Role of interleukin-33 in H1N1 Influenza A-induced airway hyperresponsiveness in C57BL/6 mice

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Abstract

Seasonal influenza A virus (IAV) continues to pose a threat to asthmatics. IAV triggers the release of IL-33, which provides a potential mechanism for airway hyperresponsiveness (AHR) and lung neutrophilia. The study aims to characterize the innate immune response triggered by IAV and elucidate its dependence on IL-33, the IL-33/IL-13 axis, neutrophilia and cysteinyl leukotrienes (CysLTs), all of which have strong associations with induction of AHR.

Administration of anti-ST2 antibody to sub-lethal PR8 H1N1 infected 8-12-week-old female C57BL/6 mice reduced AHR assessed from total respiratory system resistance and elastance following inhalation of progressively doubling concentrations of aerosolized methacholine (0 to 50 mg/mL) at peak viral replication at 3 days post infection (dpi). Anti-ST2 did not significantly affect IAV-induced increments in neutrophils, macrophages, lymphocytes, IL-33 or IL-13 levels but paradoxically further elevated CysLT levels in BAL fluid. Anti-ST2 did not alter IAV-mediated weight loss, IAV non-structural NS1 expression and viral load determined by plaque assay. Anti-ST2 did not change expression of IL-12a, TNFα, IL-6, IL-1β, CXCL1, CCL2, CCL3, CCL5, CCL7 and CCL22 on 2 and 3 dpi. Expression of lung c-maf and m-CSF was further increased on 3 dpi following anti-ST2 treatment. Anti-Ly6G neutrophil depleting antibody did not affect IAV-induced AHR 3 dpi. Flow cytometry of lung digests on 3 dpi showed no significant induction of ILCs. The monocyte-derived macrophages showed the highest percentage of $ST2^+$ cells during IAV infection. Anti-ST2 treatment reduced both absolute numbers and the M2 subset of monocyte-derived macrophages. Depletion of monocyte-derived macrophages using anti-Ly6C antibody resulted in lower trends of total respiratory resistance and elastance in response to aerosolized methacholine concentrations during IAV infection.

IAV-induced IL-33 initiated an innate immune response that causes AHR independent of the IL-33/IL-13 axis, cysteinyl leukotrienes and viral load. Anti-ST2 decreased the number of monocyte-derived macrophages during IAV infection that we postulate is the mechanism responsible for the reduction in IAV-induced AHR.

Résumé

Le virus de la grippe saisonnière A (IAV, influenza A virus) continue de poser une menace chez les asthmatiques. L'IAV déclenche le relargage d'IL-33, ce qui fournit un mécanisme potentiel pour l'hypersensibilité des voies respiratoires (AHR, airway hyperresponsiveness) et la neutrophilie pulmonaire. Cette étude vise à caractériser la réponse immunitaire innée provoquée par l'IAV et à élucider sa dépendance sur l'IL-33, l'axe IL-33/IL-13, la neutrophilie et les cystéinyl-leucotriènes (CysLT), qui ont tous de fortes associations avec l'induction de l'AHR.

L'administration d'anticorps anti-ST2 à des souris C57BL/6 femelles, âgées de 8 à 12 semaines, infectées par une dose sublétale de H1N1 PR8 réduisait l'AHR évaluée à partir de la résistance et l'élastance totales du système respiratoire à la suite d'inhalation de méthacholine aérosolisée dont les concentrations doublent progressivement (de 0 à 50 mg/mL) au pic de la réplication virale à trois jours après l'infection (dpi, days post infection). L'anti-ST2 n'a pas affecté significativement les augmentations induites par l'IAV dans les neutrophiles, macrophages, lymphocytes, IL-33 ou IL-13 mais paradoxalement a augmenté davantage les niveaux de CysLT dans le fluide de lavage broncho-alvéolaire. L'anti-ST2 n'a pas altère la perte de poids médiée par l'IAV, l'expression du NS1 non structurale de l'IAV et la charge virale déterminée par dosage de la plaque. L'anti-ST2 n'a pas changé l'expression de l'IL-12a, TNFα, IL-6, IL-1β, CXCL1, CCL2, CCL3, CCL5, CCL7 et CCL22 à 2 et 3 dpi. L'expression des c-maf et m-CSF pulmonaires était encore plus élevée à 3 dpi à la suite du traitement anti-ST2. L'anticorps appauvrissant les neutrophiles anti-Ly6G n'a pas affecté l'AHR induite par l'IAV 3 dpi. La cytométrie en flux des digestions du poumon à 3 dpi n'a montré aucune induction significative des ILCs. Les macrophages dérivés de monocytes ont montré le pourcentage le plus élevé de cellules $ST2^+$ pendant l'infection à l'IAV. Le traitement anti-ST2 a réduit à la fois les nombres absolus et le sous-ensemble M2 des macrophages dérivés de

