune disorders; a phenomenon that can be partially explained by the X chromosome in some diseases. The human X chromosome contains many immune-related genes (322). In female mammals, X chromosome inactivation (XCI) is used to generate a transcriptionally silent X chromosome (Xi) (323). The Xi is enriched with heterochromatic modifications, which allows gene expression to become equal between the sexes (323). Some groups suggest that some aspects of the enhanced immunity observed in females may be due to the Xi in human female lymphocytes partially reactivating and overexpressing immunity-related genes (324).

1.5 Rationale and Hypothesis

RSV is the most common cause of acute lower respiratory tract infections in children and causes close to 200 000 deaths annually worldwide with 99% occurring in developing countries (325). By the second year of life, nearly all children have been infected at least once by RSV (325). A strong epidemiological association between early-life RSV infection and a heightened risk of asthma development exists (208, 326-328). However, the mechanism(s) underlying this association are unclear. Importantly, severe RSV infection is associated with enhanced Th2 polarization and eosinophilic responses in the lung (329-333). In addition, IL-33 is a key innate cytokine implicated in the pathogenesis of both RSV infection and asthma (30, 334, 335). Several genome-wide association studies (GWAS) demonstrate an association between genetic variation in the genes encoding IL-33 or its cognate receptor subunit, ST2 and risk for both asthma and severe RSV disease (336, 337). Currently, no vaccines for children are available for RSV and thus novel preventative and therapeutic strategies that reduce the health burden of RSV, and subsequent development of asthma, have the potential to impact hundreds of thousands globally.

Asthma epidemiology and morbidity is different between the sexes, although the mechanisms that underlie these differences are unclear (338). Asthma is more prevalent in young males, however this trend switches around the time of puberty, when asthma becomes more prevalent in adult females (338). These clinical observations, agree with data from the Fixman and Ward labs, as well as data from others (315, 339), which show that inflammatory responses in asthma (340) and RSV models are enhanced in adult female mice. ILC2s are implicated in both

asthma and severe RSV infection and are negatively controlled by androgens (30, 63, 315, 341). Adult sex hormones, estrogen and testosterone, have often been implicated as drivers of immunological differences in men and women (252, 342). However, there is also evidence for sex bias before the onset of puberty for several autoimmune diseases and allergic disorders (343, 344), implicating perinatal hormones. Mackey et al. observed that differences in mast cell associated disease severity was driven by perinatal androgens (345).

This thesis addresses the central hypothesis that early-life RSV infection shapes the lung environment to promote induction of type 2 inflammation in response to subsequent allergen exposure. In addition, we hypothesize that these responses are selectively enhanced in females compared to males due in part to differences in perinatal hormone exposure.

1.6 Research Objectives

The first objective of this thesis was to establish a reliable and reproducible neonatal murine model of RSV infection followed by allergen exposure to imitate early-life RSV infection in infants leading to wheezing and asthma. In our model, young mice are first infected with RSV at PND10 intranasally (I.N.) as this is a time when the neonatal mouse lung is skewed towards type 2 inflammatory responses in an IL-33-dependent manner (146). Mice are then exposed acutely, on two subsequent days, through the same route to HDM.

Once we established a robust model with consistent outcomes, our 2nd objective was to identify which inflammatory events to acute HDM exposure were impacted in mice previously infected with RSV (Chapter 2). Our data show that early life exposure to RSV dramatically enhanced HDM-induced allergic lung inflammation (lung eosinophil influx and activation), as well as proportions of T cells and ILC2s expressing type 2 cytokines, IL-13 and IL-5. A large subset of these type 2 inflammatory responses were more prominent in female mice compared to male mice.

Finally, our 3rd objective was to further examine the potential mechanisms underlying the prominent sex differences by exploring the roles of perinatal androgens and estrogens (Chapter 3). When we blocked estrogen receptor we observed that the enhanced responses in females were reduced to the levels observed in males for IL-13 producing ILC2s as well as T cells. Exposure to

exogenous estrogens had reciprocal effects in male mice where the number of KLRG1^{-} ILC2s and IL-13 producing CD4^{+} T cells were enhanced.

1.7 Figures and Legends

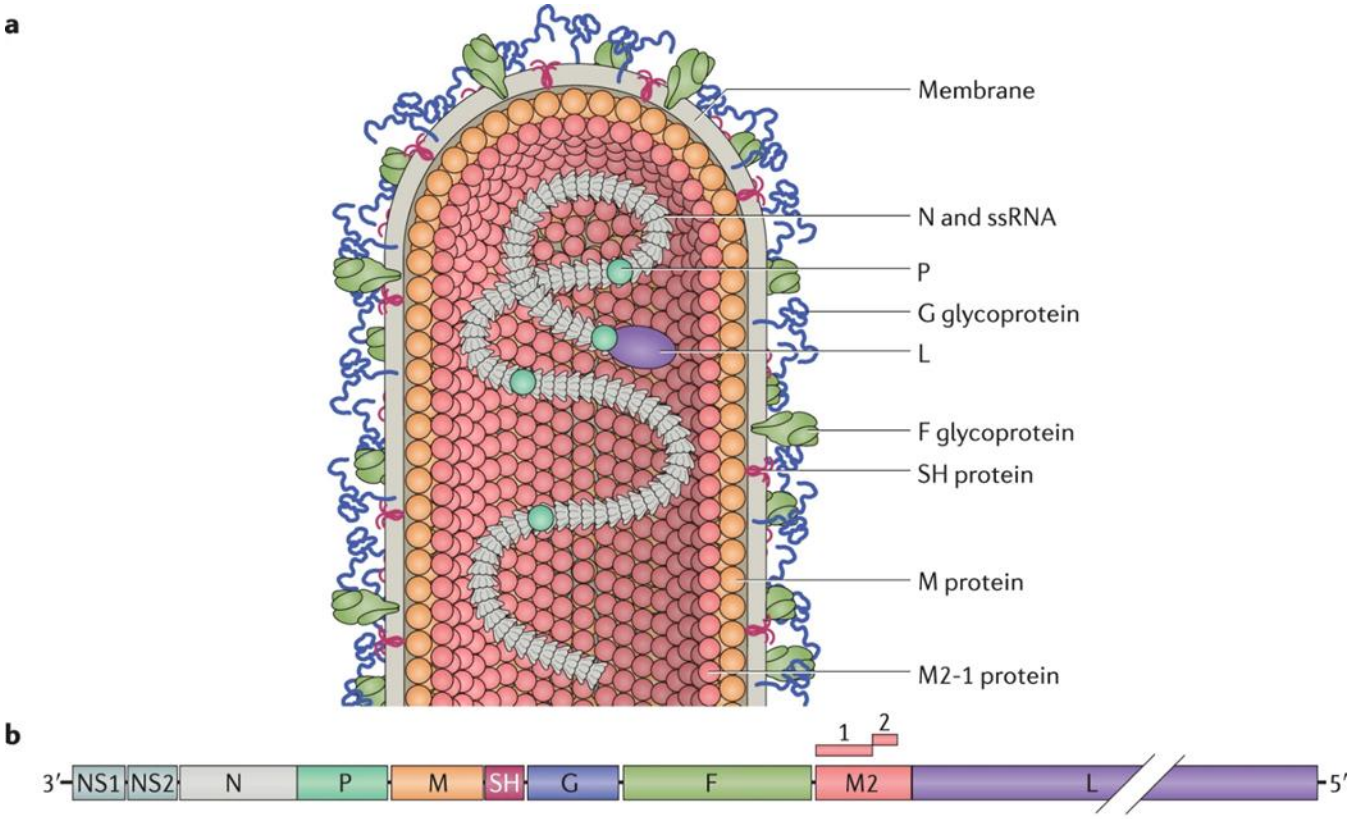

Figure 1-1. ***The morphology of the RSV virion.***

(a) The G and F glycoporteins are found embedded in the viral membrane. The SH protein is also embedded in the membrane and serves as a viroporin. Underneath the viral membrane lies a layer of M protein which gives the virion its shape. The M2-1 protein interacts with the M protein as well as the N protein which encases the viral RNA genome. L and P proteins are associated with N protein. (b) The RSV genome contains 10 genes and encodes 11 proteins.

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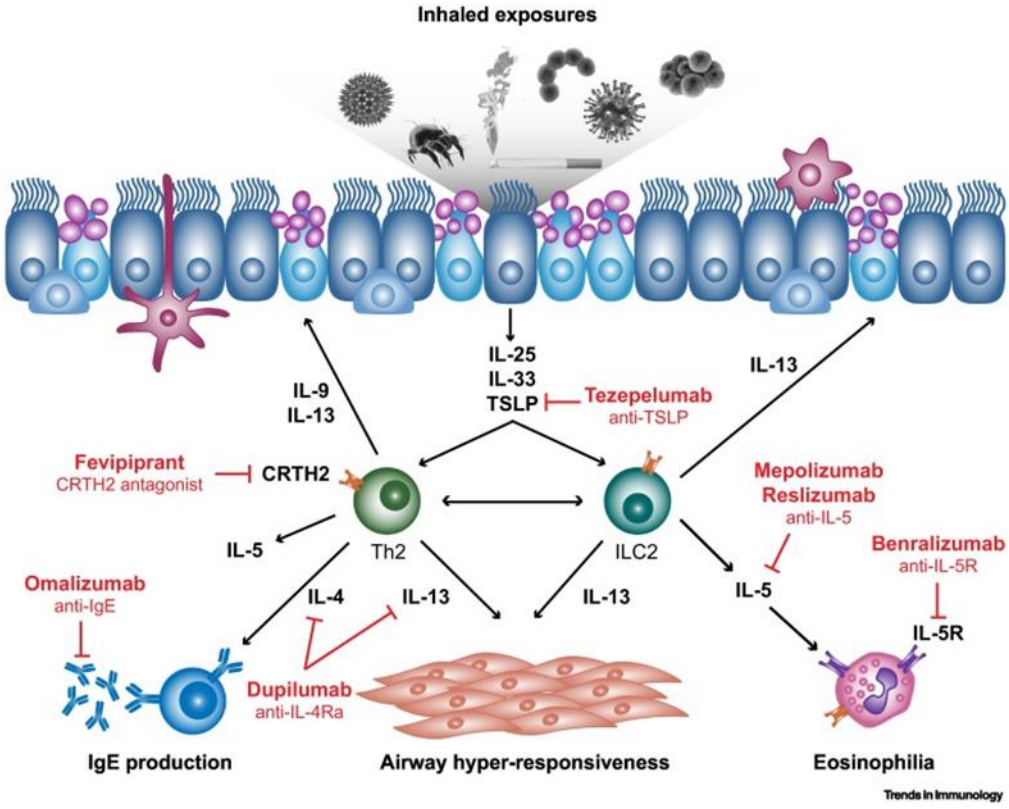

Figure 1-2. ***Emerging Biologic Agents to Target Important Type 2 Immune Pathways.***

Epithelial cells release innate cytokines which activate various immune cells to start producing type 2 cytokines, IL-13, IL-4, IL-5. Type 2 immune inflammation manifests in several ways such as eosinophilic inflammation, airway hyper-responsiveness, IgE production, and goblet cell hyperplasia. Novel biologics (highlighted in red) are designed to target these important pathways.

Reproduced from Clare M. Lloyd, Sejal Saglani, Opening the Window of Immune Opportunity: Treating Childhood Asthma, Trends in Immunology, Volume 40, Issue 9, 2019, Pages 786-798, ISSN 1471-4906, https://doi.org/10.1016/j.it.2019.07.004 with permission from Elsevier and Copyright Clearance Centre.

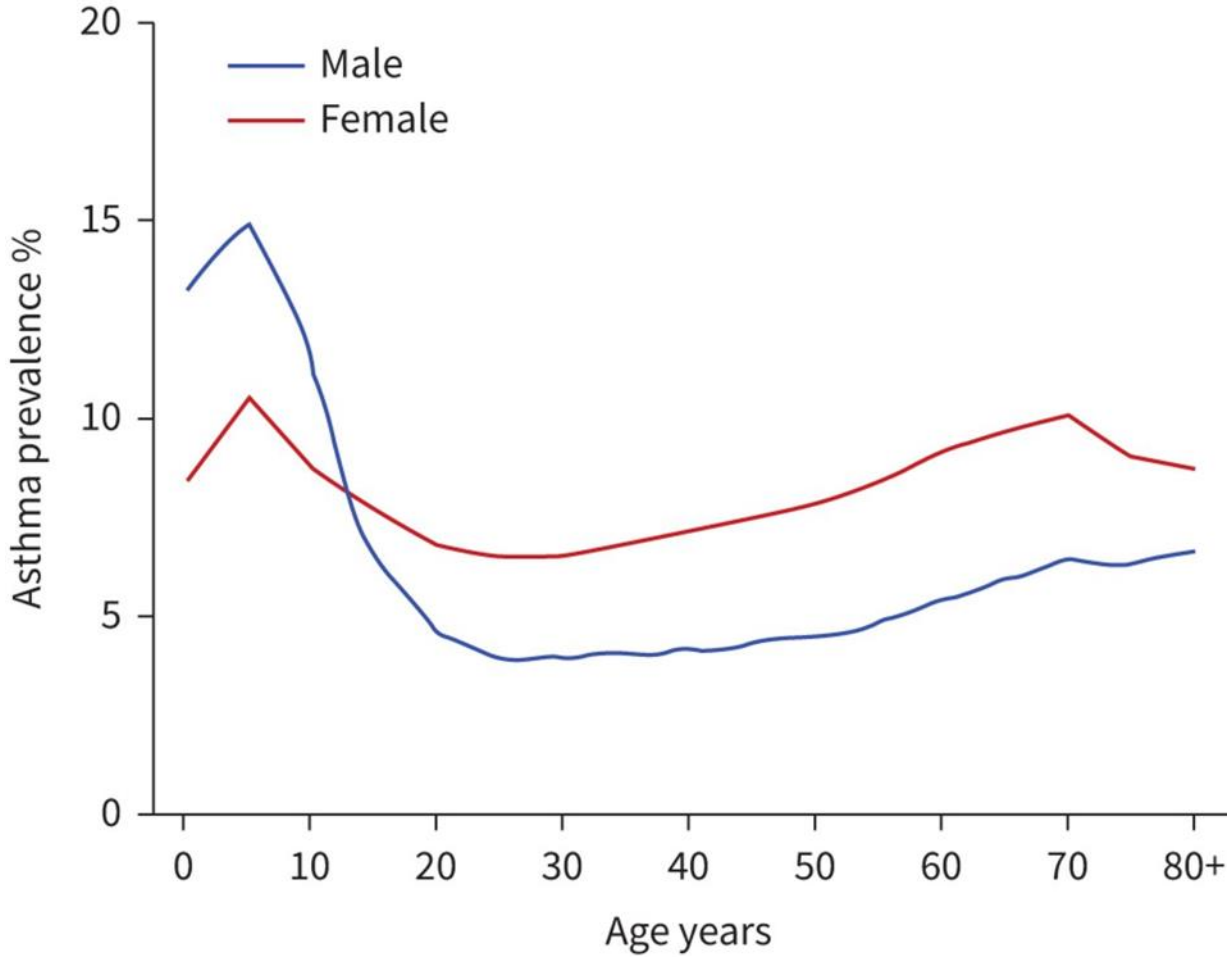

Figure 1-3. ***The prevalence of asthma by age and gender from the National Health Interview Survey in the United States.***

Asthma prevalence is higher in boys before the age of puberty (between 10 and 20 years of age), however following puberty asthma prevalence is higher in adult women.

Reproduced from F.S. Pignataro, M. Bonini, A. Forgione, S. Melandri, O.S. Usmani, Asthma and gender: The female lung, Pharmacological Research, Volume 119, 2017, Pages 384-390, ISSN 1043-6618,https://doi.org/10.1016/j.phrs.2017.02.017, with permission from Elsevier and Copyright Clearance Centre

PREFACE TO CHAPTER 2

RSV remains one of the main causes of lower respiratory tract infection, affecting mainly young children and the elderly. Early-life infection with RSV leading to severe disease is associated with an elevated risk of developing asthma. The relationship between these two diseases is poorly understood. Although severe RSV infections will now be better controlled due to the approval of 2 vaccines for use in the elderly population and one vaccine for pregnant mothers that reduces severe RSV disease in infants, the underlying mechanisms by which a viral infection is linked to asthma development still warrant further study. We hypothesized that RSV infection modifies the lung environment in such a way that type 2 inflammation, after acute exposure to allergen (HDM), is enhanced. Therefore, we developed a robust, reproducible model in young mice to study how early-life RSV infection impacts inflammatory events induced by acute allergen exposure. At PND 10, mice were infected with RSV. Ten or 30-days post-infection, mice were exposed to HDM and 3 days later, mice were euthanized. The following chapter describes the design and use of this murine model to elucidate the inflammatory events induced after early-life RSV infection and allergen exposure and the impact of sex on these responses. We examined populations of eosinophils, ILC2s, and T cells, as well as lung function and histology in these mice. We also sought to determine if IL-33 was implicated in the RSV-dependent enhancement of allergic responses.

CHAPTER 2

Early-life RSV infection modulates innate immune events to enhance allergen-induced type 2 lung inflammation

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Keywords: Respiratory syncytial virus, eosinophils, group 2 innate lymphoid cells, sex differences, IL-33, lung inflammation

2.1 Abstract

RSV causes millions of hospitalizations and several hundred thousand deaths per year globally. Early-life RSV infection is also associated with the subsequent development of wheezing and asthma with important sex-related disparities in incidence, epidemiology, and morbidity. The mechanisms that underlie these sex-specific effects are not clear. We have developed a combined infection-allergy model in which 10-day old mice are infected with RSV and subsequently exposed to a common allergen, house dust mite (HDM). We show that early-life exposure to RSV enhanced allergic lung inflammation upon HDM exposure 10 days after viral infection. Levels of the innate cytokine, IL-33, were increased in the lung 6h following HDM exposure only in mice previously infected with RSV. Accumulation of $CD11c^{med}$ eosinophils and group 2 innate lymphoid cells was more prominent in the lungs of female mice exposed to both RSV and HDM. Moreover, the numbers of IL-13^+ T cells (both $CD4^+$ and $CD8^+$) in the lung were significantly increased in mice exposed to both RSV infection and HDM, although the expression of ST2 (the cognate receptor for IL-33) was not linked to T cell cytokine production. Inflammatory responses were maintained when the interval between RSV infection and HDM exposure was extended to one month. Our results show that early exposure to RSV increased numbers of innate cells as well as T cells in response to a common allergen, whether delivered within days or after several weeks of viral infection and that most responses were enhanced in female mice. Our work highlights sex-specific impact of early-life viral infection on the developing lung, and suggests possible mechanisms to explain the subsequent female-predominant predisposition to enhanced allergic responses long after viral clearance.

2.2 Introduction

RSV is a leading cause of acute lower respiratory tract infections. Although recording is far from complete, it is thought that RSV causes at least 33 million episodes of lower respiratory infections in children under 5 years of age and approximately 118,000 deaths annually in children worldwide. Almost all (99%) of the mortality occurs in developing countries (1-3). During the first year of life, ~70% of infants are infected with RSV and nearly all children are infected at least once by two years of age (1, 4). Epidemiological data associate early-life RSV infection with a heightened risk of subsequent asthma development (5-8). Almost half of the infants hospitalized with RSV bronchiolitis develop asthma later in life (8). Roughly 5 million children in the United States alone are affected by asthma of whom 1.3 million are under the age of five (9). However, the mechanisms underlying the association between early RSV infection and asthma are unclear.

Several murine models have been developed to explore the ability of RSV infection to induce enhanced responses to allergens (10-12). Keegan et al. infected adult mice with RSV prior to challenging them multiple times with an extract of house-dust mite and demonstrated that RSV infection increased HDM-induced eosinophil influx into the airways as well as mucus production in the lung (10). Numerous groups have shown that age of initial exposure to RSV plays an important role in influencing immune responses upon RSV reinfection (13, 14). Age is also an important factor in RSV enhancement of responses in asthma models. When You et al. infected neonatal mice and subsequently sensitized and challenged these animals with ovalbumin as adults, they observed exacerbated airway hyperactivity (AHR) and inflammation with subepithelial fibrosis (12). Similarly, Malinczak et al. showed that neonatal RSV infection followed by sensitization and challenge with cockroach allergen extract (CRE) in adults increased mucus production and AHR, predominately in adult male mice (11). Severe RSV infection in children is also associated with enhanced T-helper 2 (Th2) polarization in the lung with increased IL-4 and IL-13 levels (15-19). While murine models appear to recapitulate the effect of RSV infection on adaptive immune responses following allergen sensitization and challenge in adult mice, how RSV alters innate immune events in response to allergen exposure is not known.

It is well established that sex-related differences exist in asthma epidemiology and morbidity, although the mechanisms that underlie these differences are poorly understood (20). The prevalence of asthma is greater in young boys but switches around the time of puberty, when

asthma prevalence becomes greater in young women (20). In agreement with these clinical observations, data from animal models suggest that inflammatory responses in asthma are enhanced in (adult) female mice (21-23). Notably, group 2 innate lymphoid cells, ILC2s, which are implicated in both asthma and severe RSV infections, are negatively controlled by androgens (21, 24-26).

IL-33 is a key innate cytokine implicated in the pathogenesis of both RSV infection and asthma (24, 27, 28). Genome-wide association studies (GWAS) demonstrate an association between IL-33 or its cognate receptor subunit, ST2, and risk for both asthma and severe RSV disease (29, 30). Blocking IL-33, which is released upon RSV infection in neonatal mice, reduces both ILC2 expansion in neonates and disease severity upon RSV reinfection of adults (24). Currently, no vaccines against RSV are approved for use in children and thus novel preventative and therapeutic strategies that reduce the direct health burden of RSV as well as the burden associated with subsequent development of asthma, have the potential to impact hundreds of thousands if not millions of children globally.

In this work, we have developed an RSV infection model focusing on innate events following acute allergen exposure in young mice previously infected with RSV to test the hypothesis that previous RSV infection in neonatal mice enhances type 2 innate inflammatory responses upon acute HDM exposure. Our data show that early-life exposure to RSV dramatically enhanced HDM-induced type 2 innate lung inflammation (lung eosinophil influx and activation and ILC2 expansion) as well as levels of type 2 cytokines (IL-13, IL-5) that orchestrate pathology in the asthmatic lung. Our data also demonstrate that several of these responses were more prominent in female mice. Early-life RSV infection also dramatically enhanced acute HDM-induced type 2 inflammatory responses long after viral clearance (4 weeks after initial infection). Together, these results highlight how early-life RSV infection modulates innate immunity to promote type 2 inflammation and the subsequent development of asthma.

2.3 Materials and Methods

2.3.1 Mice

Wild-type BALB/c mice were bred in-house under pathogen-free conditions (originally from Charles River Laboratories, St-Constant, QC). Breeding trios were used (2 females, 1 male). Once pregnant, dams were housed one per cage and transferred to CL2 facilities. Male and female pups used for experiments were housed with their mother until day 21 of life, when they were weaned and males and females separated into different cages. All cages were supplemented with water and irradiated food at all times. Animal studies were approved by the McGill University Animal Care Committee and performed in accordance to the guidelines of the Canadian Council on Animal Care.

2.3.2 RSV Purification

RSV A2 (ATCC #VR-1540, Manassas, VA) was used to infect Hep-2 cells (ATCC #CCL-23, Manassas, VA). The virus was added to RPMI-1640 medium containing 2.5% heat inactivated fetal bovine serum (FBS) (Wisent, St-Bruno, QC), mixed gently, then added to 70-80% confluent Hep-2 cells in a 24-well plate. While placed on a rocker at 4˚C, the virus was adsorbed to the monolayer for 90 minutes, after which the monolayer was washed with RPMI+2.5% FBS and cultured at 37°C in fresh RPMI+2.5% FBS for 3 days. Once the cell monolayer exhibited cytopathic effect (CPE) (40-50%) under light microscopy, the monolayer was disrupted by scraping. Supernatant was collected after two washes (once with H_2O, once with supernatant from the first wash, spun at 400g, 5 minutes, 4˚C) and then transferred to a T175 flask containing 80-90% confluent Hep2-cells. Virus was adsorbed for 90 minutes at 4˚C on a rocker. The monolayer was washed with RPMI+2.5% FBS and cultured at 37°C in fresh RPMI+2.5% FBS for 3 days. When CPE reached 70-80%, the cell monolayer was disrupted by scraping (as above) and the supernatant clarified from cellular debris by centrifugation at 4°C for 10 minutes at 2095 x g (31). The virus was further purified in a sucrose gradient with centrifugation at 116 000 x g for 2 hours at 4°C (32). Purified virus was aliquoted and stored at -80°C. Viral titers were determined by TCID50 as previous described (32).

2.3.3 RSV infection and HDM exposure

Wild-type BALB/c mice (ten day old males and females) were briefly anesthetized under isoflurane and treated intranasally (i.n.) with 10µL of RSV A2 (10^6 TCID50/g body weight) or

mock solution (obtained through the same process as "RSV purification", except in the initial step virus-free RPMI medium+2.5%FBS was added to Hep-2 cells). Ten or thirty days post-infection, mice were briefly anaesthetized again prior to delivery of 50µg of low-endotoxin house dust mite (HDM) extract (Stallergenes Greer Ltd, London, UK) in a 20µL solution or an equal volume of phosphate buffered saline (PBS) i.n. (equally between both nares) on each of two consecutive days.

2.3.4 Lung digestion and preparation of cells for flow cytometry

Lungs were collected and cut into small pieces using sterile scissors and digested enzymatically for 30 minutes at 37˚C, 5% CO2 with a cocktail of DNase I (200µg/mL; Sigma-Aldrich, St. Louis, MO), LiberaseTM (100µg/mL; Roche, Indianapolis, IN), hyaluronidase 1a (1mg/mL; Life Technologies, Carlsbad, CA), and collagenase XI (250µg/mL; Life Technologies, Carlsbad, CA) in RPMI-1640 as described previously (33). Cells were then washed with RPMI-1640 media containing 1% Penicillin/Streptomycin and 5% FBS. Sterile, filtered ammonium-chloride-potassium (ACK) buffer was used to lyse red blood cells. Finally, filtration through a 0.7 µM strainer was performed and the remaining viable cells were recovered.

Trypan blue exclusion was used to enumerate viable cells and samples were diluted to obtain 1×10^6 cells for eosinophil/macrophage staining, 2×10^6 cells for ILC2 staining, and $1.5x10^2$ cells for T cell staining. For T cell staining, cells were stimulated for 4 hrs, at 37˚C, 5% CO2 with phorbol 12-myristate 13-acetate (PMA) (0.5µg/mL, Sigma-Aldrich, St. Louis, MO) and ionomycin (1 µg/mL, Sigma-Aldrich, St. Louis, MO) in the presence of GolgiStop™ (0.133µL/mL; BD Biosciences, Franklin Lakes, NJ). ILC2s were incubated with only GolgiStop™ (0.133µL/mL). Cells were washed three times with PBS or FACS buffer between staining steps. All cells were incubated in the dark for 20 min with eFluor780 viability dye (eBioscience, San Diego, CA), then incubated at 4°C for 10 min with anti-CD32/16 to block Fc receptors (Mouse BD FC block, BD Biosciences, Franklin Lakes, NJ). Macrophages and eosinophils were stained together with the following antibody cocktail: BUV395-CD45.2, Alexa Fluor 700-Ly6G, APC-F4/80, Alexa Fluor 488-CD11c, PeCy7-CD11b, PE-Siglec F. Interstitial macrophages (IM) were defined as CD45.2+, Ly6G+, CD11c+, F4/80+, CD11b+ and alveolar macrophages (AM) were defined as CD45.2+, Ly6G+, CD11c+, F4/80+, CD11b- (Figure 2-8A). Eosinophils were defined as CD45.2+, Ly6G+, SiglecF+, CD11cmed or CD11c- (Figure 2-8A). ILC2s were stained using EF-450-Thy1.2, PECy7-CD127, PerCP-eF710-ST2, KLRG1-BV605, CD45.2-BUV395, BV510-

MHCII and a combination of PE-conjugated antibodies to CD3e, CD11c, CD11b, CD49b, CD45R, TCRyD, Ly6G, and FCeRa1 (Figure 2-9A). T cells were stained using FITC-CD4, PerCP-Cy5.5-CD8, V500-CD3, BUV395-CD45.2 (Figure 2-9B). Cells were then fixed with intracellular fixation buffer (eBioscience, San Diego, CA) overnight. Afterward, ILC2s and T cells were permeabilized with BD Perm/Wash™ buffer (BD biosciences, Franklin Lakes, NJ). ILC2s were stained with AF488-IL-13 and APC-IL-5 while T cells were stained with APC-IL-4, PE-IL-13, BV421-IL-5. All cells were acquired using the BD LSRFortessaTM (Immununophenotyping Core Facility, RI-MUHC) flow cytometer. Analysis was completed with FlowJo V10 (FlowJo LLC, Ashland, OR). To define positive populations, fluorescence minus one (FMO) controls were used. Additional information for the antibodies can be found in Tables 1-3.

2.3.5 Lung Explant Culture

For lung explant culture, 2 million viable cells were allocated to two conditions, saline or IL-33. In 1mL of RPMI-1640 medium with 10% FBS, 5% penicillin/streptomycin, 1mM sodium pyruvate, 1mM non-essential amino acids, and 55µM 2-mercaptoethanol containing either saline or IL-33 (20ng/mL) cells were incubated at 37˚C and 5% CO2 for 48 hours. Supernatants were then collected and ELISA was used to quantify IL-13 and IL-5.

2.3.6 IL-13 and IL-5 ELISA

IL-13 and IL-5 were quantified using Ready-SET-Go! IL13 and IL-5 ELISA kits from Thermo Fisher Scientific (Carlsbad, CA) following the instructions provided. In duplicate, samples from cells cultured in saline were used neat. Samples cultured in IL-33 from mice treated with RSV and HDM were diluted 1/100, while those from mice treated with HDM only, RSV only and PBS only were diluted 1/50 to ensure values fell within the detection limit of the assay, 3.5 to 500 pg/mL.

2.3.7 IL-33 ELISA

A piece from the lower right lung lobe, frozen in liquid nitrogen and then stored at -80˚C, was placed in cold lysis buffer (20mM Tris-HCL, 0.14mM NaCl, 10% glycerol, 10% protein inhibitor [SigmaFastTM, Oakville, On, Protease Inhibitor Cocktail Tablet diluted 1/10 in H2O to dissolve the tablet]). Tissue was homogenized using Fisher's homogenizer 150 at max speed for 10 seconds, twice after which 55 µL of 10% Igepal (CA-630, Sigma-Aldrich, St. Louis, MO) was added to each sample. Samples were centrifuged 12000rpm for 15 minutes at 4˚C. Supernatants

were collected and total protein quantified (Bradford reagent, Sigma-Aldrich, St. Louis, MO). IL-33 mouse ELISA kit from Thermo Fisher Scientific (Carlsbad, CA) was used to quantify IL-33 following the manufacturer's instructions. In duplicate, samples from mice treated with RSV and HDM or HDM only were diluted 1/20, while those from mice treated with RSV only or PBS only were diluted 1/10 to ensure that values obtained fell within the detection limit of the assay, 25-3000 pg/mL.

2.3.8 Lung Function Measurements

Lung function was assessed in adult mice using the flexiVent™ small animal ventilator (Scireq, Montréal, QC) as described previously (34, 35). Briefly, mice were anesthetized using xylazine and sodium pentobarbital and paralyzed with pancuronium bromide. Mice were attached to the flexiVent apparatus. Baseline respiratory system resistance and Newtonian resistance as well as maximal respiratory system resistance and Newtonian resistance to increasing doses of nebulized methacholine were recorded.

2.3.9 Histology

Lungs were perfused with PBS then inflated through the trachea with 10% formalin. The entire lungs were isolated and placed in formalin. Lungs were embedded in paraffin and sections of 0.5μm were cut and mounted on glass slides, which were stained with haematoxylin and eosin (H&E). Slides were examined under a microscope (Zeiss Acio Imager M2, Wetzlar, Germany).

2.3.10 Statistical Analysis

Graphpad Prism 9 Software (San Diego, CA) was used for all analyses. Data were analyzed by one-way ANOVA or two-way ANOVA as described in the Figure Legends. Tukey's post hoc test was used for multiple comparisons. A $p \leq 0.05$ was considered significant. To remove outliers Grubb's test with an alpha of 0.05 was used.

2.4 Results

2.4.1 Early-life Infection with RSV enhances lung eosinophil response to HDM in female mice

An acute RSV→HDM model was established to investigate whether early-life RSV infection enhanced HDM-dependent type 2 innate inflammatory responses (Figure 2-1A). Male and female mice were infected with RSV at day 10 of life (PND10), a time when the neonatal mouse lung is skewed toward a type 2 inflammatory response in an IL-33-dependent manner (36). Afterward, on PND20 and 21, mice were exposed to HDM. Three days later, lung inflammatory responses were assessed. While lung neutrophils were increased in all mice exposed to HDM, they were not enhanced in mice previously infected with RSV; no differences were noted in alveolar macrophages (Figure 8B, C). Although the number of interstitial macrophages was increased in RSV→HDM mice compared to Mock→HDM or RSV→PBS mice, sex differences in the number of these cells were not observed (Figure 8D). Lung eosinophil accumulation and upregulation of CD11c, as a marker of activation (37), were also examined by flow cytometry (Figure 2-1B) using a gating strategy described by Abdala-Valencia, who showed that CD11c-negative eosinophils increase in the lung where they upregulate CD11c and transit into the airway lumen (38). Our data show that activated CD11cmed eosinophils were significantly increased in RSV→HDM female mice compared to RSV→PBS or Mock→HDM mice (Figure 2-1C). The increase in CD11cmed activated eosinophils was also significantly higher in female mice compared to their male counterparts (Figure 2-1C). Eosinophils lacking CD11c expression were also significantly increased in female mice that received both RSV and HDM compared to all other groups (Figure 2-1D). Altogether, these data show that RSV infection followed by acute HDM exposure 10 days later enhanced eosinophil recruitment and activation selectively in female mice, while responses in other innate cells did not exhibit sex differences and were less pronounced.

2.4.2 Early-life RSV infection enhances HDM-induced ILC2 responses, selectively in female mice

To better understand how RSV enhanced HDM-induced eosinophil responses selectively in female mice, we examined ILC2 responses. We focused on ILC2s because these cells produce large amounts of type 2 cytokines, IL-13 and IL-5, in response to innate cytokines, including IL-33, and exhibit enhanced responsiveness in adult female mice (21, 25, 39, 40). Importantly, RSV infection in neonatal mice (PND4) is associated with an increase in ILC2 numbers (24). Our data

demonstrate that a subset of ILC2s were selectively enhanced in female mice exposed to RSV→HDM (Figure 2-2). More specifically, the number of KLRG1$^-$ ILC2s, but not KLRG1$^+$ ILC2s, was significantly increased in RSV→HDM female mice compared to RSV→PBS or Mock→HDM female mice (Figure 2-2A, B). Both KLRG1$^-$ and KLRG1$^+$ ILC2s are functional; however, interactions between E-cadherin, which is expressed by epithelial cells, and KLRG1 on ILC2s, can dampen ILC2 responsiveness, at least in vitro (41, 42). These data suggest that prior RSV infection in young female mice has a lasting impact on ILC2s, particularly those lacking expression of KLRG1, which selectively expand upon subsequent exposure to HDM. Similar responses were not observed in male mice as ILC2 number (whether KLRG1$^-$ or KLRG1$^+$), did not significantly differ in mice exposed to RSV with or without later HDM (Figure 2-2A, B). The number of IL5+ ILC2s (whether KLRG1$^-$ or KLRG1$^+$) was significantly increased only in RSV→HDM female mice (Figure 2-2C, D). While the number of IL13+ KLRG1$^+$ ILC2s was significantly increased in female, but not male, RSV→HDM mice, compared to RSV→PBS or Mock→HDM mice, there was no difference between males and females within this group (Figure 2-2E). In addition, although the number of IL-13+ KLRG1$^-$ ILC2s was greater in both male and female RSV→HDM mice, the number of these cells was still significantly greater in females (Figure 2-2F). Thus, these female-specific enhanced ILC2 responses may be responsible, at least in part, for the greater recruitment and activation of eosinophils in female mice.

2.4.3 Early-life RSV infection enhances HDM-induced release of IL-33

IL-33 is constitutively expressed in the nuclei of endothelial and epithelial cells (43). Following cellular damage or necrotic cell death, full-length IL-33 is released (44). Allergens with protease activity, including HDM, can both induce the rapid release of IL-33 as well as process IL-33 into shorter, more biologically active mature forms (40, 45, 46). In order to better understand whether IL-33 release was associated with enhanced inflammatory responses to HDM in mice previously exposed to RSV, we quantified lung IL-33 levels in mice exposed to RSV and/or HDM at various time points. Consistent with data from Lambrecht and colleagues, in untreated mice, IL-33 levels in the lung were highest on PND4, after which they decreased by PND8 and remained constant until at least PND24 (Figure 2-3A). Saravia et al have shown that after RSV infection of PND4 neonates, IL-33 levels in the lung peak at 6 hours post-infection (11). Similarly, we found that in PND10 mice infected with RSV, IL-33 levels tended to increase at 6 hours and were significantly increased at 24 hours, with a modest decline in between (Figure 2-3B). In order to

determine if the increased IL-33 levels were sustained at the time of HDM exposure, we also quantified IL-33 levels at PND20, the day in which HDM is first delivered in this model. However, there were no differences between mock-infected and RSV-infected mice at PND20 (Figure 2-3C). Likewise, there were no differences in IL-33 levels 72 hours following HDM delivery between any of the groups, providing evidence that IL-33 levels were not elevated concurrently with increased eosinophils and ILC2s (Figure 2-3E). Nevertheless, IL-33 levels were significantly increased 6h after the second HDM exposure, selectively in mice that had previously been infected with RSV, though the amount of IL-33 released did not differ between males and females (Figure 2-3D). We also quantified TSLP in these experiments, but it was not detectable at any of the above time points (data not shown).

Given the data demonstrating an impact of prior RSV infection on HDM-induced responses on eosinophils and ILC2s, both of which express ST2 and have the ability to respond to IL-33, we hypothesized that ex vivo cytokine production from total lung cells in response to IL-33 would be enhanced in RSV→HDM mice. Thus, total lung cell populations were cultured ex vivo with either PBS or IL-33 and production of IL-13 and IL-5 was quantified. While levels of each cytokine were modest in cells cultured with saline, readily detectable amounts of both cytokines were produced upon culture with IL-33 regardless of their exposure history (ie: PBS→PBS, RSV→PBS, Mock→HDM or RSV→HDM) (Figure 2-10A, B). Nevertheless, lung cells from RSV→HDM mice produced significantly greater amounts of both IL-13 and IL-5, compared to all other groups (Figure 2-10A, B). These data suggest that the profile of IL-33-responsive cells was increased in RSV→HDM mice, although cytokine production did not differ in cells harvested from males and females. Altogether, these data suggest that early-life RSV infection not only promoted acute release of IL-33 in the lung, but also shaped the lung environment so that, upon subsequent allergen exposure, larger amounts of IL-33 were released. Nevertheless, because the levels of IL-33 did not differ between males and females, these data suggest that female-specific enhanced responses are downstream of IL-33 and/or that other factors promote enhanced responses in female mice.

2.4.4 Early-life RSV infection enhances Th2 and Type 2 cytotoxic T (Tc2) cell responses to HDM

Severe lower respiratory tract infection with RSV is associated with increased Th2 polarization in the lung (3-7). Th2-type cytokines, IL-4, IL-5, and IL-13 induce Th2 differentiation; eosinophil differentiation, recruitment and activation; mucus production; and AHR (47-49). In addition, IL-33 (the levels of which were enhanced in mice exposed to RSV and HDM), directly stimulates IL-13 production by antigen-experienced $CD4^+$ T cells (50). While $CD4^+$ and $CD8^+$ T cells expressing IL-13 were increased in RSV→HDM mice compared to RSV→PBS or Mock→HDM mice (Figure 2-4A, D), few changes were noted in populations of IL-5 expressing $CD4^+$ or $CD8^+$ T cells in males and females, regardless of exposure history (Figure 2-4B, E). Interestingly, compared to $CD4^+$ T cells, there were more $CD8^+$ T cells expressing IL-4 in RSV→HDM mice (Figure 2-4C, F). On the other hand, there were larger numbers of $CD4^+$ T cells expressing IL-13 compared to $CD8^+$ T cells expressing IL-13 (Figure 2-4A, D), and these cells are the only cytokine-producing population that was greater in females compared to males. Altogether, these data suggest that early-life RSV infection increases the propensity of both $CD4^+$ and $CD8^+$ T cells to produce type 2 cytokines, most notably IL-13, a response that is enhanced in female mice upon acute HDM exposure. Thus, together with ILC2s, these cells are well-positioned to enhance eosinophil responses in RSV→HDM female mice.

We examined ST2 levels on both $CD4^+$ and $CD8^+$ T cells to determine if ST2 expression was associated with the enhanced responses in RSV→HDM mice. While exposure to either RSV→PBS or Mock→HDM was sufficient to increase the number of T cells expressing ST2 at PND24, there was no further increase in either $CD4^+$ or $CD8^+$ T cells in RSV→HDM mice (Figure 2-10F, G), suggesting that the enhanced responses in RSV→HDM mice were not associated with selective activation of ST2-expressing T cells. Finally, for both $CD4^+$ and $CD8^+$ T cells, there was no significant difference in the level of ST2 expression between any treatment group (Figure S3H, I). Similarly, no differences in ST2 expression or expansion of cytokine-positive T cells were observed (data not shown). Together, these data suggest that the enhanced T cell responses in RSV→HDM mice, as well as the greater T cell responses in female mice, were not the result of greater ST2 expression on T cells responding to IL-33.

Oliphant et al. showed that MHCII is expressed by ILC2s, allowing these cells to present antigen and activate $CD4^+$ T cells (51). When we examined MHCII expression on ILC2s in this model, there were more MHCII positive ILC2s in RSV→HDM mice, with the largest numbers in female mice, although on a per cell basis, expression of MHCII did not differ between treatment groups (Figure 2-10C, D). ILC2s were identified based on expression of ST2 (see Figure 2-2); however, ILC2 levels of ST2 did not differ between treatment groups (Figure 2-10E). Thus, following RSV→HDM exposure, ILC2s expressing MHCII are increased selectively in female mice, providing evidence that they are well-positioned to activate $CD4^+$ T cells in the lung in a sex-specific manner.

2.4.5 RSV infection-mediated enhanced type 2 allergic lung inflammation is maintained over time

To examine if the RSV-mediated enhancement of type 2 allergic responses, as well as augmented responses in females, were maintained over time, PND10 mice were infected with RSV and treated with HDM as above except that the interval between the two exposures was expanded from 10 days to one month (Figure 2-5A). Even after 30 days, the number of CD11cmed eosinophils was significantly greater in RSV→HDM female mice compared to either treatment alone and to their male counterparts (Figure 2-5B). The number of eosinophils lacking CD11c in RSV→HDM mice, however, did not differ between males and females (Figure 2-5C). The number of IL-13^+ T cells (whether $CD4^+$ or $CD8^+$) also followed the same trend as in the acute model: female RSV→HDM mice had greater numbers than RSV→PBS or Mock→HDM mice (Figure 2-5D, E). Interestingly, sex differences in IL-13-expressng T cells were now more prominent when the time between RSV infection and HDM treatment was extended. Female RSV→HDM mice had significantly more $IL13^+$ T cells compared to males of the same group (Figure 2-5D, E) and enhanced responses in RSV→HDM male mice were largely absent, unlike those in younger mice. No differences were found in T cells expressing IL-4 or IL-5 alone, in contrast to differences in the acute model (data not shown). These data demonstrate that the ability of RSV to impact allergen-induced inflammation is maintained over time, reflected in enhanced responses of eosinophils as well as T cells with the potential to produce IL-13.

2.4.6 Increased HDM-induced lung inflammation and KLRG1⁻ ILC2s in female mice infected in early life with RSV

Lung inflammation and cell infiltration into the lung were also assessed by H&E staining. While modest in all mice, inflammation was most prominent in female RSV→HDM mice, in which cell infiltration was present around both blood vessels and airways (Figure 6A), whereas inflammation in their male counterparts did not differ from untreated controls. Interestingly, RSV→PBS female mice also showed increased inflammation (Figure 2-6A). These findings agree with the unexpected increase in KLRG1⁻ (but not KLRG1+) ILC2s observed in females, but not males, infected one-month prior with RSV, whether exposed to HDM or not (Figure 2-6B, C). Similar increases in IL-13-expressing KLRG1⁻ ILC2s were also apparent in RSV→PBS female mice, though the increase did not reach statistical significance (Figure 2-6D). These data suggest that early-life RSV infection modifies the lung microenvironment such that KLRG1⁻ ILC2s increase several weeks later selectively in adult female mice.

2.4.7 AHR is present only in female mice with RSV infection prior to HDM exposure.

We examined effects of early-life RSV infection on lung function induced upon exposure to HDM in adult mice. Respiratory system resistance (Rrs) was significantly greater in RSV→HDM female mice, compared to RSV→PBS or Mock→HDM (Figure 2-7A, B). Rrs was also significantly greater in RSV→HDM females compared to their male counterparts (Figure 2-7A, B), in which no changes compared to control mice were apparent. These results show that lung function is impacted long term by early-life RSV infection. Altogether, these data provide evidence that RSV modifies the lung environment, inducing AHR and enhancing inflammatory responses upon allergen exposure, in a manner that persists for several weeks.

2.5 Discussion

No vaccines have been approved to prevent severe lower respiratory tract infection with RSV and, given the high burden of morbidity associated with childhood asthma, understanding the link between these diseases may help guide the development of effective treatments (5-8, 52). In the current study, we demonstrate that infection of young mice (PND10) with RSV followed by an acute intranasal exposure to the common allergen, HDM, promoted perivascular and peribronchial inflammation as well as eosinophil influx and activation, ILC2 expansion, and AHR. Inflammatory responses were greater in female mice compared to those in males; in particular, the subset of ILC2s lacking expression of KLRG1 and producing type 2 cytokines expanded more in female mice, in agreement with data from others showing that androgen signaling negatively regulates ILC2s and that adult female mice have greater levels of KLRG1$^-$ ILC2s (21, 25, 41). We have not, however, ruled out a role for androgen signaling in the regulation of KLRG1+ ILC2s in this model, since IL-5-producing KLRG1$^+$ ILC2s were also significantly lower in RSV→HDM male mice compared to their female counterparts.