monocytes. La déplétion des macrophages dérivés de monocytes à l'aide d'anticorps anti-Ly6C a conduit à des tendances vers une réduction de la résistance et l'élastance totales du système respiratoire en réponse aux concentrations de méthacholine en aérosol pendant l'infection à l'IAV.

L'IL-33 induite par l'IAV a initié une réponse immunitaire innée qui provoque une AHR indépendante de l'axe IL-33/IL-13, des cystéinyl-leukotriènes et de la charge virale. L'anti-ST2 a diminué le nombre de macrophages dérivés de monocytes pendant l'infection à IAV que nous postulons être le mécanisme responsable de la réduction de la AHR induite par l'IAV.

Contribution of authors

The following authors have contributed to the completion of the thesis:

1. Chronopoulos, Julia (PhD candidate) to experimental findings and assessment of data in section 3.6: Outcome of anti-IL-13 treatment on IAV-induced responsiveness to methacholine and viral load at 3 dpi.

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Chapter 1: Introduction

1.1 Asthma definition

Asthma is a heterogeneous disease characterized by chronic airway inflammation (1). The pathobiology of asthma involves excessive, unresolved airway inflammation, airway remodelling and mucus production in the lumen of airways (2). Reduction in the lumen causes decreased air flow resulting in a widening of the distribution of ventilation/perfusion (V/Q) ratios in the lung (3) thus leading to inefficient and diminished gas exchange in the alveoli. Typical symptoms include cough, sputum production, wheezing, shortness of breath and chest tightness, varying in intensity from patient to patient. Airway inflammation, remodelling and airway hyperresponsiveness (AHR) either working independently or together contribute to the clinical presentation of asthma.

Multiple phenotypes of asthma exist, each of which is characterized according to its "trigger". An asthmatic individual if exposed to one or more triggers develops an exacerbation also referred to an asthmatic attack, a sudden onset of symptoms that, if not managed properly, may require a visit to the emergency room and hospitalization. Fortunately, mortality is a rare event in most developed countries.

Over the past few years, research into asthma has moved on to elucidate its cellular and molecular manifestations as a means of better understanding its pathological mechanisms. The elucidation of key mechanisms followed by direct implication in asthma through the application of highly specific treatments (biologics) has led to the confirmation of asthma endotypes (4).

1.1.1 Prevalence of asthma

As of 2019, 300 million people worldwide have been diagnosed with asthma with further increments expected in the coming years (2). Asthma is classified as one of the common

chronic diseases worldwide; with cases among both adults and children becoming more common. Asthma, in terms of prevalence continues to increase in high-income countries with greater mortality seen in low to middle-income countries.

The prevalence of asthma in Canada has been increasing in the young population steadily since 1985. In Canada alone, over 2.8 millions over the age of 12 suffer from asthma (5). Canadian males have a greater prevalence of asthma compared to their female counterparts in the adolescent-young adult category which subsequently drops in the later age categories. In terms of quality of life, a more recent case-controlled study shows Canadian asthma patients score significantly lower in the Health-Related Quality of Life (HRQoL) scale compared to the general Canadian population (6). Greater attention must be paid to current therapies focussing on the maintenance of a good quality of life.

1.1.2 Treatments

Due to its heterogeneity and complexity, medication strategies for asthma can vary accordingly to the patient's progression and severity of the disease. There is no cure for asthma but, medications have been developed to help alleviate symptoms and to promote a superior quality of life.