In young mice previously infected with RSV and exposed to HDM 10 days later, expansion of ILC2s was enhanced in female mice. However, when ILC2s were quantified in adult mice, larger numbers of KLRG1$^-$ ILC2s were present in all female mice infected with RSV as neonates, whether they were subsequently exposed to HDM or not. Since this sex-specific expansion of ILC2s was observed in both the shorter (D10 to D24) and more prolonged models (D10 to D44), it is unlikely these effects can be attributed entirely to hormones, since gonadal hormone production is very limited in young mice (53). Data from our more prolonged model provide insight into which cells may contribute to production of several type 2 cytokines in the lung during RSV-dependent enhancement of acute HDM-induced type 2 inflammatory response. In this model, the sex differences in T cells were more apparent when the time between RSV infection and allergen challenge was extended. As speculated for the ILC2s, heightened sex differences in the more prolonged model could be attributed to the age of the mice and the effect of hormones as the mice sexually mature.

Another possibility is that RSV infection leads to an increase in levels of steady-state IL-33 in the lungs as mice mature, which could increase ILC2 levels. Lynch et al showed that BALF levels of IL-33 were significantly increased 72h after repeated exposure to CRE, specifically in

mice previously infected with pneumonia virus of mice (PVM) as neonates (54). Even in the absence of further PVM or CRE exposure, IL-33 levels continued to increase for at least 28 days. While the impact of elevated IL-33 on ILC2s was not assessed and the sex of mice was not presented in this study, their findings lead us to speculate that the increase in KLRG1$^-$ ILC2s in adult female mice could be due, at least in part, to increases in IL-33 in the lungs of adult female mice previously infected with RSV as neonates. Nevertheless, while KLRG1$^-$ ILC2s were elevated in adult female mice, even in the absence of HDM, they were not sufficient to induce a change in AHR, which was increased in female mice, but only after HDM exposure. In fact, AHR in these mice was more closely aligned with elevated numbers of CD4$^+$ and CD8$^+$ T cells expressing IL-13. Further work is required to define mechanisms by which AHR develops selectively in female mice following acute HDM exposure and to better understand why ILC2s increase in female mice over time following early-life RSV infection.

Elevated levels of CD8$^+$ T cells expressing IL-4 in nasal aspirates are associated with increased disease severity in RSV-infected infants (55). In our murine model, CD8$^+$IL-4$^+$ T cells triggered by RSV infection may respond to IL-33 released by epithelial cells following acute HDM exposure (56), in an antigen-independent manner through ST2 signaling (57). The increase in IL-13$^+$ CD4$^+$ T cells we observed agrees with the findings from Guo et al. and Endo et al. who showed, respectively, that effector Th2 cells and effector-memory Th2 cells promote type 2 inflammation by producing IL-13 and/or IL-5 in a TCR-independent manner in response to IL-33 (50, 58). However, although IL-33 levels in the lung were significantly increased within hours of HDM delivery in RSV treated young mice, the increase in IL-33 did not appear to directly impact T cell expansion as ST2$^+$ T cell numbers, as well as the quantity of ST2 expressed per cell, were not greater in RSV→HDM mice, nor was their number greater in females compared to males.

The role for other innate cytokines in enhanced responses to HDM in RSV-exposed mice has not, however, been excluded. IL-25 has been shown to play an important role in regulating the inflammatory response to RSV (59, 60). For example, data from Peterson et al. showed that IL-25 and its receptor IL-17RB are expressed in the lungs of RSV-infected BALB/c mice and that knocking out IL-17RB reduces pathology in these mice (59). Moreover, Lee et al. showed that the innate cytokine, TSLP, is elevated in the lungs of RSV-infected mice and that TSLP receptor (TSLPR) knock-out mice have reduced levels of IL-13 as well reduced airway resistance (61). In

skin inflammation models, $CD4^+$ T cell specific expression of TSLPR is critical for infiltration of both $CD4^+$ T cells as well as eosinophils (62). Thus, in our model, the expansion of T cells could be in part due to the release of other innate cytokines upon RSV infection leading to larger numbers of T cells in the lung. Another possibility is that the effects of IL-33 on T cells may be indirect. For example, enhanced production of IL-13 by ILC2s, particularly in ILC2s lacking expression of KLRG1, in female mice exposed to RSV→HDM could promote dendritic cell activity (63, 64), enhancing expansion and cytokine production of both $CD4^+$ and $CD8^+$ T cells. In this scenario, increased T cell responses would correspond primarily to RSV-specific effector/memory T cells in combination with a smaller contribution from newly differentiating HDM-specific T cells.

Our data show that following RSV infection, IL-33 levels increase in the lung, peaking at 24 hours post-infection. Saravia et al. performed similar experiments and found that IL-33 increased rapidly, peaking at 12 hours post-infection, and gradually decreasing thereafter. During the alveolar phase of lung development (PND4 to PND21), the lung experiences a gradual accumulation of type 2 innate cells (e.g. ILC2s, eosinophils) peaking at PND14 (36). Therefore, the differences in IL-33 levels in our data and those of Saravia may be due to the time at which mice are infected during this sensitive phase of lung development (PND5 for Saravia et al. and PND10 in our model).

Our cumulative results demonstrate that, compared to males, female mice are impacted to a greater extend by early-life RSV infection prior to acute HDM exposure. Our data show greater numbers of eosinophils, ILC2s, and T cells, as well as increased lung infiltration and AHR in females compared to males. Malinczak et al. showed that RSV infection prior to CRE sensitization and challenge leads to increased airway hyperactivity, mucus production, and IL-13 mRNA levels in male mice compared to females (11). These differences could be attributed to the strain of RSV used, RSV A2 with Line 19 fusion protein in their work compared to RSV A2 in ours. RSV A2 with Line 19 fusion protein leads to greater viral load, pulmonary IL-13 and mucin, and lung resistance compared to the more traditional A2 strain (65). Understanding how RSV strain differences and sex interact, both in neonates and adults, will be an important consideration to better understand effects of lower respiratory tract RSV infection in both children and adults.

Altogether, data from this RSV→HDM model suggest that the increased number of $KLRG1^-$ $IL\text{-}13^+$ ILC2s as well as $IL\text{-}13^+$ T cells, may be responsible for the increased numbers of activated

CD11cmed eosinophils in lungs of RSV-infected neonates subsequently exposed to acute HDM delivery. This may be through interactions with innate cytokines such as IL-33 though our data do not rule out a role for other innate cytokines, such as IL-25 and TSLP. Larger numbers of $IL5^+$ ILC2s in female mice may help explain why recruitment of $CD11c^-$ eosinophils was greater in these mice. Moreover, there were larger numbers of T cells producing type 2 cytokines in response to acute HDM exposure in mice infected 10 days prior with RSV. We speculate that these T cells respond to a type 2 skewed lung environment "primed" by RSV infection (66), driving them to respond more strongly to a second encounter with a similar innate cytokine milieu induced by HDM exposure. The precise antigen specificity of these T cells is not yet known. This body of work also sheds light on important sex differences that occur in type 2 inflammatory responses associated with early-life RSV infection and furthers our understanding of how "training" of cells of the innate immune system by virus infection may have enduring local effects on immune responses. As the prevalence and severity of asthma has been on the rise for many years and current treatment options for severe RSV disease are limited, a better understanding of the connection between early-life events and subsequent allergen exposures can contribute to the development of better prophylactic and/or treatment strategies to mitigate these risks that may differ between the sexes.

2.6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.7 Author Contributions

LL and EDF were responsible for designing the experiments as well as writing the manuscript. Experiments were performed by LL with assistance from HA, RK, and VG. BJW participated in experimental design.

2.8 Funding

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2.10 Data Availability Statement

Datasets are available on request:

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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2.12 Figures and Legends

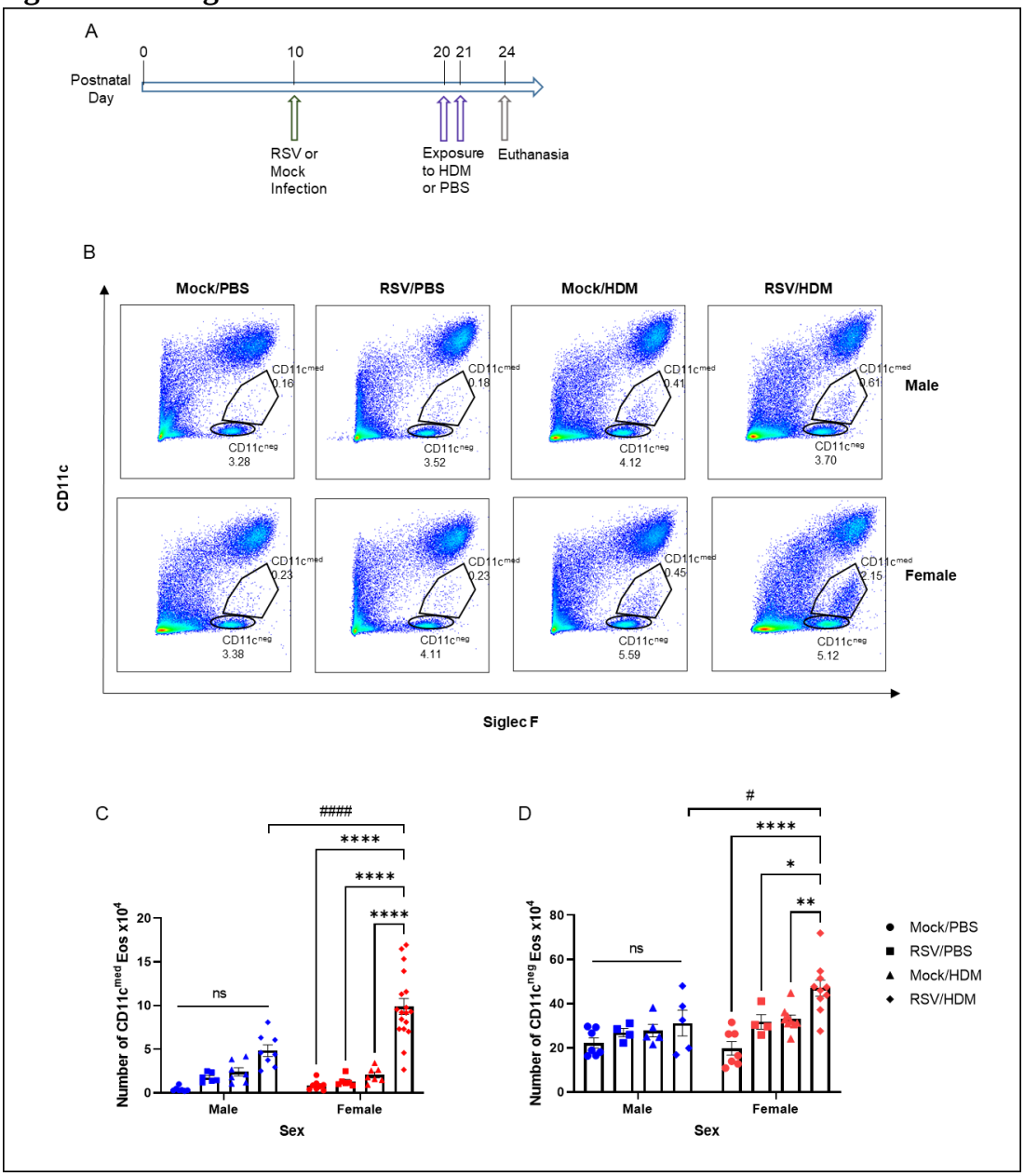

Figure 2-1. ***Early -life infection with RSV enhances lung eosinophil responses to HDM in females.***

(A) Mice were mock infected or exposed to RSV intranasally on PND10 and then treated with PBS or HDM (50ug per dose) intranasally on PND20 and PND21. Seventy-two hours later, lungs were harvested. **(B)** Representative flow cytometry panels highlighting $CD11c^{-}$ and $CD11c^{med}$ eosinophil populations for each treatment in each sex. **(C, D)** Absolute count of $CD11c^{med}$ and $CD11c^{-}$ eosinophils in the lung. Blue for male, red for female. Data are from the combination of four independent experiments (n=4-18 per group). Outcomes for C and D are presented as mean ± SEM assessed by two-way ANOVA, Tukey's post hoc test. ns=not significant, * $p \leq 0.05$, **or ## $p \leq 0.01$, **** or #### $p \leq 0.0001$.

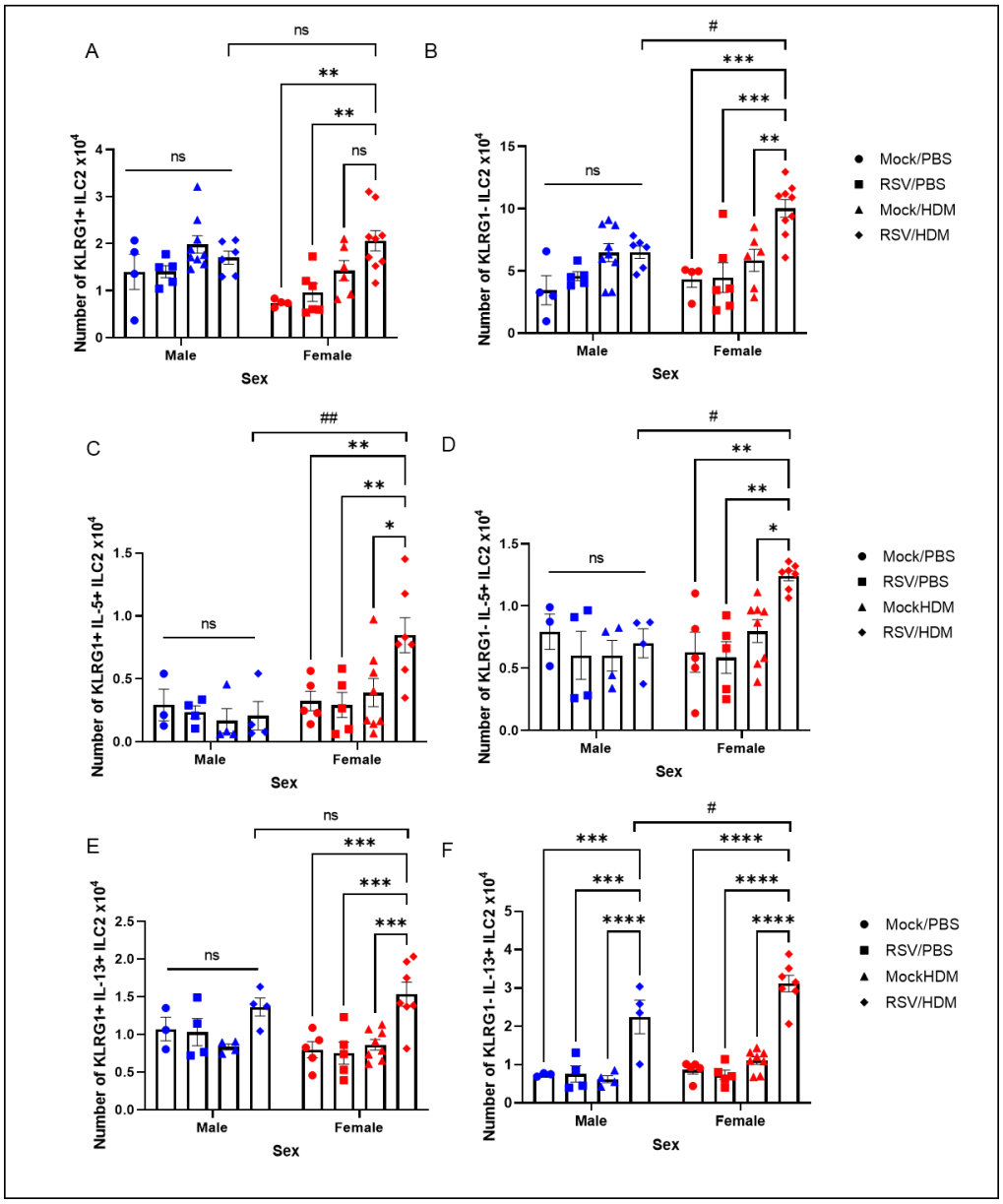

Figure 2-2. ***Early-life RSV infection enhances HDM-induced ILC2 responses, selectively in females.***

Mice were treated as in Figure 2-1A. Absolute count of **(A)** KLRG1$^+$ ILC2s and **(B)** KLRG1$^-$ ILC2s in males and females. Absolute count of KLRG1$^+$ ILC2s expressing **(C)** IL-5 or **(E)** IL-13 in males and females. Absolute count of KLRG1$^-$ ILC2s expressing **(D)** IL-5 or **(F)** IL-13 in males and females. Blue for male, red for female. Data are from the combination of two independent experiments (n=4-7 per group). Outcomes are presented as mean ± SEM assessed by two-way ANOVA, Tukey's post hoc test. ns=not significant, *or # $p \leq 0.05$, **or ## $p \leq 0.01$, *** $p \leq 0.001$, **** or #### $p \leq 0.0001$.

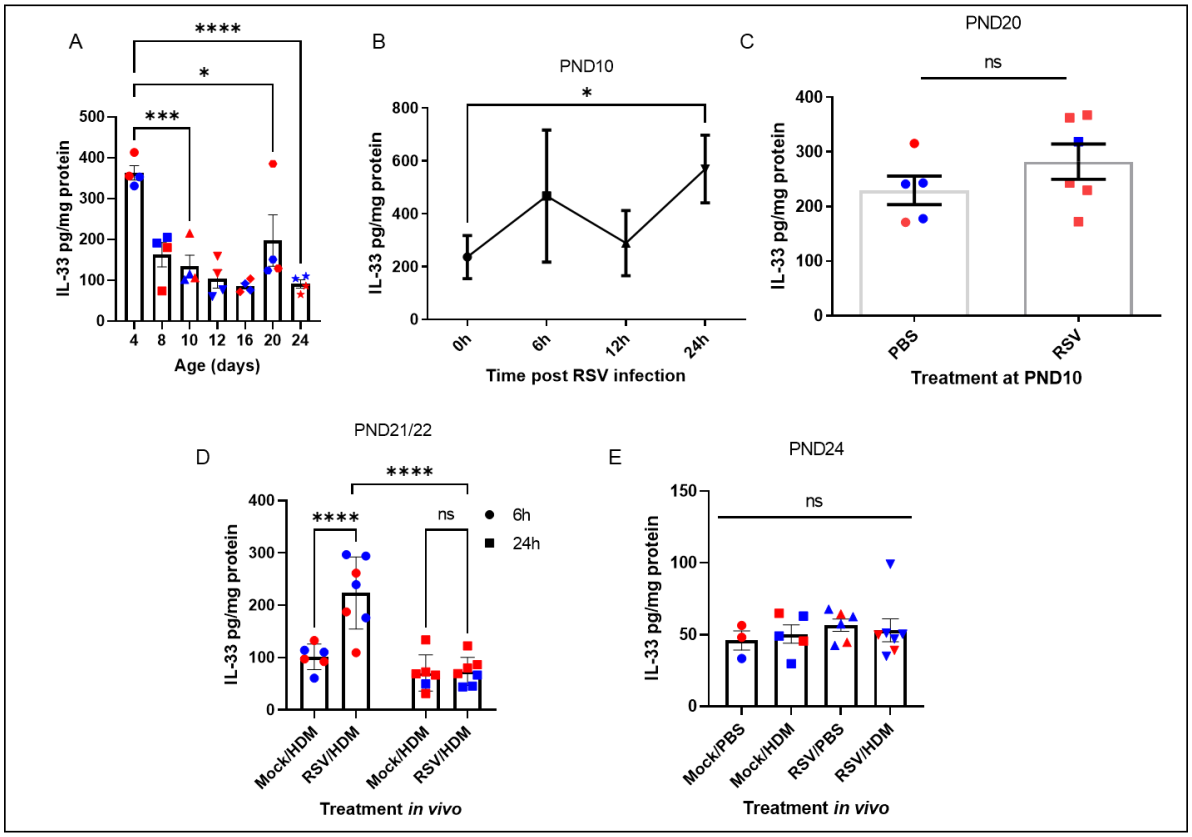

Figure 2-3. ***RSV infection enhances HDM-induced release of IL-33.***

(A) Levels of IL-33 in the lung of untreated mice at various ages, indicated on the x-axis. **(B)** Levels of IL-33 in the lung of PND10 mice treated with PBS or exposed to RSV and sacrificed 6, 12, or 24h later. **(C)** Levels of IL-33 in the lung of PND20 mice treated with PBS or infected with RSV on PND10. **(D)** Mice were treated as in Figure 2-1A, except they were sacrificed 6h or 24h after HDM to quantify levels of IL-33 in the lung. **(E)** Mice were treated as in Figure 2-1A and levels of IL-33 in the lung quantified 72h after HDM. Blue for male, red for female. Data are from a single experiment for each panel with n=4-9 per group in each experiment. Outcomes for A, B, C and E are presented as mean ± SEM assessed by one-way ANOVA, Tukey's post hoc test. Outcomes for D are presented as mean ± SEM assessed by two-way ANOVA, Tukey's post hoc test. ns=not significant, * $p \leq 0.05$, *** $p \leq 0.001$ **** $p \leq 0.0001$.

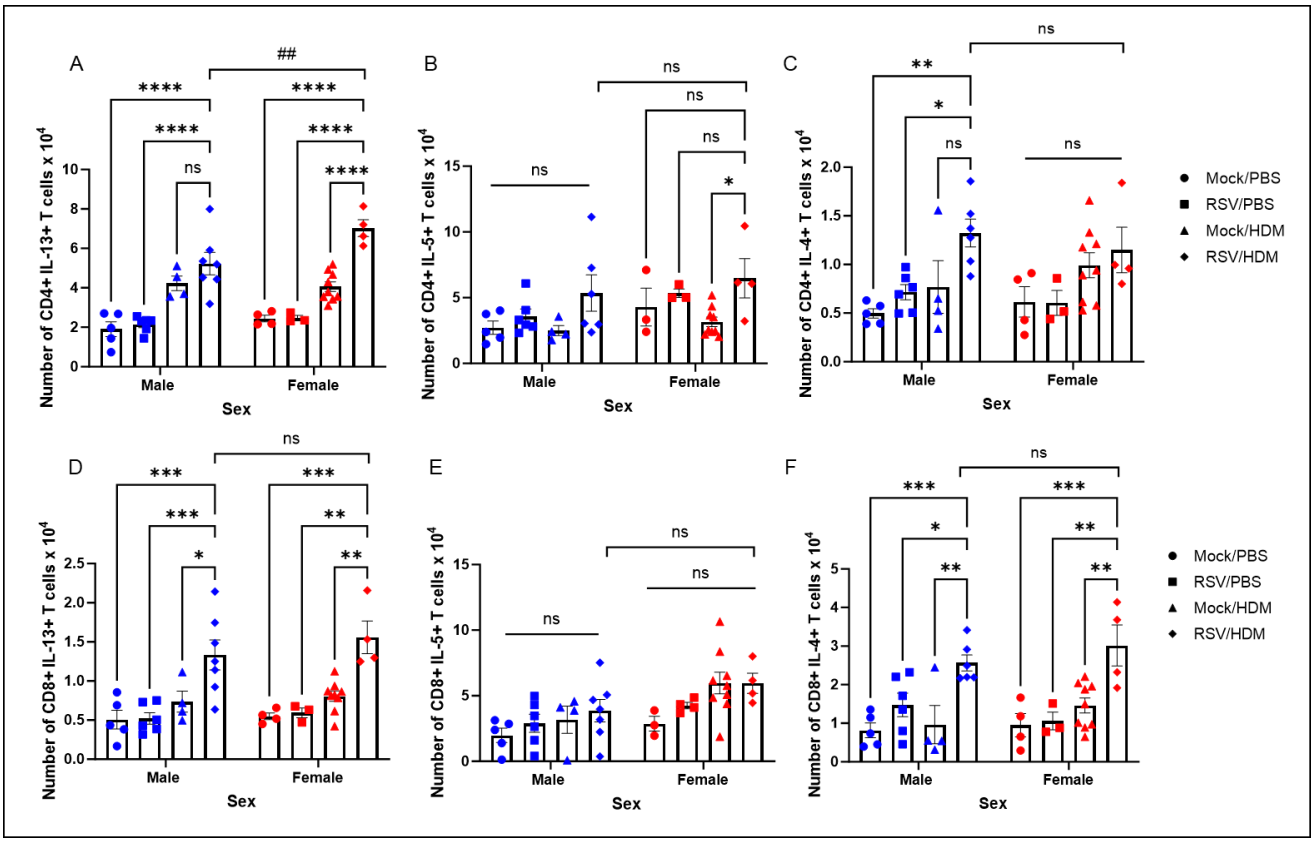

Figure 2-4. ***Early-life RSV infection enhances Th2 and Tc2 cell responses to HDM.***

Mice were treated as in Figure 2-1A. Absolute count of $CD4^+$ T cells **(A-C)** or $CD8^+$ T cells **(D-F)** expressing **(A, D)** IL-13, **(B, E)** IL-5, **(C, F)** IL-4. Blue for male, red for female. Data are from a combination of two independent experiments (n=3-9 per group). Outcomes are presented as mean ± SEM assessed by two-way ANOVA, Tukey's post hoc test. ns=not significant, * $p \leq 0.05$, **or ## $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

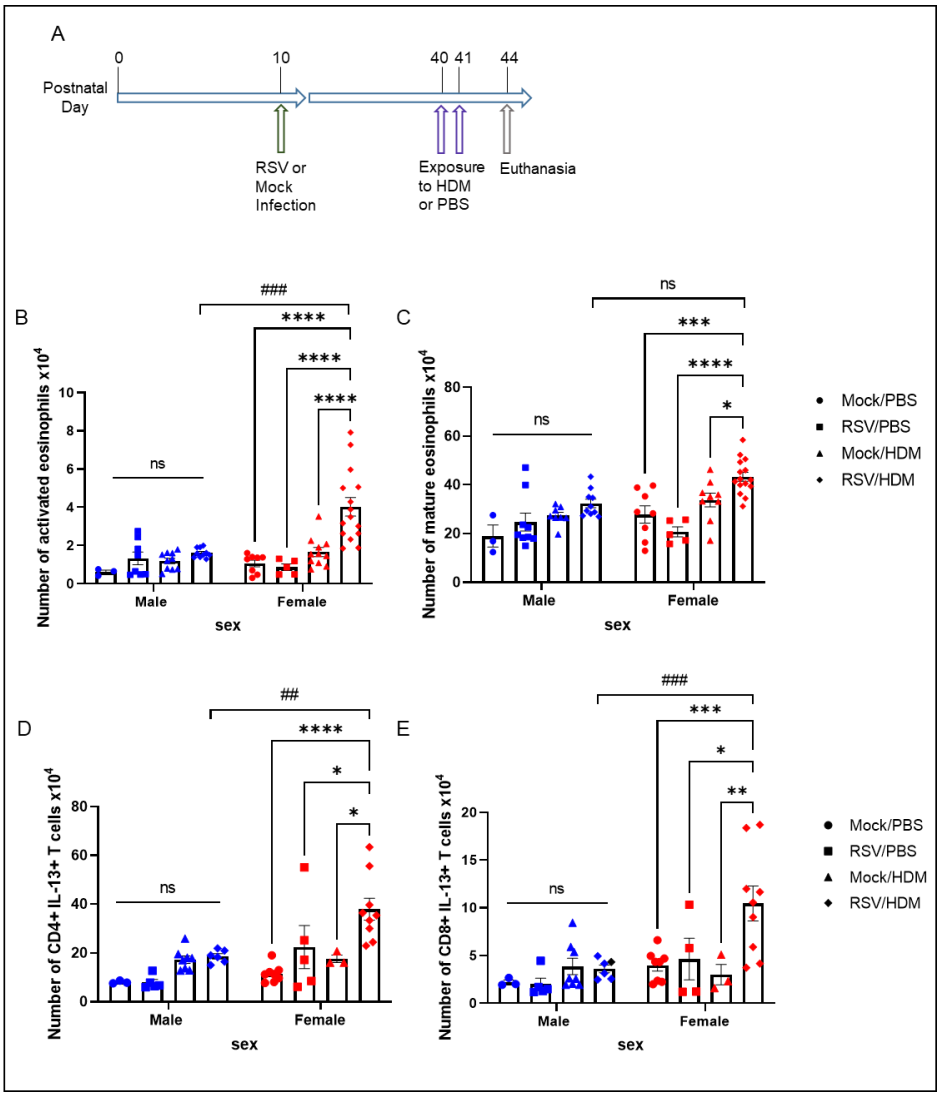

Figure 2-5. ***Early-life RSV infection-mediated enhanced type 2 allergic lung inflammation is maintained over time.***

(A) Mice were mock infected or exposed to RSV intranasally on PND10 and then treated with PBS or HDM (50ug per dose) intranasally on PND40 and PND41. Seventy-two hours later, lungs were harvested. **(B, C)** Absolute count of $CD11c^{med}$ and $CD11c^{-}$ eosinophils in the lung. Absolute count of IL-13 expressing **(D)** $CD4^{+}$ T cells or **(E)** $CD8^{+}$ T cells. Blue for male, red for female. Data are from the combination of two independent experiments (n=3-9 per group). Outcomes for B to E are presented as mean ± SEM assessed by two-way ANOVA, Tukey's post hoc test. ns=not significant, * $p \leq 0.05$, **or## $p \leq 0.01$, ***or ### $p \leq 0.001$, **** or #### $p \leq 0.0001$.

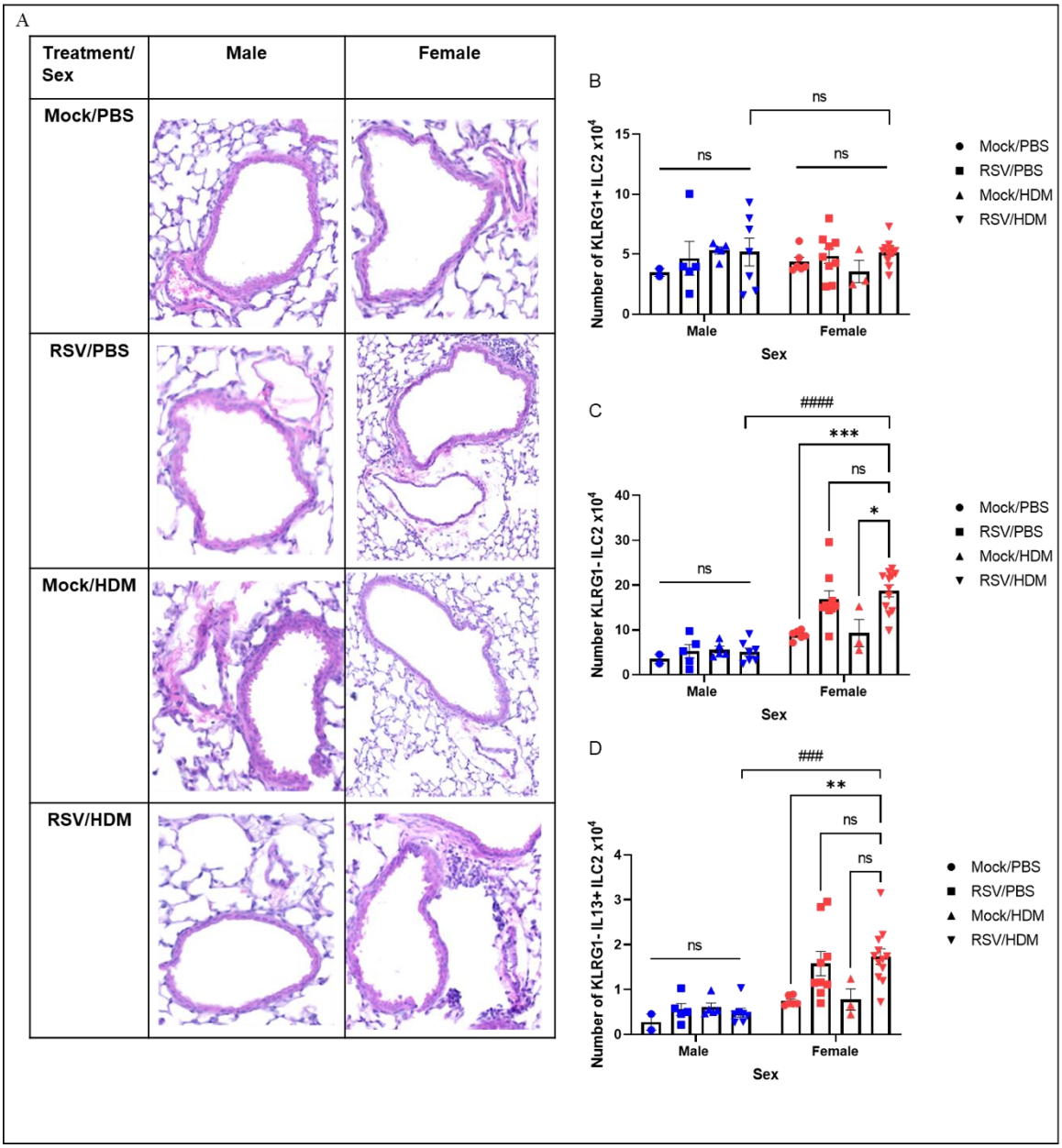

Figure 2-6. ***Increased HDM-induced lung inflammation and KLRG1⁻ ILC2s in female mice infected in early life with RSV.***

Mice were treated as in Figure 2-5A and lungs harvested for **(A)** histology or **(B-D)** flow cytometry 72h after HDM exposure. **(A)** H&E staining showing representative sections from male and female mice in each group. Images are representative of 16 mice from two independent experiments. Absolute count of **(B)** KLRG1$^+$ ILC2s, **(C)** KLRG1$^-$ ILC2s, and **(D)** KLRG1$^-$ ILC2s expressing IL-13 in males and females. Blue for male, red for female. Data in B-D are from the combination of two independent experiments (n=3-12 per group). Outcomes for B to D are presented as the mean ± SEM assessed by two-way ANOVA, Tukey's post hoc test. ns=not significant, * $p \leq 0.05$, ** $p \leq 0.01$, ***or ### $p \leq 0.001$, **** or #### $p \leq 0.0001$.

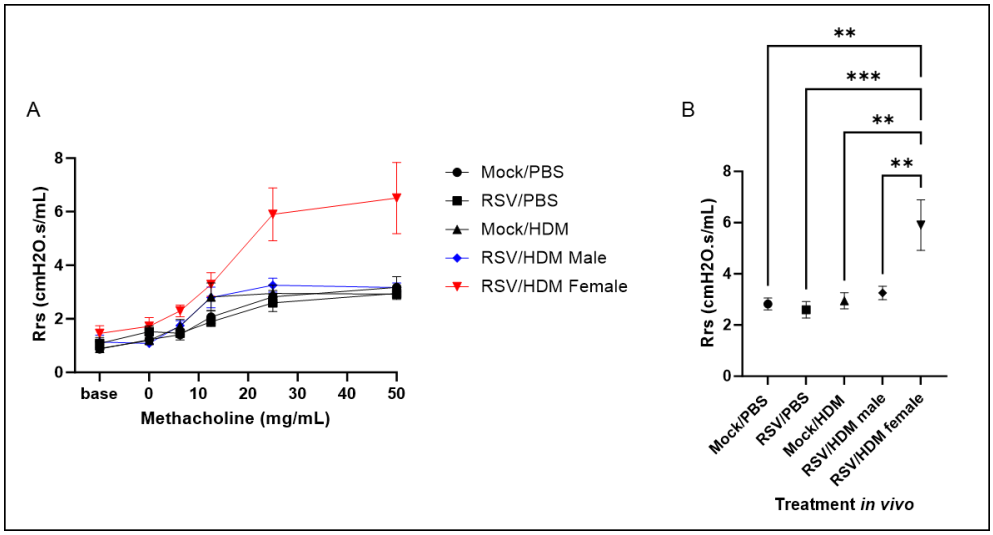

Figure 2-7. ***Early-life RSV infection is required for AHR induction following acute HDM exposure only in female mice.***

Mice were treated as in Figure 2-5A and lung function assessed 72h after HDM exposure. Respiratory system resistance (Rrs): **(A)** methacholine dose response and **(B)** 25mg/ml dose. Data are from the combination of two independent experiments (n=6-8 per group). In mock/PBS, RSV/PBS and mock/HDM no sex differences were observed; therefore, all mice were combined in these groups. Outcomes for B presented as the mean ± SEM assessed by one-way ANOVA, Tukey's post hoc test. ** $p \leq 0.01$, ***= $p \leq 0.001$.

2.13 Supplementary Figures and Legends

Antibody Tables

Table 1. Antibodies used for eosinophil and macrophages multi-color flow cytometry.

Antibody	Conjugation	Company	Clone Number
Viability	EF780	eBioscience	NA
CD45.2	BUV395	BD Biosciences	104
Ly6G	AlexaFluor700	BioLegend	1A8
F4/80	APC	eBioscience	BM8
CD11c	AlexaFluor488	eBioscience	N418
CD11b	PeCy7	eBioscience	M1/70
SiglecF	PE	BD Biosciences	E50-2440

Table 2. Antibodies used for ILC2 multi-color flow cytometry.

Antibody	Conjugation	Company	Clone Number
Viability	EF780	eBioscience	NA
Thy1.2	EF-450	eBioscience	53-2.1
CD127	PECy7	BioLegend	A7R34
ST2	PerCP-ef710	eBioscience	RMST2-33
KLRG1	BV605	BioLegend	2F1/KLRG1
MHCII	BV510	BioLegend	M5/114.15.2
CD45.2	BUV395	BD Biosciences	104
CD3e	PE	BioLegend	145-2C11
CD11c	PE	BioLegend	N418
CD11b	PE	eBioscience	M1/70
CD49b	PE	BD Biosciences	DX5
CD45R	PE	eBioscience	RA3-6B2
TCRyD	PE	BD Biosciences	GL3
Ly6G	PE	BioLegend	1A8

FCeRa1	PE	BioLegend	MAR-1
IL-5	APC	BD Biosciences	TRFK5
IL-13	AF488	eBioscience	eBio13A

Table 3. Antibodies used for T cell multi-color flow cytometry.

Antibody	Conjugation	Company	Clone Number
Viability	EF780	eBioscience	NA
CD45.2	BUV395	BD Biosciences	104
CD4	FITC	eBioscience	GK1.5
CD8	PerCP-Cy5.5	eBioscience	53-6.7
CD3e	V500	BD Biosciences	500A2
IL-13	PE	eBioscience	eBio13A
IL-5	BV421	BioLegend	TRFK5
IL-4	APC	eBioscience	11B11

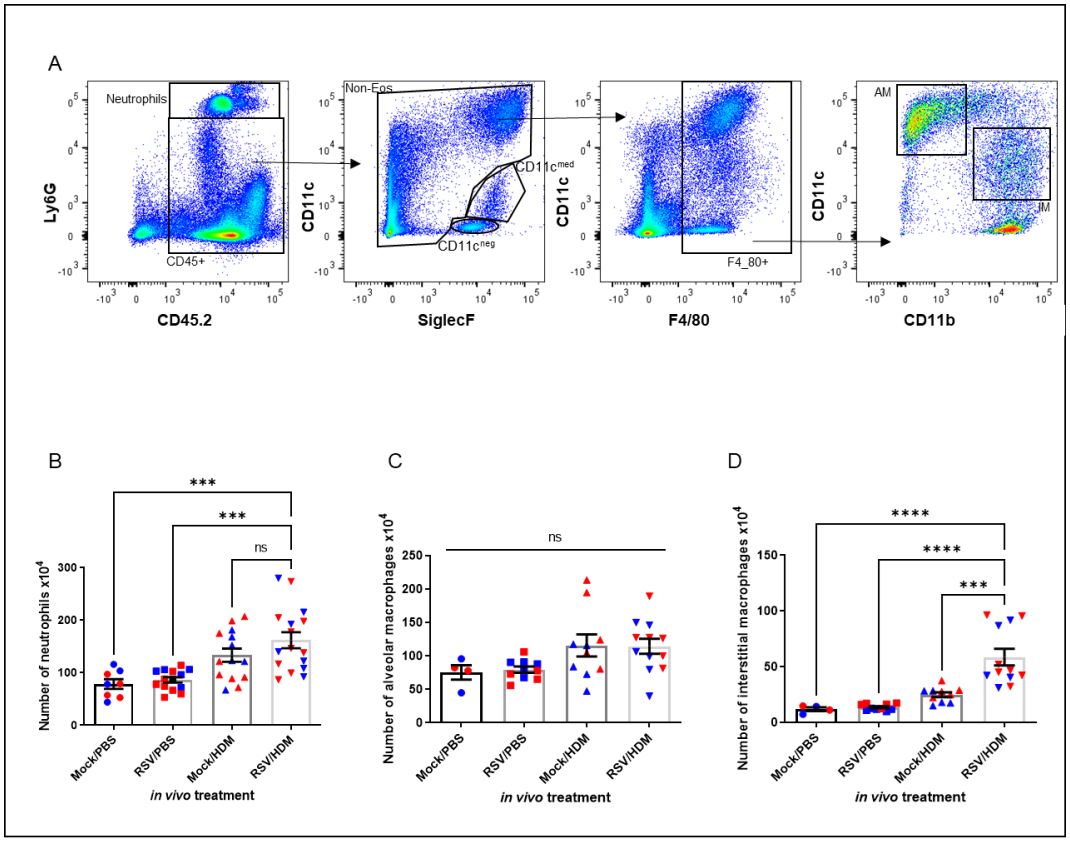

Figure 2-8. ***Interstitial macrophages, but not neutrophils or alveolar macrophages, are increased by acute HDM delivery in mice infected with RSV in early life.***

Mice were treated as in Figure 2-1A. **(A)** Flow cytometry gating strategy to identify neutrophils, alveolar macrophages and interstitial macrophages. Absolute count of **(B)** neutrophils, **(C)** alveolar macrophages (AM), and **(D)** interstitial macrophages (IM). Blue for male, red for female. Data are from the combination of two (C, D) or three (B) independent experiments (n=4-16 per group). Outcomes for B to D are presented as mean ± SEM assessed by one-way ANOVA, Tukey's post hoc test. ns=not significant, *** $p \leq 0.001$, **** $p \leq 0.0001$.

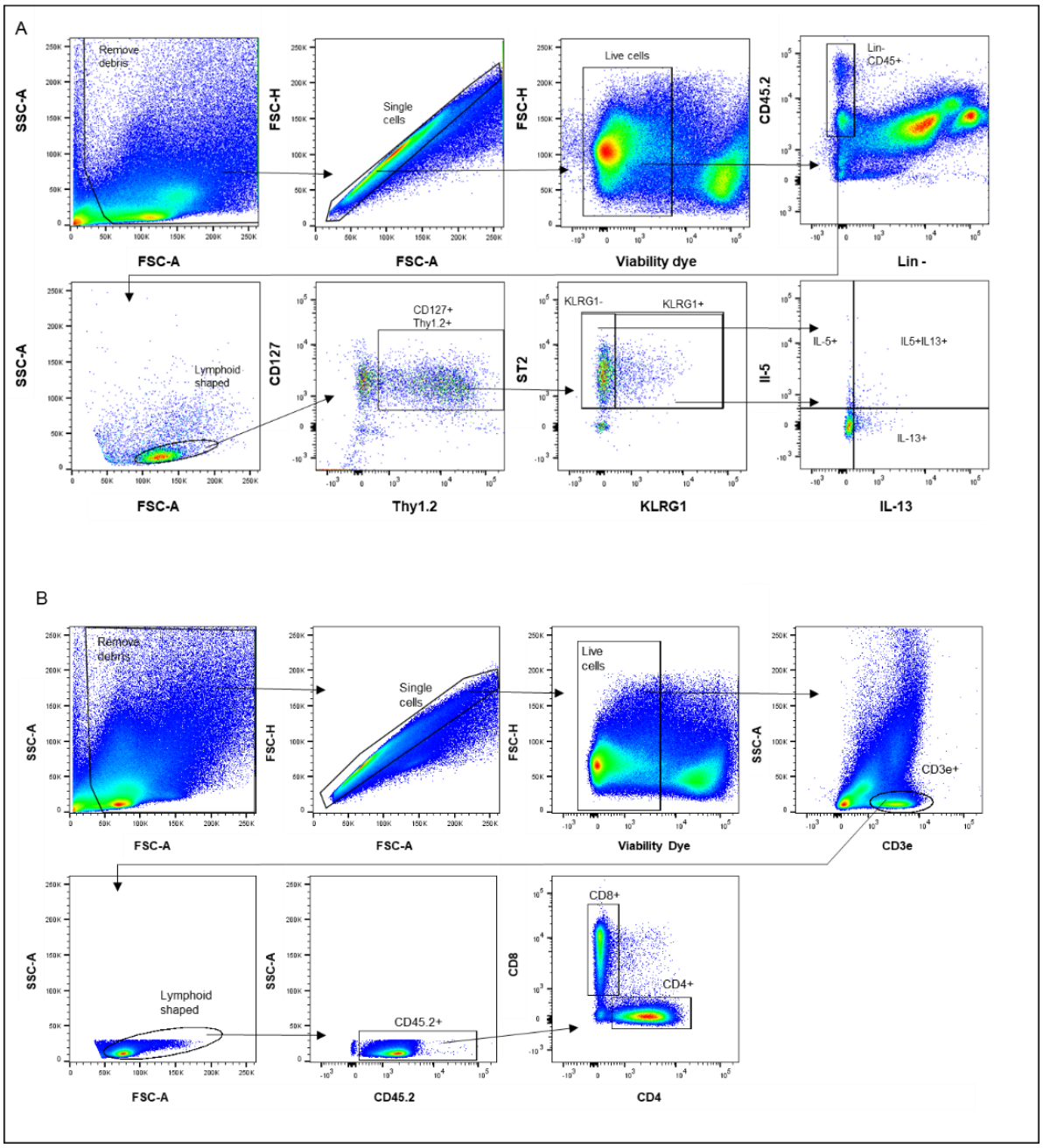

Figure 2-9. ***Flow gating strategy to identify type 2 immune cells***.

(A) ILC2 were stained using AF488-IL-13, APC-IL-5, EF-450-Thy1.2, PECy7-CD127, PerCP-eF710-ST2, KLRG1-BV605, CD45.2-BUV395, and a combination of PE-conjugated antibodies to CD3e, CD11c, CD11b, CD49b, CD45R, TCRyD, Ly6G, and FCeRa and gated as depicted. **(B)** T cells were stained using FITC-CD4, PerCP-Cy5.5-CD8, V500-CD3, BUV395-CD45.2 and gated as depicted.