Treatments include commonly prescribed short- and long-term beta-adrenergic agonists (β-agonists) which collectively reduce bronchoconstriction by stimulating smooth muscle cell relaxation (1). Short and long-term anticholinergics block parasympathetic signals from muscarinic M3 receptors expressed on ASM cells reducing the extent of bronchoconstriction (7). Inhaled corticosteroids (ICS) function by binding to glucocorticoid receptors (GR) and altering expression of various gene targets (8). Overall, expression of pro-inflammatory cytokines, chemokines, peptides, enzymes, and adhesion molecules is reduced along with upregulation of anti-inflammatory cytokines. The effect of ICS extends to structural cells as

well. Examples of such effects include reduction in inflammatory cytokine production from epithelial and airway smooth muscle (ASM) cells, maintenance of epithelial barrier integrity and reduction in mucus secretion from goblet cells (8). Lastly, medications can also target specific inflammatory mediators/cells. Examples of such medications are tumour necrosis factor (TNF)-α inhibitors, leukotriene receptor antagonists and leukotriene synthesis inhibitors, mast cell stabilizers and monoclonal antibodies (8). Among the monoclonal antibodies are anti-IL-4, 5, 13 and IgE that collectively function to reduce the impact of the Th2 immune response prominent in allergic asthma and against alarmins like TSLP and IL-33 to prevent the initiation of underlying downstream asthmatic pathology (9).

Currently, ICS are the mainstay of therapy and are used in conjunction with leukotriene antagonists (LTRA), with long-acting beta agonists (LABA) and muscarinic antagonists added on for high risk and uncontrollable exacerbations (10). The use of biologics such as anti-IL-5 and anti-IgE is reserved for severe cases of asthma wherein constant monitoring of the immunological phenotype utilizing blood and sputum levels of eosinophils, total IgE and both aeroallergen and exhaled nitric oxide (FeNO) testing are required to judge the severity and management status (11). The administration of biologics has to be tailored according to the characteristics of the patient in addition to a failure of elevated, long-term doses of ICS, LABA, LTRA, tiotropium and short-term beta agonist (SABA) to achieve control (12).

Clinically, the treatment choices for asthma require a trial-and-error approach therefore, physicians opt for combinatorial interventions like the Maintenance and Reliever (MART) therapy that includes a steroid and a bronchodilator. The treatment decisions are based on the historical severity of the disease and the assessment of the patient's treatment response (13). Lastly, even non-pharmacological interventions like physical and breathing exercises, asthma education and allergen avoidance can be beneficial in improving the quality of life of asthmatic patients.

1.2 Pathogenesis of asthma

Asthma involves a wide range of pathological processes that include airway inflammation, airway hyperresponsiveness, airway remodeling and mucus hypersecretion. These processes may vary in their contributions according to the type of asthma in question. Each of these contributors to the pathobiology of asthma will be considered in the following sections.

1.2.1 Airway inflammation

The inflammatory response is pleiomorphic with the infiltration of the airways by various immune cells. These cells further promote inflammation in conjunction with the inflammatory mediators released from epithelial cell layer damage and shedding (14). Immune cells enter the microenvironment in response to both allergic and non-allergic stimuli, the latter independently of the need for sensitization. The inflammatory mediators in turn attract more immune cells to the milieu, thus, perpetuating an inflammatory process which is more prominent in chronic cases of asthma (15).

Early human autopsies and bronchoscopy studies reported widespread inflammation affecting large airways of asthmatics (16). Subsequent post-mortem studies have confirmed immune cell infiltration even in the small, peripheral airways (17). The inflammatory cells comprise eosinophils, mast cells, $CD4^+$ lymphocytes and neutrophils. The distribution of inflammatory cells within the airway tree is non-uniform, with elevated lymphocytes and macrophages in bronchoalveolar lavage fluid (BAL) and neutrophils and eosinophils (when present) predominating in proximal bronchial washes in humans (17). The severity of the disease influences the predominance of inflammatory cells (17). For instance, eosinophils are present in both central and peripheral airways in moderate asthma whereas, neutrophils are often the dominant cell type in more severe cases (18).