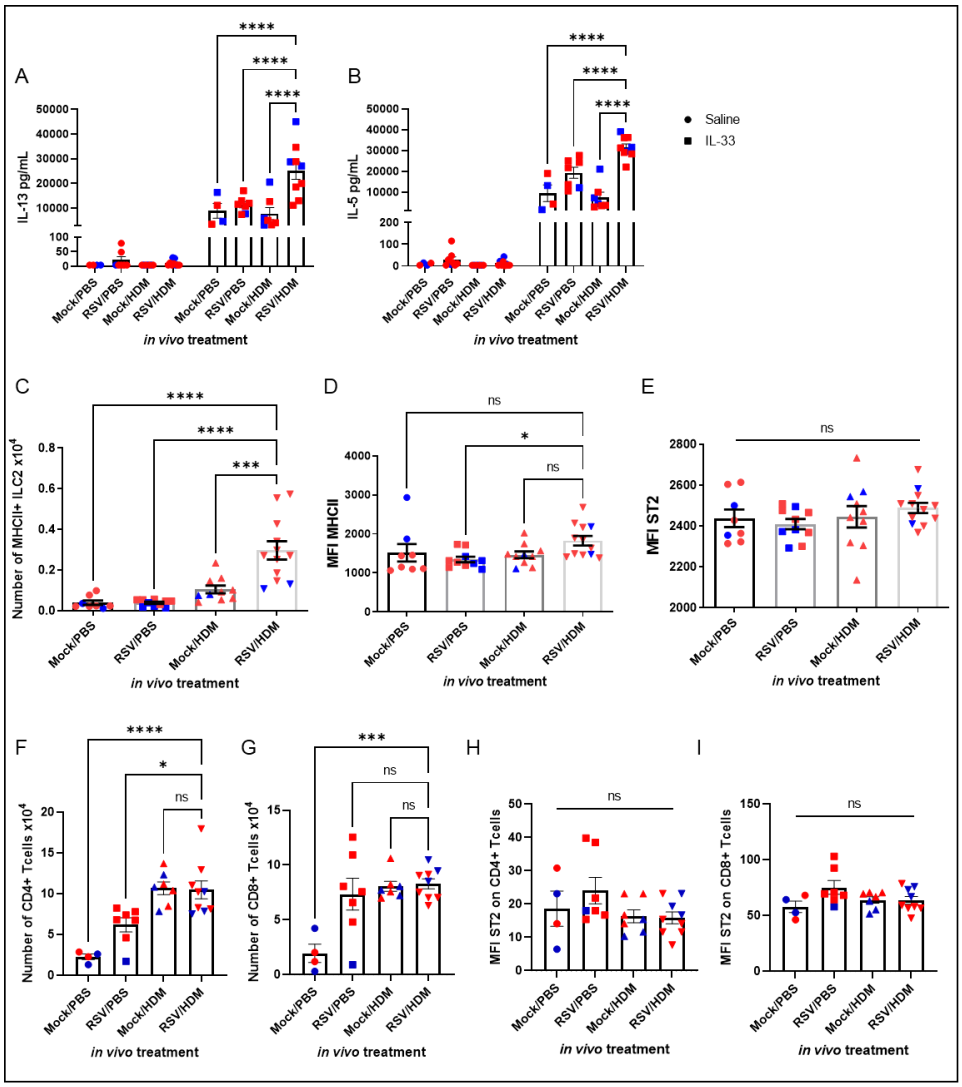

Figure 2-10. ***Lung cell cytokine production and characterization of ILC2 and T cell phenotype*.**

Mice were treated as in Figure 2-1A. Lungs were harvested and **(A, B)** cultured ex vivo or **(C-I)** assessed by flow cytometry. Quantity of **(A)** IL-13 or **(B)** IL-5 from saline or IL-33 cultured lung cells. **(C)** Absolute count of KLRG1^{-} ILC2s expressing MHCII. Median fluorescence intensity (MFI) of **(D)** MHCII or **(E) ST2** on ILC2s. Absolute count of **(F)** CD4^{+}or **(G)** CD8^{+} T cells expressing ST2. MFI of ST2 on **(H)** CD4^{+}or **(I)** CD8^{+} T cells. Blue for male, red for female. Data are from the combination of two independent experiments (n=4-12 per group). Outcomes are presented as mean ± SEM assessed by one-way ANOVA, Tukey's post hoc test. ns=not significant, *p ≤0.05, **p ≤0.01, *** p ≤0.001, ****p ≤0.0001

BRIDGE TO CHAPTER 3

In chapter 2, we demonstrated, using an early life RSV→HDM model, that ILC2s and eosinophils, as well as T cells are increased in response to acute allergen exposure when mice have been infected with RSV in early-life. Many responses were significantly greater in female mice compared to males, including AHR as well as numbers of activated eosinophils and KLRG1^{-} ILC2s expressing IL-13. These findings encouraged further work to understand the mechanisms driving these sex differences in the RSV→HDM model. Sex differences are well established in several diseases and many adult studies have demonstrated that sex hormones are responsible for establishing differences between male and female immune responses. However, even though they are essential for proper physical and behavioral development in mice, prepubertal hormones are often ignored as potential drivers of sex differences. Therefore, in chapter 3 we explored the role(s) of sex hormones, estrogen and testosterone, in modulation of inflammatory responses in the RSV→HDM model. This chapter describes the impact of modulating sex hormones prior to RSV infection. We examined effects of blocking androgen signalling, using DEHP, as well as providing exogenous testosterone propionate. We also explored effects of blocking estrogen signalling, using tamoxifen, and providing exogenous 17β-estradiol.

CHAPTER 3

Perinatal Estrogens Contribute to Sex Differences in RSV-Enhanced Type 2 Inflammation in the Murine Lung

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Keywords: Respiratory syncytial virus, eosinophils, group 2 innate lymphoid cells, perinatal hormones, estrogen, testosterone

3.1 Abstract

Differences in immune responses between men and women have been well defined and the concept that females mount stronger immune responses, and particularly antibody responses, to many stimuli than males is supported by numerous studies. Adult sex hormones are often the central focus in understanding sex differences in diseases. However, the onset of many diseases occurs before puberty and before sex hormones have reached peak levels. The possible impact of prepubescent hormones on immunity remains poorly understood. Severe RSV disease in infants and the heightened risk for the development of asthma thereafter are a significant burden on the healthcare system. Previously, we have shown that even in sexually immature mice, type 2 responses including eosinophil activation, airway hyperresponsiveness and increases in IL-13 producing cells are greater in females following early-life exposure to RSV and HDM. Unlike young female mice, young male mice experience a surge of testosterone during the perinatal period, which powerfully shapes their overall physiological development and adult behaviour. We hypothesized that perinatal hormones regulate the enhanced female responses to RSV→HDM exposure. Surprisingly, blocking androgens prior to early-life RSV infection had no effect in RSV→HDM males, and only eosinophils were reduced in similarly-treated female pups. Responses to RSV→HDM exposure in males were unaffected by exogenous androgens but IL-13+ T cells were reduced in females. On the other hand, blocking estrogen receptors reduced the number of ILC2s and T cells with the ability to produce IL-13 in RSV→HDM females and exogenous 17β-estradiol enhanced the number of these cells in RSV→HDM males. In summary, our data demonstrate the impact of perinatal hormones, particularly estrogens, on shaping the early innate immune environment and suggest that such effects may contribute to enhanced type 2 inflammation upon exposure to allergens after early-life RSV infection.

3.2 Introduction

Sex differences in immunological responses are well established. Compared to men, women tend to have more robust humoral immune responses, which likely predisposes them to various disorders such as food allergy and intolerance, migraines, irritable bowel syndrome, among others (1-5). The incidence, prevalence and severity of asthma are all higher in adult women compared to men (6). Although such enhanced responses appear to be detrimental, they may also provide better protection against some viral, bacterial, and parasitic infections (7).

In adults, sex hormones such as estrogen and testosterone are often considered drivers of sex differences. However, adulthood is not the only developmental stage at which sex differences are apparent (8, 9). Sex biases in several immune disorders, such as autoimmune diseases, hay fever, and migraines, exist in children prior to the onset of puberty (5, 10, 11). For example, the occurrence of wheezing and asthma is up to two-fold greater in young boys compared to young girls (12). The biological mechanisms and roles for hormones in establishing sex differences in young children are poorly understood.

Responses in group 2 innate lymphoid cells (ILC2s), which produce large amounts of type 2 cytokines, particularly IL-5 and IL-13 (13, 14) are hormonally regulated. Kadel et al. showed that in mice, androgens suppress the functional capacity of ILC2s and reduce a subset of ILC2s that lack expression of the killer-cell lectin like receptor G1 (KLRG1). While males and females have similar numbers of ILC2s that express KLRG1, KLRG1^{-} ILC2s are present at much higher levels in females, findings we have confirmed (15, 16). Similarly, data from Laffont et al. demonstrated that ILC2 progenitors express the androgen receptor (AR), which upon activation reduces maturation of ILC2 progenitors (17). Whether androgens modulate ILC2s in young mice is not known. While estrogens have no direct effect on ILC2s, they increase levels of IL-33 in the lung, thereby enhancing allergen-induced type 2 inflammation (18). Consistent with these findings from mice, women with moderate to severe asthma have larger numbers of circulating ILC2s compared to men (19).

By the age of two, almost all children have been infected with respiratory syncytial virus (RSV) (20). Most cases lead to mild disease, but of those hospitalised with severe lower respiratory tract infection up to 48% will develop asthma later in life (21). While the mechanisms linking severe RSV infection and asthma are poorly understood, ILC2s in nasal aspirates are elevated in

infants hospitalized with RSV bronchiolitis and elevated numbers of lung ILC2s are present in mice exposed to RSV followed by cockroach allergen (22, 23), supporting a role for ILC2s in linking RSV to asthma development. Other innate cells, such as alternatively activated macrophages, and adaptive cells, such as $CD4^+$ and $CD8^+$ T cells, have also been proposed as important mediators linking RSV infection and asthma development (24-26).

In our previous work we showed that inflammatory responses to a common allergen, house dust mite (HDM), were enhanced in mice previously infected with RSV in early life. We established a model in which postnatal day (PND) 10 mice were infected with RSV and then, 10 days later, exposed twice to HDM. Controls were exposed to RSV or HDM only (chapter 2). These experiments revealed that, even in sexually immature mice, eosinophil and ILC2 numbers are increased selectively in females, and only after exposure to both RSV and HDM. ILC2s and T cells expressing IL-13 are also preferentially increased in female mice, where they are associated with airway hyperresponsiveness (chapter 2).

The developing testes in male mice secrete testosterone in two perinatal surges, that occur late in gestation and then on the day of birth (27, 28). These testosterone bursts contribute to the physiological attributes of male mice as well as the neural circuits underlying sex-specific behaviors (27-29). Alcohol and stress have been previously reported as factors that can modulate testosterone levels at these key development periods in murine models (30). In addition, viruses such as influenza and COVID-19 can also impact testosterone levels in infected adults (31, 32), though a role for viral infection in modulation of testosterone in neonates does not appear to have been addressed.

Here we demonstrate that, unlike what is observed in adult mice, modulating androgens in neonatal mice prior to RSV infection, had no effect on inflammatory responses to RSV and HDM in males (or females). Interestingly, blocking estrogen receptor signalling with tamoxifen prior to RSV infection reduced the number of ILC2s and T cells with the ability to produce IL-13 selectively in young female mice, whereas providing exogenous 17β-estradiol enhanced the number of these cells selectively in male mice. Overall, we demonstrate that modulation of female sex hormones in prepubertal mice alters the inflammatory response initiated by RSV infection: blocking estrogen receptor reduces inflammation in young females, while providing 17β-estradiol enhances the inflammatory response in young males.

3.3 Materials and Methods

3.3.1 Mice

Wild-type BALB/c mice were bred in-house under pathogen-free conditions (originally from Charles River Laboratories, St-Constant, QC). Mice were bred in trios (2 females, 1 male). Male mice were removed from the breeding trios after 2 weeks. Once visibly pregnant, dams were housed one per cage. Following the birth of a litter of pups, cages were transferred to containment level 2 (CL2) facilities. Male and female pups were housed with their mothers until the experimental endpoint was reached (PND24). All cages were supplemented with water and irradiated food at all times. Animal studies were approved by the McGill University Animal Care Committee and performed in accordance to the guidelines of the Canadian Council on Animal Care.

3.3.2 RSV Purification

Hep-2 cells (ATCC #CCL-23, Manassas, VA) were infected with RSV A2 (ATCC #VR-1540, Manassas, VA). The virus was added to RPMI-1640 medium containing 2.5% heat inactivated (HI) fetal bovine serum (FBS) (Wisent, St-Bruno, QC) and gently mixed. Medium containing virus was then added to 70-80% confluent Hep-2 cells in a 24-well plate. At 4˚C, the virus was adsorbed to the monolayer for 90 minutes on a rocker. RPMI+2.5% HI-FBS was used to wash the monolayer which was then cultured for 3 days at 37°C in fresh RPMI+2.5% HI-FBS. When the cell monolayer exhibited cytopathic effect (CPE) over 40-50% of the surface under light microscopy, scraping was used to disrupt the monolayer. Two washes of the wells were performed, once with H2O to disrupt any intact cells followed by supernatant from first wash, both spinning at 400g for 5 minutes at 4˚C, to remove intact cells and debris. Supernatant was transferred to a T175 flask containing 80-90% confluent Hep2-cells. At 4˚C, virus was adsorbed for 90 minutes on a rocker. Cells were washed with RPMI+ 2.5% HI-FBS, and the monolayer was cultured at 37°C in fresh RPMI+2.5% HI-FBS for 3 days. When CPE reached 70-80%, the cell monolayer was disrupted by scraping (as above). Centrifugation at 4°C for 10 minutes at 2095 x g was used to clear the supernatant of cellular debris (33). A sucrose gradient with centrifugation at 116 000 x g for 2 hours at 4°C further purified the virus (34). The final purified virus was aliquoted and stored at -80°C. To determine viral titers, TCID50 was used as previous described (34).

3.3.3 Perinatal Testosterone propionate (TP) exposure

Following daily verification, day 1 of gestation was determined by the presence of a copulatory plug in female mice. Starting on gestational day 16 until parturition, pregnant females were treated subcutaneously on the dorsum of the back with 100µg of testosterone propionate (TP) (Health Canada, Ottawa, Ontario) in 50µL of sesame oil (SO) or vehicle alone. TP or vehicle injections at the same dose as above were administered intraperitoneally to pups on alternate days from PND1 to PND7 using a 30-gauge needle (35). By using both prenatal and postnatal androgenization, the natural perinatal androgen surges were recapitulated as previously described (36, 37).

3.3.4 Perinatal Anti-androgen di-(2-ethylhexyl) phthalate (DEHP) exposure

Starting on gestational day 16 until PND7, dams consumed half of a Cocoa Puff (Nesquick brand) covered in corn oil (CO) containing 200 mg/kg body weight of anti-androgen di-(2-ethylhexyl) phthalate (DEHP) (Sigma-Aldrich, St. Louis, MO) or vehicle alone as previously described (38). Females were monitored until the entire piece of Cocoa Puff was consumed (approximately 30 minutes).

3.3.5 Neonatal Tamoxifen exposure

Pups were given 1mg/kg of tamoxifen (Sigma-Aldrich, St. Louis, MO) suspended in a peanut oil/lecithin/condensed milk mixture (2:0.2:3 v/v) at a dose volume of 5µL/g body weight or vehicle alone from PND2 to PND5 by oral gavage (39).

3.3.6 Neonatal 17-β estradiol (E2) Exposure

Each pup was treated with 6.25ng of 17-β estradiol (E2) (Sigma-Aldrich, St. Louis, MO) dissolved in 0.03µL DMSO and diluted in olive oil (final volume of 50µL per pup) or vehicle alone intraperitoneally as above on alternate days from PND1 to PND7 (40).

3.3.7 RSV infection and HDM exposure

PND10 wild-type BALB/c pups (males and females), under brief anesthesia with isoflurane, were treated intranasally (i.n.) with 10µL of RSV strain A2 (10^6 TCID50/g body weight) or mock solution as previously described (chapter 2). Ten days post-infection, mice were briefly anaesthetized with isoflurane and then administered i.n. (equally between both nares) 50µg of low-endotoxin house dust mite (HDM) extract (Stallergenes Greer Ltd, London, UK) in a 20µL solution or an equal volume of phosphate buffered saline (PBS) on each of two consecutive days.

3.3.8 Lung digestion and preparation of cells for flow cytometry

Following collection using sterile scissors, whole lungs were cut into small pieces and then digested enzymatically for 30 minutes at 37˚C, 5% CO2 with a cocktail of DNase I (200µg/mL; Sigma-Aldrich, St. Louis, MO), LiberaseTM (100µg/mL; Roche, Indianapolis, IN), hyaluronidase 1a (1mg/mL; Life Technologies, Carlsbad, CA), and collagenase XI (250µg/mL; Life Technologies, Carlsbad, CA) in RPMI-1640 as described previously (41). Cells were then washed with RPMI-1640 media containing 1% Penicillin/Streptomycin and 5% FBS. To lyse red blood cells sterile, filtered ammonium-chloride-potassium (ACK) buffer was used. Finally, cells were filtered through a 0.7 µM strainer and the remaining viable cells were recovered.

To enumerate viable cells, trypan blue exclusion was used and then samples were diluted to obtain 1×10^6 cells for eosinophil/macrophage staining, 2×10^6 cells for ILC2 staining, and $1.5x10^6$ cells for T cell staining. For T cell staining, cells were stimulated for 4 hrs, at 37˚C, 5% CO2 with phorbol 12-myristate 13-acetate (PMA) (0.5µG/mL, Sigma-Aldrich, St. Louis, MO) and ionomycin (1µg/mL, Sigma-Aldrich, St. Louis, MO) in the presence of GolgiStop□ (0.133µL/mL; BD Biosciences, Franklin Lakes, NJ). ILC2s were stimulated with only GolgiStop□ (0.133µL/mL). Cells were washed three times with PBS. All cells were incubated in the dark for 20 min with eFluor780 viability dye (eBioscience, San Diego, CA), then incubated at 4°C for 10 min with anti-CD32/16 to block Fc receptors (Mouse BD FC block, BD Biosciences, Franklin Lakes, NJ). Macrophages and eosinophils were stained together with the following antibody cocktail: BUV395-CD45.2, Alexa Fluor 700-Ly6G, APC-F4/80, Alexa Fluor 488-CD11c, PeC7-CD11b, PE-Siglec F. Interstitial macrophages (IM) were defined as CD45.2+, Ly6G+, CD11c+, F4/80+, CD11b+ and alveolar macrophages (AM) were defined as CD45.2+, Ly6G+, CD11c+, F4/80+, CD11b- (Figure S1A). Eosinophils were defined as CD45.2+, SiglecF+, CD11c medium. ILC2s were stained using EF-450-Thy1.2, PECy7-CD127, PerCP-eF710-ST2, KLRG1-BV605, CD45.2-BUV395 and a combination of PE-conjugated antibodies to CD3e, CD11c, CD11b, CD49b, CD45R, TCRyD, Ly6G, and FCeRa1. T cells were stained using FITC-CD4, PerCP-Cy5.5-CD8, V500-CD3, BUV395-CD45.2. Then cells were fixed overnight with intracellular (IC) fixation buffer (eBioscience, San Diego, CA). Afterward, ILC2s and T cells were permeabilized with BD Perm/Wash™ buffer (BD biosciences, Franklin Lakes, NJ). ILC2s were stained with AF488-IL-13 and APC-IL-5 while T cells were stained with APC-IL-4, PE-IL-13, BV421-IL-5. All cells were acquired using the BD LSRFortessaTM (Immunophenotyping Core Facility, RI-

MUHC) flow cytometer. Analysis was completed with FlowJo V10 (FlowJo LLC, Ashland, OR). Fluorescence minus one (FMO) controls were used to define positive populations. Additional information for the antibodies can be found in Tables 1-3 in chapter 2.

3.3.9 Statistical Analysis

All analyses were performed using Graphpad Software Prism 10 (San Diego, CA). Two-way ANOVA was used to analyse data as described in the Figure Legends. Tukey's post hoc test was used for multiple comparisons. A $p \leq 0.05$ was considered significant. Grubb's test with an alpha of 0.05 was used to remove outliers.

3.4 Results

3.4.1 Blocking androgens does not alter male responses to RSV→HDM exposure

During the organizational period of sexual development, which includes embryonic and fetal periods, male mice experience prenatal and post-natal androgen surges on embryonic days 16 and 17 as well as PND 0 (27, 42). While estrogen levels are not different in males and females during development, androgen levels are markedly higher in males compared to females (42). Therefore, we hypothesized that perinatal androgens protect even very young males from enhanced responses to HDM following RSV infection and thus, that blocking androgens would feminize the male-pattern responses. Mackey et al. observed that protection from mast cell-associated anaphylaxis severity was driven by perinatal androgens; perinatal exposure to an antiandrogen, DEHP, increased disease severity in males into adulthood (35). We used a similar strategy to explore the effect of androgens on enhanced type 2 inflammation to HDM following early-life RSV infection (Fig 3-1). Androgen signaling was blocked using DEHP as described in Fig 1A. As shown previously, the number of eosinophils in female mice exposed to RSV→HDM was elevated compared to male mice (Fig 3-1B) (Labrie et al.). Surprisingly, DEHP had no effect on eosinophils in males, but reduced their numbers in female mice, suggesting that androgens promote HDM-induced eosinophil responses selectively in young female mice previously infected with RSV (Fig 3-1B). Alveolar macrophages and interstitial macrophages were not affected by DEHP in males or females (Fig 3-6A, 3-6 E). Similarly, DEHP delivery did not alter ILC2 responses in males or females. As shown previously (chapter 2) in mice exposed to RSV and HDM, numbers of $KLRG1^+$ ILC2s did not differ between males and females and $KLRG1^-$ ILC2s were elevated in females (though the difference was not statistically different in vehicle-treated control mice). Moreover, the number of $KLRG1^-$ ILC2s expressing IL-13 was elevated in females compared to males and unaffected by DEHP treatment (Fig 3-1E). ILC2s expressing IL-5 did not differ between males and females exposed to RSV→HDM and were not affected by DEHP delivery (Fig 3-7A). $CD4^+$ T cells expressing IL-13 or IL-5 followed a similar trend, where females showed higher numbers of cells compared to males while no differences were apparent upon DEHP treatment, regardless of sex (Fig 3-1F, Fig 3-8A). $CD4^+$ T cells expressing IL-4 did not differ between males and females exposed to RSV→HDM and were not affected by DEHP exposure (Fig 3-8E). CD8+ IL-13^+ expressing T cells showed no differences between any groups (Fig 1G). These data show

that eosinophils, but not ILC2s, were modulated only in RSV→HDM treated female mice upon inhibition of androgen signalling.

3.4.2 Exogenous androgens masculinize female T cell responses to RSV→HDM exposure

Although blocking androgen signaling with DEHP did not enhance the male response to RSV→HDM, we hypothesized, given that ILC2s are negatively regulated by androgen signalling and are found in greater numbers in females (15, 17), that having lower levels of perinatal androgens in females would contribute to the greater responses observed to RSV→HDM and that administration of exogenous androgens would reduce type 2 inflammation, particularly in female mice. Thus, testosterone propionate (TP) was administered as described in Fig 3-2A. Macrophage numbers were unaltered by TP exposure (Fig 3-6B, 3-6F). While the number of eosinophils in vehicle-treated females was significantly greater compared to their male counterparts, no TP-dependent differences in males or females were apparent (Fig 3-2B). Similarly, TP delivery did not alter ILC2 responses in males or females. As shown previously (chapter 2), in mice exposed to RSV and HDM, numbers of $KLRG1^+$ ILC2s did not differ between males and females and $KLRG1^-$ ILC2s were elevated in females (Fig 3-2C, D). Moreover, the numbers of $KLRG1^-$ ILC2s expressing IL-13 were elevated in females compared to males and were unaffected by TP treatment (Fig 3-2E). ILC2s expressing IL-5 did not differ between males and females exposed to RSV→HDM and were not affected by TP delivery (Fig 3-7B). $CD4^+$ T cells expressing IL-13 (Fig 3-2F), but not IL-5 or IL-4 (Fig 3-8B, 3-8F) were significantly decreased in females treated with TP compared to vehicle-treated females. Although not significantly different, a similar trend was also present in males treated with TP (Fig 3-2F). Similar trends were also noted in $CD8^+$ T cells expressing IL-13 (Fig 3-2G). These data demonstrate that only a subset of cells, particularly T cells expressing IL-13, but not ILC2s, were decreased in female RSV→HDM mice upon exposure to exogenous androgen. By providing TP, IL-13^+ T cells in females resembled those in males.

3.4.3 Blocking estrogen receptor decreases IL-13 producing ILC2 and T cell numbers in female mice

Given the increase in $KLRG1^-$ ILC2s in females in mice exposed to RSV→HDM (Fig 3-1D, 3-2D; chapter 2), that ILC2s in the lung express estrogen receptor alpha (Erα) (43), and that

estrogen receptor signalling is linked to an increase in allergen-induced IL-33 release (18), we examined the effects of perinatal female hormones on enhanced type 2 responses to HDM following early-life RSV infection. We first examined the effects of blocking the estrogen receptor using tamoxifen as depicted in Fig 3A. As shown previously (Fig 1B, 2B and Labrie et al.), the number of eosinophils was significantly greater in females compared to males (Fig 3-3B), though tamoxifen had no effect in male or female mice, similar to findings in macrophages (Fig 3-6C, 3-6G). Interestingly, ILC2s, expressing KLRG1 or not, were significantly reduced with tamoxifen treatment, but only in females (Fig 3-3C, 3-3D). As shown above (Fig 3-1,3- 2) and previously (chapter 2), IL-13 producing KLRG1$^-$ ILC2s, CD4$^+$ T cells, and CD8$^+$ T cells were elevated in RSV→HDM females (Fig 3-3E, 3-3F, 3-3G). These were not changed upon tamoxifen treatment in males; however, each was significantly reduced in tamoxifen-treated RSV→HDM females (Fig 3-3E, 3-3F, 3-3G). Nevertheless, CD4$^+$ T cells and CD8$^+$ T cells expressing IL-5 or IL-4 were not altered by tamoxifen treatment in males or females (Fig 3-8C, 3-8G). ILC2s producing IL-5 were also significantly decreased upon tamoxifen exposure in females, with a trend for a decrease in males as well (Fig 3-7C). These results suggest that ILC2s and T cells that express IL-13 are regulated by estrogen signaling in female mice, but not males. By blocking estrogen signaling, the number of cells expressing IL-13 were reduced to the levels in male mice.

3.4.4 Exogenous 17β-estradiol increases KLRG1$^-$ ILC2s and IL13+ T cells in male mice

To further understand the effect of perinatal female hormones on type 2 inflammation induced by HDM following early-life RSV infection, we next examined the effect of delivering exogenous estrogen to young mice. 17β-estradiol was delivered as shown in Fig 3-4A. No significant differences were found in eosinophils in these mice, though there was a trend for eosinophils to be higher in vehicle-treated females compared to their male counterparts (Fig 3-4B). Interstitial macrophages, but not alveolar macrophages, were increased in both males and females treated with 17β-estradiol (Fig 3-6D, 3-6H). While the number of KLRG1+ ILC2s were decreased in females treated with 17β-estradiol, males showed no change (Fig 3-4C). No changes were apparent in KLRG1$^-$ ILC2s in female mice; however, 17β-estradiol-treated males had significantly more KLRG1$^-$ ILC2s, levels that were similar to those in vehicle-treated RSV→HDM female mice (Fig 3-4D). Surprisingly, IL-13 expressing KLRG1$^-$ ILC2s were not different between control and treatment groups for either sex (Fig 3-4E) and KLRG1$^-$ ILC2s

producing IL-5 were significantly decreased upon 17β-estradiol exposure in females, with a similar trend in males (Fig 3-7D). Both $CD4^+$ T cells and $CD8^+$ T cells expressing IL-13 followed similar trends, with 17β-estradiol-treated males showing an increased number of IL-13^+ T cells compared to vehicle-treated males, though a significant increase was present only for $CD4^+$ IL-13^+ T cells (Fig 3-4F, 3-4G). In both males and females, 17β-estradiol increased $CD4^+$ IL-5^+ numbers and decreased $CD4^+$ IL-4^+ numbers (Fig 3-8D, 3-8H). These results demonstrate that the RSV→HDM response in males can be partially feminized by the addition of exogenous 17β-estradiol.

Altogether, our data demonstrate that the RSV-dependent enhancement of HDM responses are modulated by perinatal estrogen signaling. Although androgen exposure reduced the IL-13^+ T cell response in females, no other responses were significantly affected upon manipulation of perinatal androgens, most notable ILC2s, which are targeted by androgens in adults (17, 19). In contrast, blocking perinatal estrogen signaling reduced the levels of ILC2s and T cells producing IL-13 in females to levels observed in males and upon exposure to exogenous estrogen, KLRG1^- ILC2s and $IL13^+$ T cell numbers in males were increased to levels present in females.

3.5 Discussion

Although sex differences have been highlighted in several diseases and are often attributed to hormonal differences in adult men and women, the impact of perinatal hormones on diseases that begin in childhood remain largely unexplored. RSV is a major health burden to both young children and older adults and few accessible anti-viral therapies are available (1, 2). Because of the association between lower respiratory tract RSV infection in infants and development of wheezing and asthma, reducing severe RSV disease may improve clinical outcomes associated with asthma later in life. Our previous work demonstrated that, compared to males, female mice exhibit greater perivascular and peribronchial inflammation as well as eosinophil influx and activation, ILC2 expansion, and AHR following early-life RSV infection followed by a short 2-day course of HDM delivery to the lungs. Here we demonstrate that the mechanisms underlying several of these sex differences are linked to perinatal estrogens. Exposure to tamoxifen, prior to RSV infection, reduced responses in females to levels found in males for IL-13 producing ILC2s as well as both $CD4^+$ and $CD8^+$ T cells producing IL-13. Conversely, exposure to exogenous 17β-estradiol in early life enhanced responses in males: the number of $KLRG1^-$ ILC2 and IL-13 producing $CD4^+$ T cells were enhanced. Although perinatal estrogens had a significant impact on ILC2 and T cell responses, by either reducing responses in females when estrogens were removed or enhancing responses in males when estrogens were provided, eosinophils in females were reduced, surprisingly, when androgens were blocked. On the other hand, $IL\text{-}13^+$ $CD4^+$ T cells were reduced selectively in females upon delivery of exogenous testosterone propionate. Unlike findings in adult males, modulation of androgens through delivery of DEHP or testosterone propionate had no impact on these inflammatory responses in young males.

One key observation made during testosterone propionate use in young mice was that all pups were born with physical features commonly attributed to males. Females lacked visible teats throughout their development (day 0 to day 24) and the distance between the external genitalia and the anus were nearly identical in both males and females (at day 24 both males and females had fur between the external genitalia and anus). In order to avoid incorrect sexing of the mice, at the time of euthanasia all mice were examined for the presence of internal gonads. Mice were identified as female upon locating the uterine horns, and ovaries (3). This change in the physical features of females highlights the importance of perinatal androgens in shaping the physiological appearance of mice and demonstrates that these interventions were successfully administered.

Nevertheless, only modest changes in lung inflammation, described above, were apparent in these mice. Another key observation was that the total lung cell numbers between models varied quite dramatically and thus it is not appropriate to make directly comparisons between the different models. The difference is likely attributable to the different vehicles (eg: oil olive, corn oil, condensed milk, cocoa puff) used in each of the four models and their effects on the pregnant dams and/or their pups. For example, dams that were fed cocoa puffs coated in corn oil with or without DEHP were larger than dams in other models and pups that were given a condensed milk mixture with or without tamoxifen were larger than pups in other models. These variations in the dam's or the pup's size could effect the number of total lung cells.

Eosinophils express estrogen receptor α (ER-α) but do not express androgen receptor (AR) (4-6). However, despite this, eosinophils are often androgen responsive due to their regulation by other androgen-sensitive cells, including ILC2s, which are, at least in adults, negatively regulated by androgens (7, 8). Our data show that ILC2 numbers and their cytokine production were not decreased in female mice upon DEHP treatment. Therefore, it is unlikely that changes in ILC2s can explain the decrease in eosinophil numbers in DEHP-treated females. Dihydrotestosterone (DHT), an androgen, has been linked to increased M2 macrophage polarisation, which promotes eosinophil infiltration into the lung (9, 10). In the DEHP model, a lack of M2 polarised macrophages, specifically in female mice, may be responsible for the decrease in eosinophil migration to the lung. Why this decrease was not apparent in the male pups in our study is not clear. Perhaps, cell intrinsic differences exist between macrophages in male and females, at least during the neonatal period.

Upon manipulation of estrogens, either by blocking the estrogen receptor using tamoxifen or providing exogenous 17β-estradiol, IL-5^+ ILC2s decreased in female mice. The tamoxifen-mediated decrease is similar to data from Cephus et al. showing that, following allergen challenge, IL-5^+ ILC2s are reduced in gonadectomised female mice (lacking estrogen and progesterone) (7). On the other hand, Warren et al. reported that administering estrogen reduces ILC2 numbers following allergen challenge (11). Thus, it may be that ILC2s are sensitive to very high or very low concentrations of estrogens, whereas steady-state levels of estrogens increase ILC2 activity, those above or below this concentration reduce ILC2 activity. In addition, whether the signalling occurs through ER-α or ER-β has an impact on the immune response. When signalling occurs

through ER-α, airway hyperresponsivness (AHR), IL-33 production, type 2 cytokine production and eosinophil infiltration are all increased, whereas when signalling occurs through ER-β eosinophil infiltration and AHR decrease (10, 12, 13).

Treatment with 17β-estradiol decreased KLRG1$^+$ ILC2s in female mice. Although lung ILC2s are largely unaffected by steady-state levels of estrogens, despite expressing ER-α and ER-β (14), ILC2s located in the uterus are regulated by female sex hormones (15). In our study, 17β-estradiol may have influenced the migration of KLRG1$^+$ ILC2s and led to an accumulation of KLRG1$^+$ ILC2s in the uterus as opposed to other tissues, such as the lung, upon RSV infection. In future studies, examining the quantity of both ERα and ERβ expressed by ILC2s throughout the development of neonatal mice would shed light on intrinsic differences in male ILC2s, which may explain differences in sensitivity to estrogen exposure.

In summary, our work highlights an important role for perinatal estrogens in mediating the RSV-dependant enhancement of HDM responses in young mice through IL-13 producing cells and the subpopulation of ILC2s lacking expression of KLRG1. Future research targeting a better understanding of the mechanisms by which perinatal estrogens influence responses to RSV and subsequent development of asthma could allow for the development of novel prophylactic and/or treatment options to reduce the enhanced responses in females. Exploring the role of perinatal hormones may shed light on the long unstudied sex-specific mechanisms contributing to several childhood diseases.

3.6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.7 Author Contributions

LL and EDF were responsible for designing the experiments as well as writing the manuscript. Experiments were performed by LL with assistance from RK. BJW participated in experimental design.

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3.10 Data Availability Statement

Datasets are available on request:

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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3.12 Figures and Legends

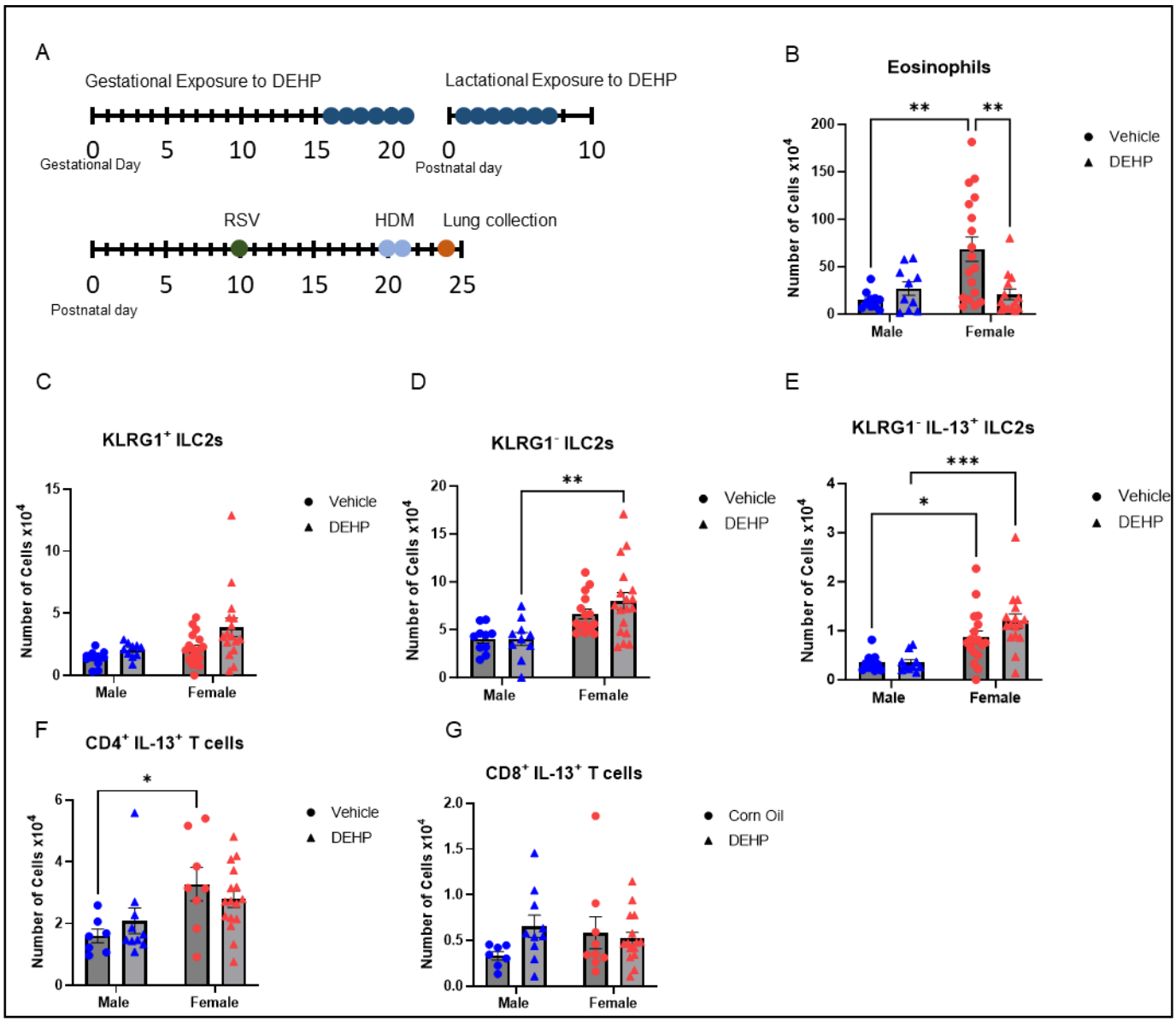

Figure 3-1. ***Blocking androgens does not alter male responses to RSV→HDM exposure***

(A) Adult female were administered DEHP (200 mg/kg body weight in corn oil) or vehicle via cocoa puff daily from day 16 of pregnancy until post-natal day 7. Young pups were treated with PBS or exposed to RSV on PND 10. Pups were treated 10 days later with PBS or HDM (50ug) intra-nasally on PND 20 and 21. Seventy-two hours later (PND 24), lungs were harvested. Number of activated eosinophils (B), KLRG1$^+$ ILC2 (Th1.2+, CD127+ and ST2+) (C), KLRG1$^-$ ILC2 (D), KLRG1$^-$ ILC2 expressing IL-13 (E), CD4$^+$ T cells expressing IL-13 (F) or CD8$^+$ T cells expressing IL-13 (G). Data are from the combination of two independent experiments (n=7-17 per group). Outcomes for B to G are presented as mean ± SEM assessed by two-way ANOVA, Tukey's post hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

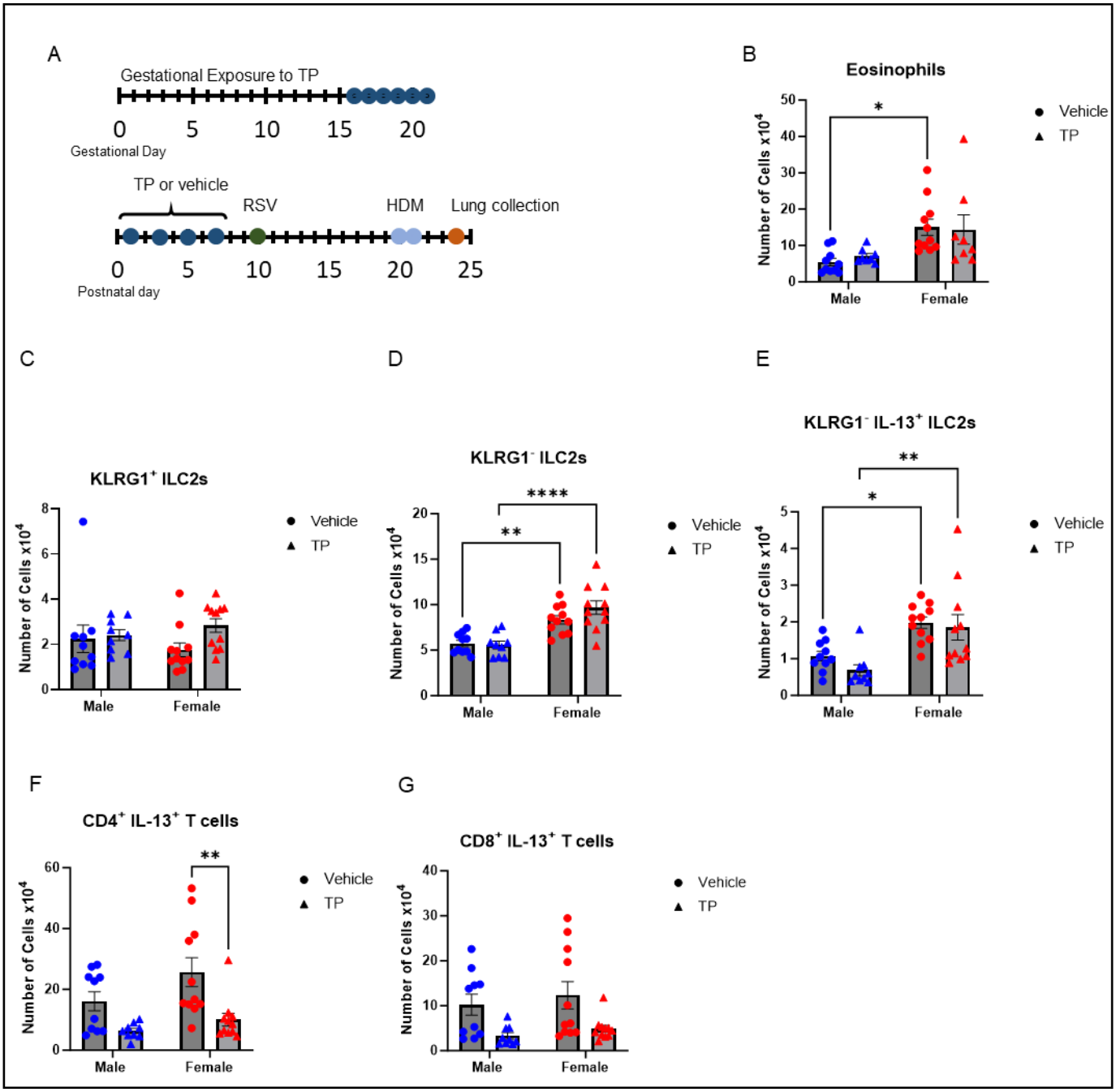

Figure 3-2. ***Exogenous androgens masculinize female T cell responses to RSV→HDM exposure.***

(A) Adult female were administered TP (100µg) in sesame oil or vehicle via subcutaneous injection day 16 of pregnancy until parturition. Pups with administered TP (100µg) intraperitoneally on alternate days from PND 1 to 7. Young pups were treated with PBS or exposed to RSV on PND10. Pups were treated 10 days later with PBS or HDM (50ug) intra-nasally on PND 20 and 21. Seventy-two hours later (PND 24), lungs were harvested. Number of activated eosinophils (B), KLRG1$^+$ ILC2 (Th1.2$^+$, CD127$^+$ and ST2$^+$) (C), KLRG1$^-$ ILC2 (D), KLRG1$^-$ ILC2 expressing IL-13 (E), CD4$^+$ T cells expressing IL-13 (F) or CD8$^+$ T cells expressing IL-13 (G). Data are from the combination of two independent experiments (n=10-11 per group). Outcomes for B to G are presented as mean ± SEM assessed by two-way ANOVA, Tukey's post hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

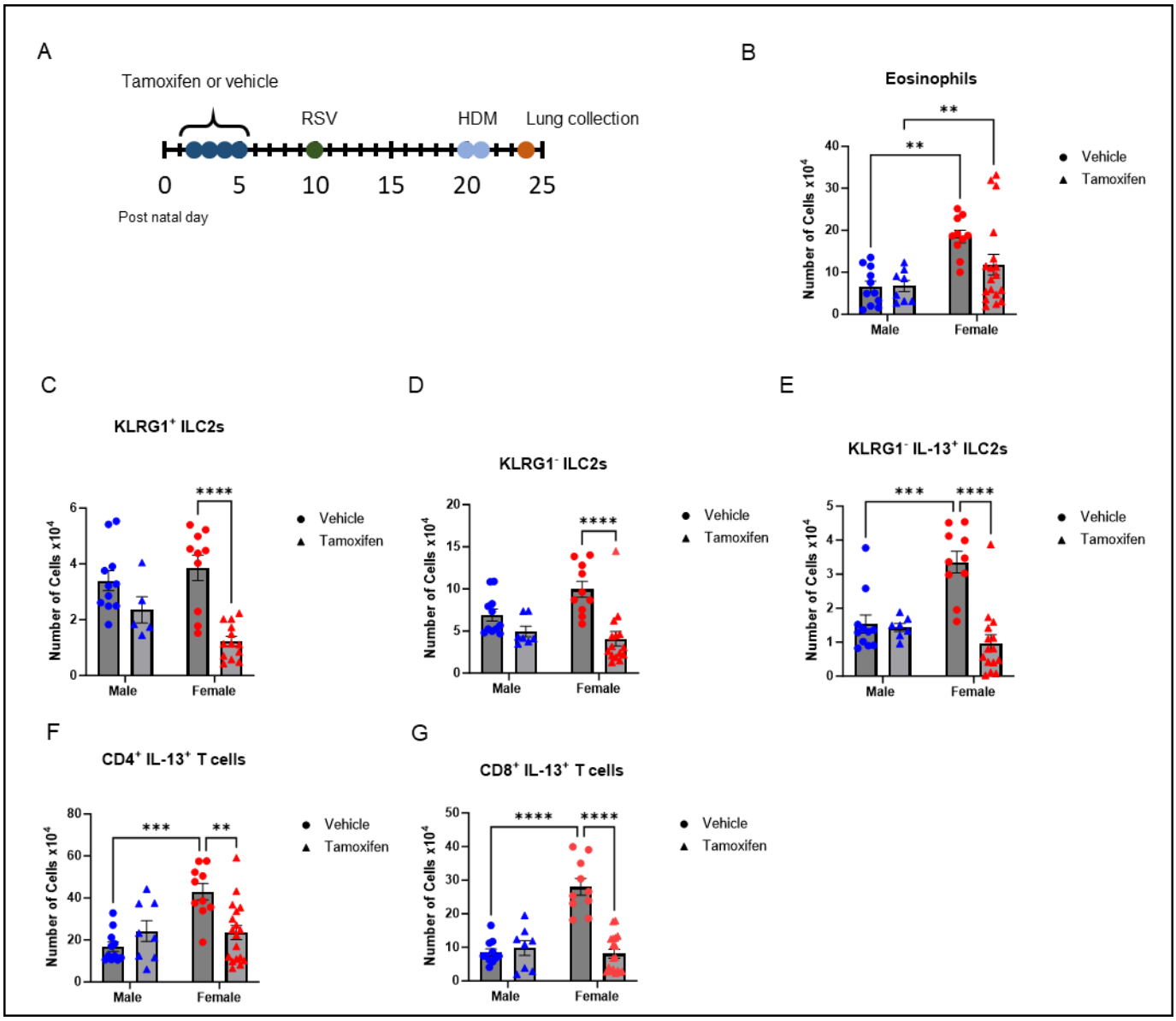

Figure 3-3. ***Blocking estrogen receptor decreases the number of IL-13 producing ILC2 and T cells in female mice.***