The association between airway inflammation and airway dysfunction is not always evident. A lack of correlation between sputum inflammatory cytokine levels and declining lung function of severe asthmatics has been reported (19). In contrast, inflammation has been shown to predict lung function as evidenced by a correlation between increased lymphocytes and eosinophils and worsening lung function (16, 20). This correlation was observed among stable asthmatic subjects during night-time. Cluster analysis of a cohort of asthmatic patients has revealed significant discrepancies among measurement of airway inflammation, symptoms and ling function (21). Thus, airway inflammation cannot be used as a proxy for lung function assessment. However, eosinophilic inflammation is a strong predictor of risk of asthma exacerbations (22).

1.2.2 Airway hyperresponsiveness

AHR is an important characteristic used to confirm a diagnosis of asthma when there is doubt based on clinical data. AHR is described as an exaggerated response in the airways to non-specific airway constrictive agents like histamine and methacholine, highlighting the predisposition to bronchoconstriction occurring in the diseased state of asthma (23). The presence of AHR predisposes to airway narrowing impeding normal ventilation. The association between alteration in ASM cell properties and AHR are undeniable. However, the causative role of the interactions of immune cells and/or their mediators with ASM cells in AHR is yet to be fully understood.

The causes of AHR can be divided into two categories: persistent airway structural changes and acute inflammatory mediators acting on the airways (24). Both of these factors may work independently of each other but can also work synergistically to cause AHR. Various environmental triggers such as allergens and viral infection stimulate the release of inflammatory mediators transiently causing AHR. Generally, a Th2 based inflammatory

reaction produces mediators such as IL-4 and IL-13, cytokines that have been heavily implicated in causing AHR as confirmed by studies by *in vitro* and *in vivo* studies (25, 26). However, other mediators are being investigated especially considering the heterogeneity of the disease.

As for structural changes, asthma causes the architecture of the lung to change. Specifically, chronic changes involve alterations to the normal structure and function of the pseudostratified airway epithelium, ASM cells and extracellular matrix (ECM) (27). The structural alterations, in particular the increase in airway smooth muscle mass, are what are predicted to promote the continued persistence of AHR that has been observed in severe or longstanding forms of asthma (28).

1.2.3 Airway remodelling

Structural remodelling is a complex cellular and molecular process resulting in increased ASM cell mass, goblet cell metaplasia, epithelial cell hyperplasia, basement membrane thickening and angiogenesis (29). All of these alterations thicken both the small and large airway walls thus further promoting airway occlusion. In terms of causality, ongoing inflammation can co-exist with such structural changes however, there have been post-mortem asthmatic subjects analysed wherein structural changes have been independent of airway inflammation (30).

Epithelial cell damage and desquamation or alterations in normal epithelial-mediated signalling conducive of permanent airway architectural changes occur (31). An example of epithelial signalling alterations can be seen in an allergic OVA murine model overexpressing Smad2 causing transforming growth factor beta (TGF-β) signalling and in consequence promoting endothelin-1 and fibroblast growth factor (FGF) expression. Endothelin-1 and FGF are implicated in driving the synthesis of ECM and ASM hyperplasia (32). Excessive dysregulation in goblet cell differentiation in the epithelium is strongly associated with mild and moderate

asthma promoting occlusion of the airways (33). Other phenotypic changes observed in the epithelium include disruption of tight junctions (TJs) by reducing expression of proteins necessary for maintenance of epithelial integrity (34). The consequent effect of loss of TJs is better highlighted in animal models where a deficiency in E-cadherin prevents epithelial repair by disrupting the epithelial architecture and shows some features reminiscent of asthma such as mucus overproduction and infiltration of innate immune cells like eosinophils and dendritic cells (35).

The architecture of the ECM changes with disease progression. ECM changes include greater deposition of certain subtypes of collagen, fibronectin and proteoglycan that collectively contribute to increasing the thickness of the airways and the basement membrane (36). Myofibroblasts affect deposition and organization of collagen upon TGF-β1 stimulation *ex vivo* (37). Hence, fibroblasts are playing a contributory role in promoting airway remodelling. The increased proliferation and migration of ASM cells are also thought to contribute to disease pathology (38, 39). Studies have shown both Th2 and Th17 immune responses to be implicated in airway remodelling. Examples of such studies include the prevention of the action of Th2 cytokines in murine allergic models with anti-IL-5 and anti-IL-13 antibodies that reduce airway fibrosis and ECM deposition (40, 41). Likewise murine allergic models have shown lipopolysaccharide activated dendritic cells produce a Th17 response by virtue of releasing IL-17A and inducing TGF-β signalling in fibroblasts (42). TGF-β is viewed as one of the key factors in the initiation and maintenance of airway remodelling by driving proliferation of ECM expression stimulated by ASM cells.

Some authors have placed emphasis on decoupling airway inflammation and remodelling in asthma. For instance, it has been hypothesized that epithelial-mesenchymal trophic units (EMTU) mediate the airway remodelling process irrespective of Th2-cytokines in asthma (43). EMTUs entail cellular communication between damaged and stressed epithelial cells and

fibroblasts that promote mesenchymal cell proliferation that normally exist to recover from damage caused to the epithelium. It is believed that trophic units excessively facilitate continued communication between the airway epithelial cells and underlying mesenchyme to drive expression of growth factors such as amphiregulin, transforming and epidermal growth factors, TGF-β and EGF and inhibitors of matrix metalloproteases to collectively promote excessive wound repair signals leading to airway remodelling. Currently, mesenchymal cell-derived fibronectin and vimentin are being explored for their contributions to airway remodelling and are purported to be reliable EMT markers (44).

As insightful as the animal studies might be, due to a lack of wide-scale observational studies, it is yet to be confirmed whether *in vivo* observations are applicable to airway remodelling among humans.

1.2.4 Mucus hypersecretion

One of the more characterised consequences of asthmatic structural alterations is mucus hypersecretion. Goblet cell hyperplasia and luminal mucus in airways have been observed in morphometric analyses of lungs from autopsied patients who died from an acute, severe asthma attack (45). Additionally, mucus hypersecretion has been associated with poorer asthma control in asthma patients as excessive mucus secretion contributes to airway occlusion (46). Mucus plugs when quantified are correlated with spirometric measurements such as the forced expiratory volume in one second (FEV_1). In addition, there is a correlation between eosinophil numbers in sputum and the mucus plug score, suggesting an important role for these cells (47).

Goblet cell hyperplasia has been reproduced in murine allergic models. For instance, OVA-challenged mice show goblet cell proliferation following underlying exocrine-like club cell hyperplasia and not epithelial cell damage (48). Furthermore, various *in vitro* human epithelial cell culture studies have shown the effects of asthma associated Th2-cytokines like IL-4, IL-5

and IL-13in inducing the expression of the mucin (MUC) gene, MUC5AC (49). MUC5AC is a prominent gene member driving goblet cell proliferation in the epithelium and its elevated expression is associated with the presence of asthma among humans (50).

1.3 Endotypes of asthma

In recent years, the understanding of asthma has evolved to a molecular basis for disease and to a mechanistic perspective that relates to the clinical presentation. Independent observational cluster studies such as the ADEPT study, U-BIOPRED study and a study by Woodruff et al. have aimed to better elucidate the overall immune phenotype among asthmatics based on endobronchial biopsies and biomarkers from blood and sputum (51-53). The common conclusion from all studies is that asthma can be divided into two distinct categories: Th2 high and Th2 low.

A Th2-high response is initiated by the release of epithelial-derived alarmins: IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) in response to a trigger (54). These alarmins, along with antigen presentation by antigen presenting cells (APCs), achieve Th2 differentiation of $CD4^+$ T cells bringing about the synthesis and secretion of Th2 cytokines, IL-4, IL-5, IL-9 and IL-13 that collectively stimulate innate cells such as eosinophils, mast cells and basophils. T follicular helper (Tfh) cells are essential cells in regulating IgE production (55). Both the Th2 cytokines and innate cell mediators like cysteinyl leukotrienes and prostaglandin D2 augment Th2 responses resulting in AHR, mucus hypersecretion and airway remodelling (56). In addition, the alarmins can also stimulate the influx of innate lymphoid type 2 cells (ILC2) which alone can carry out a major proportion of T2 effector functions early in the pathology without the requirement for an adaptive response.