(A) Mice were administered tamoxifen (1mg/kg in peanut oil/lecithin/condensed milk mixture) or vehicle orally on PND 2,3,4 and 5. Mice were treated with PBS or exposed to RSV on PND10. Mice were treated 10 days later with PBS or HDM (50ug) intra-nasally on PND 20and 21. Seventy-two hours later (PND 24), lungs were harvested. Number of activated eosinophils (B), KLRG1$^+$ ILC2 (Th1.2$^+$, CD127$^+$ and ST2$^+$) (C), KLRG1$^-$ ILC2 (D), KLRG1$^-$ ILC2 expressing IL-13 (E), CD4$^+$ T cells expressing IL-13 (F) or CD8$^+$ T cells expressing IL-13 (G). Data are from the combination of two independent experiments (n=7-17 per group). Outcomes for B to G are presented as mean ± SEM assessed by two-way ANOVA, Tukey's post hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

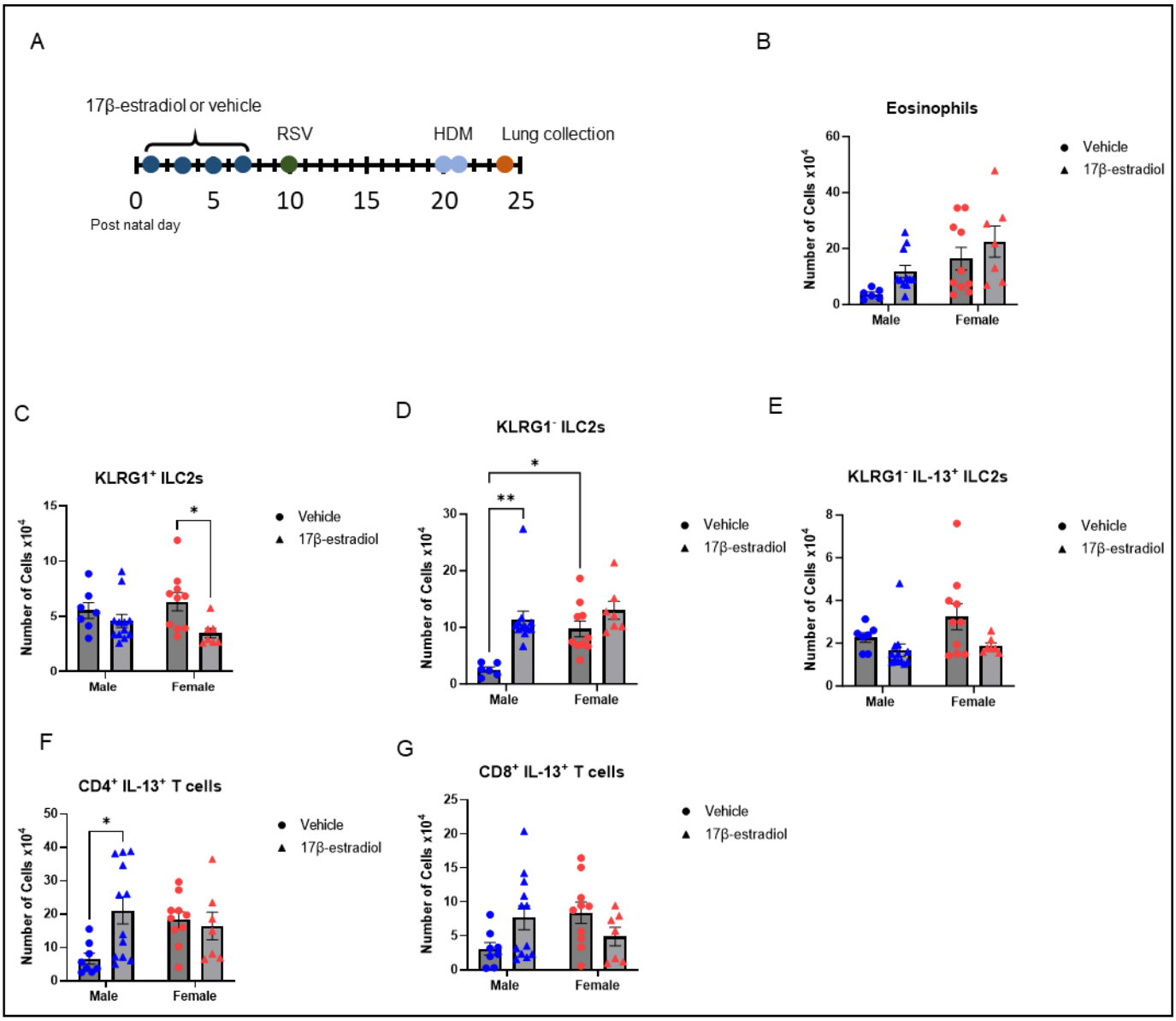

Figure 3-4. ***Exogenous 17β-estradiol increases KLRG1⁻ ILC2s and IL13⁺ T cells in male mice.***

(A) Mice were administered 17B-estradiol (6.25ng in olive oil) or vehicle intraperitoneally on PND 1, 3, 5 and 7. Mice were treated with PBS or exposed to RSV on PND 10. Mice were treated 10 days later with PBS or HDM (50ug) intra-nasally on PND 20 and 21. Seventy-two hours later (PND 24), lungs were harvested. Number of activated eosinophils (B), KLRG1$^+$ ILC2 (Th1.2$^+$, CD127$^+$ and ST2$^+$) (C), KLRG1$^-$ ILC2 (D), KLRG1$^-$ ILC2 expressing IL-13 (E), CD4$^+$ T cells expressing IL-13 (F) or CD8$^+$ T cells expressing IL-13 (G). Data are from the combination of two independent experiments (n=7-11 per group). Outcomes for B to G are presented as mean ± SEM assessed by two-way ANOVA, Tukey's post hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.13 Supplementary Figures and Legends

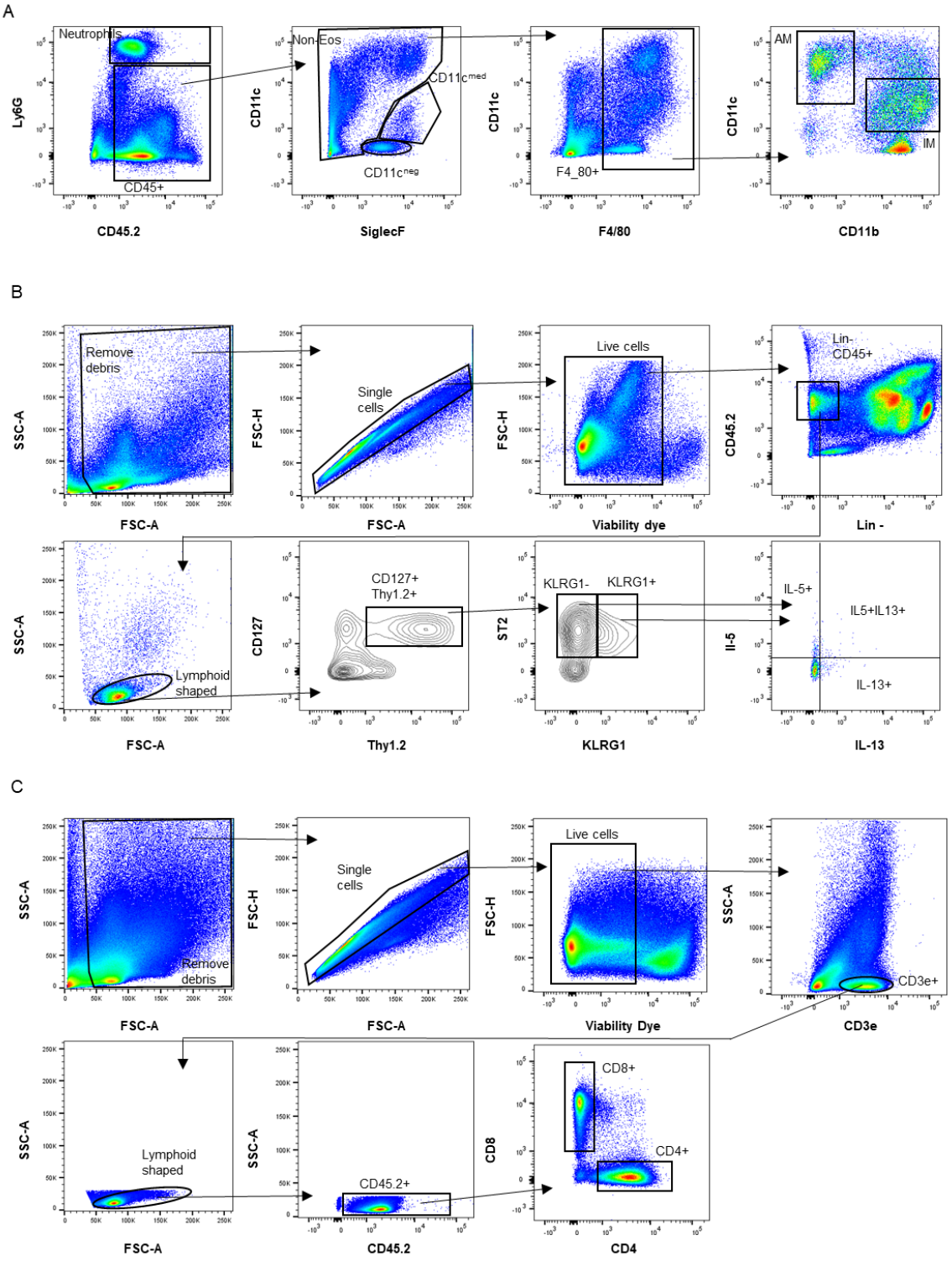

Figure 3-5. ***Gating strategies for eosinophils, macrophages, ILC2s and T cells.***

See methods and materials for details on antibodies used.

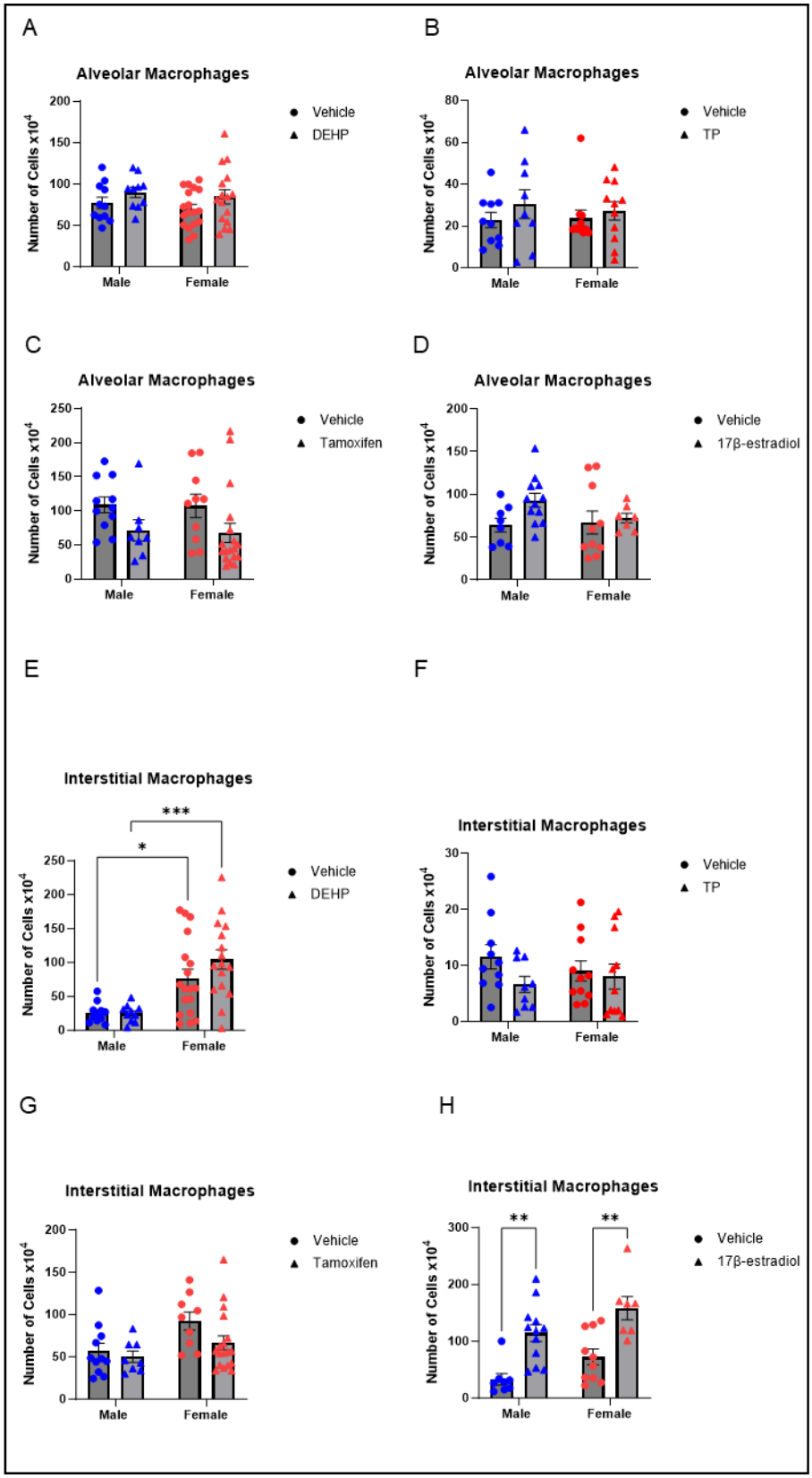

Figure 3-6. ***Interstitial macrophages increase in both sexes upon exogenous estrogen exposure.***

Number of alveolar macrophages (A) mice treated as in Figure 3-1, (B) mice treated as in Figure 3-2, (C) mice treated as in Figure 3-3, (D) mice treated as in Figure 3-4. Number of interstitial macrophages (E) mice treated as in Figure 3-1, (F) mice treated as in Figure 3-2, (G) mice treated as in Figure 3-3, (H) mice treated as in Figure 3-4. Data are from the combination of two independent experiments (n=7-17 per group). Outcomes are presented as mean ± SEM assessed by two-way ANOVA, Tukey's post hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

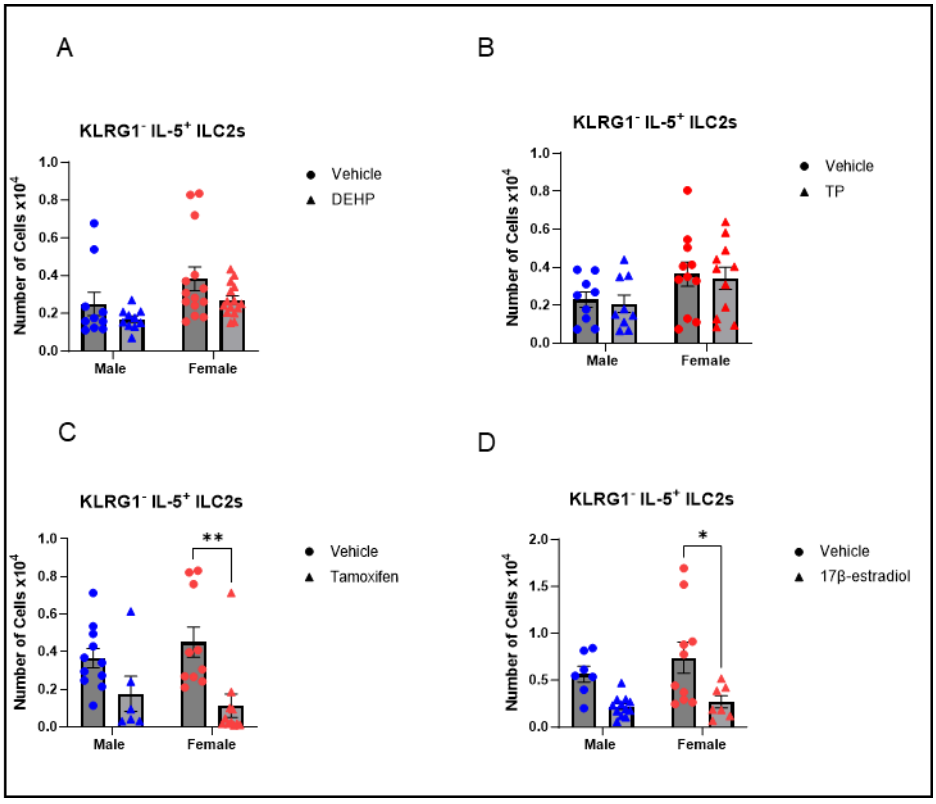

Figure 3-7. ***KLRG1⁻ IL-5⁺ ILC2s are decreased upon tamoxifen or exogenous estrogen exposure.***

Number of IL-5$^+$ ILC2s (A) mice treated as in Figure 1, (B) mice treated as in Figure 2, (C) mice treated as in Figure 3, (D) mice treated as in Figure 4. Data are from the combination of two independent experiments (n=7-17 per group). Outcomes are presented as mean ± SEM assessed by two-way ANOVA, Tukey's post hoc test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

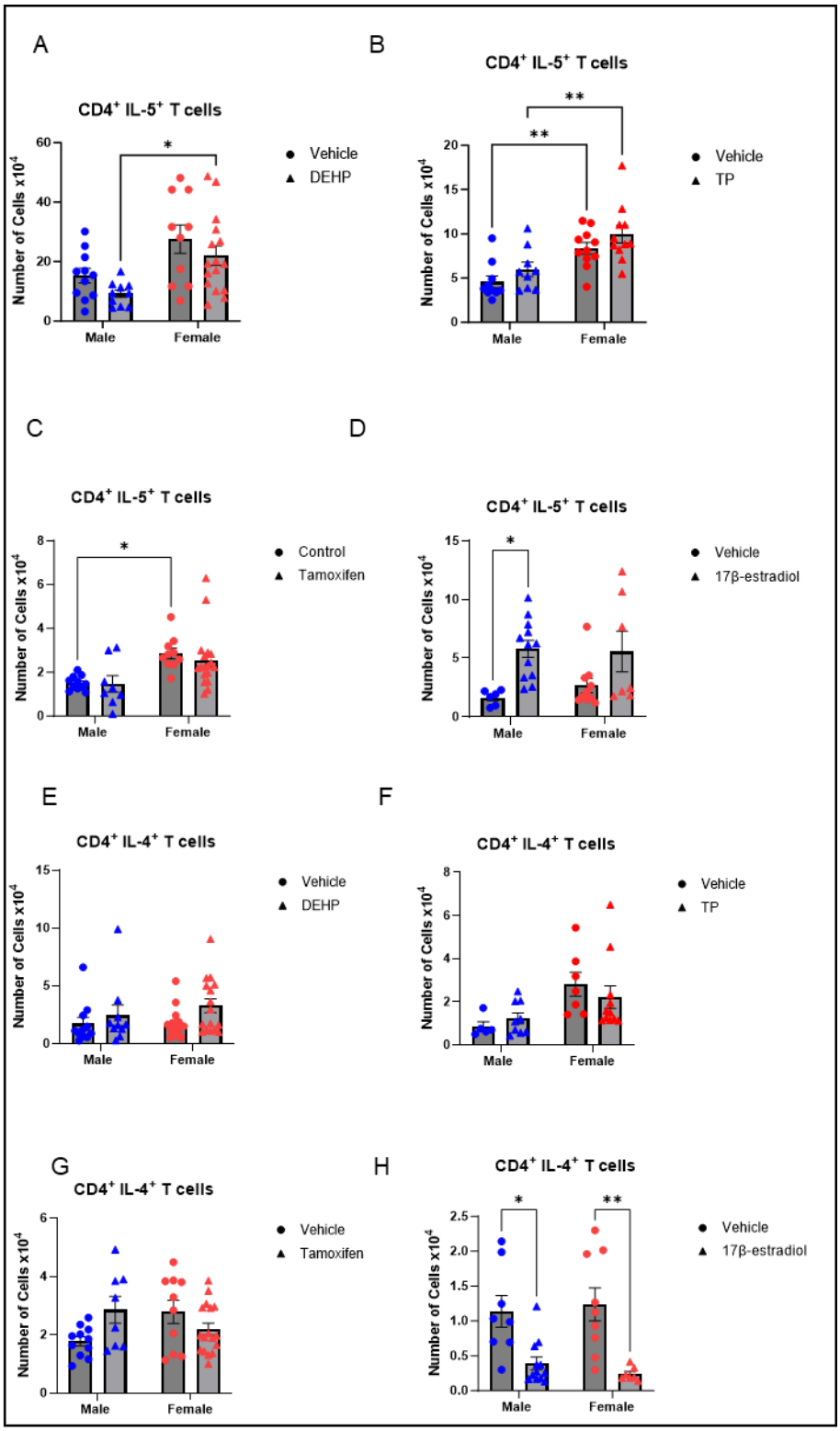

Figure 3-8. ***IL-5$^+$ CD4$^+$ T cells are increased while IL-4$^+$ CD4$^+$ T cells are decreased by exogenous estrogen exposure.***

*Number of IL-5$^+$ CD4$^+$ T cells (A) mice treated as in Figure 1, (B) mice treated as in Figure 2, (C) mice treated as in Figure 3, (D) mice treated as in Figure 4. Number of IL-4$^+$ CD4$^+$ T cells (E) mice treated as in Figure 3-1, (F) mice treated as in Figure 3-2, (G) mice treated as in Figure 3-3, (H) mice treated as in Figure 3-4. Data are from the combination of two independent experiments (n=7-17 per group). Outcomes are presented as mean ± SEM assessed by two-way ANOVA, Tukey's post hoc test. * p ≤0.05, ** p ≤0.01, *** p ≤0.001, **** p ≤0.0001.*

CHAPTER 4

General Discussion

In children, RSV remains the leading viral cause of acute LRTI (28, 346). Most children experience their first infection by 2 years of age (26). While many experience only mild symptoms, others require hospitalisation due to severe infection (23-25). In developed countries, life-saving supportive care can be provided. However this is not the case worldwide and therefore roughly 200 000 children succumb to the disease each year (28, 346). In addition to the symptoms experienced during LRTI with RSV such as bronchiolitis and pneumonitis, several studies indicate a heightened risk for RWEC and/or asthma later in life following hospitalisation with RSV (198, 199). Asthma during childhood can be a debilitating disease. In some cases, asthma can be difficult to adequately control with available treatments (116, 117). Innate immunity is implicated in the pathogenesis of both RSV and asthma. Innate cytokines such as IL-33 are elevated in infants with severe RSV LRTI and in patients with asthma (63, 126). Innate cells such as eosinophils and ILC2s are implicated in the pathogenesis of both RSV and asthma (47, 48, 63, 142, 152). However, our understanding of how innate immunity may link these two diseases is still unclear. Until this year, no vaccines for RSV were approved. In recent months, two RSV vaccines for individuals over 65 years of age were approved and one vaccine for pregnant mothers was approved (104-106). While these vaccines may now help to protect the elderly population and young infants from severe RSV infection, our understanding of how early-life viral infections contribute to immune events later in life remains to be fully elucidated.

Sex differences are apparent in the prevalence of asthma. Before puberty, the prevalence of asthma is greater in young males compared to young females. However around the time of puberty, the prevalence becomes greater in adult females compared to adult males (268). The reasons for these sex differences are not fully understood. Sex differences in health outcomes in adults are often attributable to adult sex hormones. However, the role of hormones before puberty should not be ignored. The primary goal of this thesis was to elucidate effects of early-life RSV infection on type 2 immunity following acute allergen exposure and to explore the impact of sex and sex hormones on these responses. This work adds to our understanding of how viral infection

early in life can impact responses to allergens later in life and how sex hormone exposure long before puberty can shape responses in the lung to viral infection.

4.1 Main findings

In chapter 2, we describe the inflammatory responses that occur in an early-life neonatal model of RSV infection followed by acute exposure to a common allergen, HDM. Our goal was to better understand the immune events underlying the association between RSV infection early in life and subsequent asthma development. Using a reproducible model in which mice were infected with RSV at PND 10 and exposed to HDM acutely 10 days after that, we demonstrated through flow cytometry that lung eosinophil responses to RSV→HDM were increased preferentially in female mice while interstitial macrophage numbers increased in both sexes. Similarly, we demonstrated that cytokine-expressing ILC2s were elevated following RSV→HDM exposure in both sexes. We showed that female mice had significantly greater numbers of KLRG1^{-} ILC2s expressing either IL-13 or IL-5 compared to males, which may partially explain the enhanced eosinophil response in females. In examining IL-33 levels using ELISA, we demonstrated that while IL-33 increased rapidly following HDM exposure in mice previously infected with RSV, no sex difference was apparent. We also demonstrated that Th2 and Tc2 responses were increased upon RSV→HDM exposure in both males and females, with females having greater numbers of Th2 cells producing IL-13. We further demonstrated that these responses were maintained even after HDM exposure was delayed by 20 days. In addition, prior RSV exposure was associated with increased lung inflammation only in females upon examination of histological samples. However, AHR, assessed by flexiVent analysis, was observed only in RSV→HDM females. Given the lack of increase in outcomes in the RSV→PBS group, we conclude that responses in the RSV→HDM group are not building upon an established RSV-specific inflammatory response. These results instead demonstrate that RSV alters the lung environment, such that subsequent acute exposure to an allergen, leads to enhanced responses, including AHR selectively in females. Due to the striking sex differences observed, we next sought to understand if they were linked to hormonal differences in early-life.

In chapter 3, sex hormones were either supplemented or blocked during the perinatal period. Several groups have demonstrated that in adult male mice, ILC2s are negatively regulated by androgen signaling (314, 315). In addition, there is a perinatal surge of androgens in male mice.

Given this, we hypothesised that blocking perinatal androgens would increase ILC2s and enhance inflammation in males in response to RSV→HDM exposure. However, blocking androgens did not alter male responses to RSV→HDM, although it did increase the number of eosinophils in female mice. Similarly, upon providing TP, we demonstrated that male responses to RSV→HDM were unaffected, and only IL-13^{+} T cells, but not eosinophils or ILC2s, were decreased in females to levels observed in males. Changes in female responses upon androgen modulation were not expected as females lack the early androgen surge observed in males. These findings in female mice could potentially be explained by intrinsic differences in a particular cell type, such as macrophages, early in life that we have yet to explore. As androgens did not alter responses in males, we next examined the effects of manipulating estrogen signaling. When estrogen signaling was blocked, female mice had reduced numbers of ILC2s and T cells producing IL-13 upon RSV→HDM exposure. However, when exogenous estrogens were given, KLRG1^{-} ILC2s and IL-13^{+} T cells were increased selectively in male mice upon RSV→HDM exposure. In our model, we have yet to determine the precise source of the estrogens contributing to these enhanced responses. Estrogen levels remain low and stable in female mice until after the time of weaning (PND25) (347). We hypothesis that the estrogens are maternally derived. During nursing, mice receive estrogen from their mothers through their milk. These results show that blocking estrogens leads to a more masculinised response to RSV→HDM in females, while providing exogenous estrogens leads to a more feminised response to RSV→HDM in males. Together, chapter 2 and chapter 3 demonstrate that early events following virus infection in the neonatal lung contribute to enduring immunological changes that are dependent on perinatal hormones.

4.2 Limitations

The findings presented in this thesis support the use of murine models to elucidate immune events that underlie human disease. However, additional factors must be considered as research in this area continues, such as the limitations in the animal model chosen, the variability between strains of RSV, and the challenges that accompany hormone manipulation.

4.2.1 Animal model

Throughout this work, the animal model chosen for all *in vivo* experiments was the BALB/c mouse strain. There are several inherent differences between laboratory mice and humans in terms of their immune responses to pathogens and how they are exposed to external factors. Ideally, we

wish to extrapolate some of these murine results and apply them to humans, but in order to do so, species differences must be considered and alternatives should be evaluated for future work.

4.2.1.1 Differences in immune responses: Mice Versus Humans

Innate and adaptive immune responses are not identical in mice and humans. Mice and humans differ in their expression of TLRs, surface expression of differential markers on lymphocytes, production of cytokines and chemokines, and ratios of neutrophils to lymphocytes (348). In addition, with regard to RSV infection, in humans, neutrophils are the most abundant cell type recruited to the lungs (349). However, this is not seen in mice where neutrophil recruitment to the lung is limited in response to RSV infection (350).

4.2.1.2 Other animal models of RSV infection

RSV undergoes only minimal replication in murine hosts and therefore RSV is not a true infectious pathogen for this species (351, 352). As such, mice are not always the ideal animal model for human RSV studies, although they are the most accessible and cost-effective animal model currently available. Abundant data regarding mechanisms by which RSV modulates lung immunity from mice have been replicated in humans and contribute to our knowledge of RSV pathogenesis.

Cotton rats are an alternative rodent model. They are semi-permissive to RSV, roughly 100-fold more so than mice (353). RSV can be detected in both the upper and lower respiratory tract of cotton rats, although no clinical signs of LRTD are observed (353). Although cotton rats are susceptible to RSV at all ages, 3-day-old rats experience the highest viral titers that persist for the longest duration in the nasal passages (354). Compared to mice, there are considerably fewer immunological reagents available and no gene-deleted strains are available (355).

One alternative to rodents is sheep. Lambs are susceptible to RSV infection (356, 357) and in lambs, viral replication is high and clinical signs are moderate (352). Upon intratracheal inoculation with RSV, neonatal lambs display clinical manifestations of URTI on day 6 as well as LRTI, such as bronchiolitis, as the infection progresses (356). Unlike rodents, many structural features of the respiratory tract in sheep resemble those of humans. For example, the size of the nasal cavity and airways is similar in both humans and sheep (358). One caveat to the lamb model is that molecular tools such as antibodies are much more limited. In addition, these animals require considerably more space and care, and expense, compared to rodents.

Another animal model alternative to mice, which would be the closest alternative to human studies, is the chimpanzee. One major advantage to using chimpanzees is their genetic and anatomical similarities to humans. In fact, the first human RSV isolate was purified from chimpanzees in 1956 in Washington (359). RSV readily replicates in chimpanzees and mild clinical symptoms such as sneezing and coughing are observable (360, 361). Unfortunately, similar to sheep, there are very few reagents designed for experimental use in chimpanzees, and the cost of housing and caring for these animals is high, leading to very small experimental sample sizes.

4.2.1.3 Non-reflective conditions

While well-controlled experimental conditions allow us to address specific questions of interest, this is usually accomplished using reductionist models that do not fully reflect natural conditions. Humans are genetically diverse and are exposed to a wide variety of viruses as well as a plethora of allergens during their day-to-day life, regardless of age.

In this work, inbred mice were housed under pathogen-free conditions, and given irradiated food. Inbred mice are used in research to reduce genetic variability; however, these conditions are obviously dramatically different from human populations that are genetically complex and live in a world filled with environmental exposures (362). Studies show that in 35-40% of RSV infections, other respiratory viruses, such as RV, are also present (363, 364). It is unclear if these co-infections impact the severity of RSV-associated disease. One systemic review comparing mono-infection and RSV co-infection found no differences in clinical severity between RSV mono-infection and co-infection with RV. However, co-infection with human metapneumovirus was associated with an increased risk and duration of hospitalisation (365).

The current housing system of the mice used in our experiments also limits the variety of allergens they are exposed to. Humans are exposed to airborne allergens on a daily basis. At home, humans are exposed to allergens such as HDM, cockroach, pet dander, mammalian pest dander, tobacco smoke, and mold (366). At work, humans can be exposed to additional occupational allergens such as bakery dust, detergents, fish proteins, latex, and wood dust (366). Finally, seasonally, humans can be exposed to pollen from ragweed, trees, grass, and shrubs (366). On any given day, humans may be exposed to multiple allergens. Such a setting is difficult to replicate in the laboratory and adds an additional level of complexity to experiments. Therefore, although great effort is put into replicating human experiences, a true natural environment cannot be achieved.

We do not know how exposure to multiple different allergens simultaneously would affect our findings or how it currently impacts the ability of RSV to enhance allergic responses.

4.2.2 Differences between RSV Strains

In our models we use the A2 strain of RSV. However, other subgroup A strains are also used in murine models such as Line19 and the chimeric RSV A2 strain with recombinant Line19 F. The A2 strain was first isolated from an infant in Melbourne, Australia in 1961 (367). This strain is used in various *in vitro* and *in vivo* models, and is the most commonly used strain in vaccine development (368). The Line19 strain, which was obtained from an ill infant at the University of Michigan in 1967 (369), is often used in animal models to demonstrate severe disease characterised by AHR (370). In murine models, findings demonstrate that different RSV strains from the same antigenic subgroup elicit different immune responses. The phenotypic expression of RSV-induced illness varies between strains (371).

In comparing A2 and Line19 strains, Line19 induces greater AHR and significantly more goblet cell hyper/metaplasia in a dose-dependent manner (371). Line19 also significantly increases *IL-13* mRNA and protein, while A2 does not (372). Both strains demonstrate similar growth curves. However, *in vivo* levels of infectious A2 are generally higher compared to Line19 and in Hep-2 cells A2 replicates to higher titers than Line19 (370). The chimeric RSV A2 with recombinant Line19 F induces a similar phenotype to the Line19 strain, demonstrating the importance of this genetically unique fusion protein in immunopathology (373). These distinct phenotypes between strains may explain some of the differences between our results and those obtained from Malinczak et al. who use the chimeric RSV A2 with recombinant Line19 F. They show that neonatal RSV infection leads to greater disease severity in male mice upon allergen exposure later in life (225). In many of the comparison studies addressed above, sex of the mice is not considered. Therefore, different RSV strains may potentially have different phenotypes depending on the sex of the infected host as well.

4.2.3 Hormone models

In our models where hormones were manipulated, it is important to note that complete inhibition of the target hormones cannot be guaranteed with the use of tamoxifen or DEHP. The binding energy of DEHP to AR is similar to that of testosterone (374). However, while DEHP has

a strong ability to disrupt AR signalling, it also has other off-target effects such as activating nuclear receptors in the liver (375).

In addition, although tamoxifen is a potent estrogen inhibitor in breast tissue, its effects on lung tissue have yet to be fully defined. In the breast tissue, tamoxifen blocks the effects of estrogen by competing with estrogen for binding sites (376). However, in the uterus, bone, and hypothalamus, tamoxifen can act as an estrogen agonist (376, 377). For this reason, tamoxifen is called a selective estrogen receptor modulator (SERM). Given the precarious nature of tamoxifen, pure antiestrogen drugs such as fulvestrant could be an alternative for future studies to confirm the results obtained in this work. That stated, in our model, tamoxifen did appear to act as an estrogen antagonist. When tamoxifen was given to female and male mice, responses that were generally increased in female mice such as the number of IL-13^{+} cells, were reduced to levels observed in male mice.

4.3 Future perspectives

We have demonstrated thus far that early-life RSV infection increases type 2 immunity following acute allergen exposure and that neonatal estrogen contributes to these enhanced responses. However, we have not yet determined the mechanism(s) behind these results. Moving forward, the mechanisms by which ILC2s, IL-33, and T cells modulate the inflammatory responses to RSV→HDM should be determined. In addition, more complete strategies should be employed to confirm the involvement of neonatal hormones. Keeping translational research in mind, the results obtained in this work should be considered in the context of human research currently taking place and how it may contribute to the development of future treatments for human disease.

4.3.1 ILC2s

Given the results from our models, the role of ILC2s should not be undervalued. These innate cells are likely directly involved in the RSV-dependant enhancement of type 2 responses. However, in this work we did not fully elucidate by which mechanism(s) these cells would do so. In future studies, more detailed experiments could focus on ILC2 memory as well as their ability to cross-talk with other cells, such as T cells.

Chromatin immunoprecipitation (ChIP) assays could be performed in ILC2s to determine if epigenetic chromatin modifications (e.g. H3-K4 trimethylation, H3K9 acetylation and/or H3-K27 trimethylation) correlate with sex differences we have observed. The key loci of interest

would be *Il13* and *Il5* in ILC2s in fluorescence-activated cell sorting (FACS)-sorted lung cells from female and male mice exposed to RSV→HDM.

4.3.1.1 Memory-like functions

Martinez-Gonzalez et al. demonstrated that IL-33 or allergen-experienced ILC2s can acquire memory-like properties in an antigen-independent manner and contribute to the enhancement of allergic lung inflammation (378). Similar findings were shown in an *Alternaria alternata* model (379). In the context of our model, RSV-experienced ILC2s may respond more potently to later exposure to allergens due to their unique gene expression profile and/or responsiveness to IL-25 (a cytokine that we have not yet assessed in this work). Memory-like ILC2s upregulate IL-25 receptor, which could be used as a marker to identify this unique subset via flow cytometry (378).

4.3.1.2 Cross-talk with T-cells (via MHCII)

In chapter 2 supplemental figure 3, we show that an increased number of ILC2s expressing MHCII are present following RSV→HDM exposure, indicating the potential for ILC2s to interact with T cells. In a helminth infection model, Olpihant et al. showed that when ILC2s are depleted, Th2 cell responses are reduced (380). Their work demonstrated that ILC2s expressing MHCII can interact with T cells, leading to IL-2 production by T cells, which in turn leads to ILC2 production of IL-13 (380). Similarly, Symowski at al. demonstrated using a *Nippostrongylus brasiliensis* model, that in response to IL-4 and IL-13 production by T cells, ILC2s proliferate and accumulate in the lung (381). In addition, they showed that MHCII expression on ILC2s was enhanced by STAT6 signaling, which is activated through IL-4 and IL-13 (381)..

4.3.2 IL-33

The findings from chapter 2 demonstrate that IL-33 is increased for a short period of time following HDM exposure in mice previously infected with RSV. Although no sex differences were observed in these results, they do indicate that IL-33 may be a mechanism underlying RSV-mediated enhancement of type 2 responses to allergens. Therefore, it would be of interest to explore in further detail at which time-point following RSV infection cells are producing IL-33 and if this is different in males compared to females. IL-33 production could be quantified in the lungs of RSV infected mice at various time points post-RSV infection by assessing mRNA levels via quantitative polymerase chain reaction (qPCR) and protein levels by ELISA.

4.3.3 Depletion studies

In order to determine which cell type(s) is (are) the major driver(s) behind the RSV-dependant enhancement of type 2 inflammation, several depletion studies would need to be performed.

4.3.3.1 CD8+ T cell depletion

In chapters 2 and 3, we showed that $CD8^+$ T cells, specifically those producing IL-13 or IL-4, were increased following early-life exposure to RSV and HDM. In addition, in humans, Siefker et al. showed that during severe RSV infection in infants, elevated levels of $CD8^+$ T cells expressing IL-4 are detectable in nasal aspirates compared to infants with moderate disease (81). To determine if the presence of $CD8^+$ T cells is necessary to enhance type 2 responses to allergens after prior RSV infection, depletion experiments could be utilised. This could be accomplished in two ways. First, the majority of $CD8^+$ T cells can be depleted using commercially available monoclonal antibodies (mAb) that target either the alpha or the beta subunit of CD8, such as anti-CD8α mAb, clone 53–6.7, and anti-CD8β, clone 53–5.8 (382, 383). However, it is important to consider that the use of mAbs cannot deplete all $CD8^+$ T cells and this treatment leaves behind a population of $CD8^+$ T cells with unique functional attributes and phenotypes which can impact interpretation of results (384). Alternatively, CD8α knock-out mice are available and provide reliable results (385). However, they are generally on a C57Bl/6 background, therefore our findings would first need to be confirmed in the C57Bl/6 background before proceeding to studies in these knock-outs.

4.3.3.2 CD4+ T cell depletion

Similar to $CD8^+$ T cells, we demonstrated that $CD4^+$ T cells that are positive for cytokines such as IL-13 are elevated in RSV→HDM exposed mice compared to control groups. To determine the necessity of these cells, various forms of depletion experiments could be used. First, monoclonal antibodies targeting CD4, such as GK1.5, can be used to transiently reduce the number of $CD4^+$ T cells in murine models (386, 387). Second, CD4 knock-out mice are available on the background of various murine strains, including BALB/c, and have successfully shown the effects of CD4-deficiency in mice (388). Given the more complete depletion of targeted cell types in knock-out mice compared to mAbs, CD4 knock-out mice on a BALB/c background would be the

most reliable option to examine the importance of $CD4^+$ T cells in our model of RSV-dependent enhancement of type 2 responses.

4.3.3.3 ILC2 depletion

ILC2s can be depleted in several ways. First, treatment with anti-CD90.2 antibodies can remove ILC2s from peripheral tissues, although T cells are also removed using this method and adoptive transfers of T cells would be required to evaluate ILC2 depletion alone (389-391). Second, inducible knock-out mice can be used to transiently deplete ILC2s (380). These mice are generated by inserting a floxed diphtheria toxin (DTx) receptor (DTR) gene into the *Icos* locus. This allows a CD4-cre-mediated excision of the DTR gene in T cells, but not in ILC2s therefore administering DTx to these mice removes ILC2s, but leaves T cells unaffected (380). Third, ILC2 deficient mouse strains were developed to deplete ILC2s (380, 392). Retinoid-related orphan receptor alpha (RORα) is a transcription factor that is critical in ILC2 development (393). Therefore, one ILC2 knock-out strain was developed by intercrossing $Rora^{fl/sg}$ mice with IL-7 receptor-cre mice (380). Another group developed an ILC2 knock-out mouse strain using $Klrg1^{cre}$-mediated *Gata3* deletion since GATA3 is required for maintenance of ILC2s and KLRG1 is expressed by a large proportion of ILC2s (392). Although this strain may be useful to draw conclusions about ILC2s that express KLRG1, in chapter 2 and chapter 3, we highlight the importance of $KLRG1^-$ ILC2s in our models. Using these mice would not allow the depletion of $KLRG1^-$ ILC2s. In addition, these knock-out strains are on a C57Bl/6 background. Thus, our findings would have to be confirmed in C57Bl/6 mice prior to assessing effect of knock-outs. Alternatively, the targeted knock-outs could be transferred to the BALB/c strain.

4.3.4 Castration and ovariectomy studies

Given the limitations of hormone modulation through drugs such as tamoxifen and DEHP, castration and ovariectomies could be performed to remove the effects of male and female hormones. While these surgeries are routinely done in adult mice, they were not performed in this work as they are complex, require training, and can be more difficult on neonates. Before undertaking such invasive surgeries on neonates, we sought to first determine using more simple techniques if any role existed for prepubertal hormones in our model. Given the promising results presented in this thesis, it is now justified to examine hormones using more reliable and complete methods, even if they require more challenging techniques. Early after birth, male and female

neonates could be castrated or ovariectomised (394, 395), and a similar model as the one described in chapter 2 could be used to assess hormonal effects on responses to RSV→HDM exposure.

4.3.5 Human studies and applications

Looking forward to translating this work from murine models to human application, several key studies would be of interest. Given the recent approval of a RSV vaccine for use in pregnant mothers (32 to 36 weeks of pregnancy)(105), important longitudinal studies should be conducted on infants born from these vaccinated mothers to assess whether vaccination against RSV also correlates with reduction in diagnosis of RWEC and/or asthma in these infants. If the frequency of asthma is reduced in infants born from RSV-vaccinated mothers compared to unvaccinated mothers, this would be an additional line of evidence demonstrating a causal role for severe RSV infection leading to asthma. In addition, as a precaution, the infants born from RSV-vaccinated mothers should be followed long term as a recent murine study of maternal RSV vaccination indicated that even though the offspring of RSV-vaccinated dams were protected following primary RSV infection, the offspring also developed enhanced type 2 cellular responses following secondary exposure to RSV (396). Although maternal RSV vaccination may present a viable solution to prevent severe (primary) RSV infection in infants, it may also delay negative responses at a later age. Follow-up studies will be essential to evaluate the long-term safety of maternal RSV vaccination.

Potential therapies for humans to prevent RSV-enhanced type 2 responses could target cells such as ILC2s and Th2 cells, which rely on STAT6 signalling for proliferation and cytokine production (397). Over the last several years, the Fixman and Ward laboratories have studied a peptide designed to inhibit STAT6 signalling. Data from our laboratories show that this immunomodulatory peptide, STAT6-IP, inhibits type 2 inflammatory responses that contribute to both allergic airways disease and RSV immunopathology in mice (398-400). Delivery of STAT6-IP during neonatal RSV infection markedly diminishes both structural and functional changes in the lungs of mice upon subsequent RSV reinfection of adults (400). Currently, through *in vitro* experiments, STAT6-IP modulation of human ILC2s is being assessed (unpublished). This peptide could potentially be used to prevent RSV-dependent enhancement of type 2 allergic responses. A long-term goal would be to further understand the mechanism(s) by which STAT6-IP reduces type

2 inflammation in order to develop inhibitor(s) that work(s) equally well in males and females or sex-specific inhibitors.

4.4 Concluding remarks

In this thesis, we explore the ability of early-life RSV infection to enhance subsequent responses to allergen exposure in a murine model. Using two models, differing only in the time between RSV infection and exposure to allergen, we demonstrate the long lasting effects of early-life RSV infection on type 2 immunity in the lung following acute allergen exposure. We also demonstrate that several responses, such as eosinophil activation and expansion of the KLRG1^{-} ILC2 population, were greater in females compared to males. These unexpected sex differences, led us to investigate whether neonatal hormones were a driving factor in RSV→HDM exposed mice. By modulating hormones early in life, we demonstrate that estrogens present in the neonate promote RSV-enhanced type 2 inflammation in response to acute HDM exposure. Considerable work remains to identify the precise mechanism(s) behind the RSV-enhancement of type 2 inflammation as well as the mechanism(s) by which neonatal estrogens promote these responses. Overall, we hope the findings from this research will contribute to a better understanding the long-lasting effects of early-life viral infections in humans of both sexes.

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