In a Th2-low asthmatic milieu, epithelial-derived alarmins such as TSLP and IL-33 are also present. Clinical studies have shown that anti-TSLP treatment (tezepelumab) and blocking the

action of IL-33 (anti-ST2) reduced asthma exacerbations, even among asthmatics with low levels of eosinophils (57, 58). Released alarmins act on APCs such as dendritic cells. Dendritic cells, in coordination with macrophages function to skew $CD4^+$ T cells towards $CD4^+$ Th1 and Th17 cells which secrete IFN-γ, TNF-α, IL-17 and IL-22 (59). Furthermore, other lymphoid cell subsets including ILC1 and ILC3 also synthesize IFN-γ and IL-17, which further augment their respective Th1 and Th17-responses (60). Collectively, such cytokines function by stimulating neutrophil recruitment and consequently bring about airway hyperresponsiveness, remodelling and mucus hypersecretion. The cytokines themselves can stimulate certain features of asthmatic pathology. For instance, IL-17 can increase smooth muscle proliferation and drive collagen deposition (61). Attenuation of IL-22's action in fungal allergen-exposed mice leads to reduced AHR (62). As for IFN-γ, it has been shown to be associated with asthma severity but its mechanistic role in asthma remains uncertain (63). Mechanisms of Th2-low asthma are still obscure with no reliable biomarkers as yet described (59).

While Th2 high and low are the two main cluster of asthma, there is also a smaller population of asthmatics who show a more mixed pathology of both Th2 high and low immune cells or in populations where the asthmatic presentation occurs with low levels of inflammation.

1.4 Phenotypes of asthma

The mechanistic pathophysiology of asthma presents complexity with respect to both the immune cells and the cytokine/chemokine and inflammatory mediators involved. This is due to the heterogeneity as seen from sputum cytological studies among the asthmatic population (64).

1.4.1 Eosinophilic asthma

Both atopic (triggered by sensitization to allergens) and non-atopic asthma, early-onset or later onset in adulthood, are influenced by the presence of eosinophils (65). Eosinophilic asthma

continues to remain prevalent in older patients with additional complications such as fixed airway obstruction, among other comorbidities. Dendritic cells take up and process allergens to present to T cells to initiate a Th2-response with the induction of IL-4, IL-5, IL-9 and IL-13 cytokines (66). IL-5 particularly induces maturation of eosinophils and with chemokines CCL11 and CCL24 mediates chemotaxis of eosinophils (67). Furthermore, IL-13, in coordination with IL-4, mediates class-switching in B cells to IgE antibody and its subsequent production. The combined action of both IL-4 and IL-13 stimulates mucus hypersecretion by inducing goblet cell metaplasia and enhances contractility of ASM cells (68). IL-9 can attract and activate mast cells which further mediate inflammation through the degranulation process stimulated by the crosslinking of IgE antibodies released by B cells (69). Although allergic eosinophilic asthma is primarily attributable to adaptive immunity, innate immunity may also participate through ILC2s in the T2-mediated pathophysiology. This is the case because allergens can stimulate the release of the epithelial derived alarmins, IL-25 and IL-33 (70) activating ILC2s to secrete IL-5 and IL-13, thus acting in coordination to achieve the same effects as $CD4^+$ Th2 cells.

The non-allergic form of eosinophilic asthma follows the release of alarmin cytokines such as IL-33, IL-25 and TSLP from damaged and/or dead airway epithelial cells caused by a non-allergic stimulus (examples are air pollutants, smoke, irritants). These alarmins specifically mediate pathology by activating ILC2s which release T2-cytokines such as IL-5 and IL-13 (66). ILC2-mediated synthesis of IL-5 and IL-13 carry out the same functions as adaptive Th2 cells in the allergic form of eosinophilic asthma. It is possible that non-allergic asthma doesn’t require the action of Th2 cells. Evidence for this is suggested by studies showing ozone-exposed mice developing AHR independently of Th2 cells (71) and *Alternaria alternata* exposed naïve mice showing dependence exclusively on ILC2s to drive eosinophilia and AHR (72).

1.4.2 Neutrophilic asthma

Neutrophilic asthma is associated with obesity, smoking, chronic bacterial infection/colonization of organisms such as *Streptococcus* pneumonia infections (73). Th17-high asthma is present in both adult and elderly populations (65). Neutrophilic infiltration generally involves a Th17-skewed immune reaction with cytokines IL-17A, IL-17F, IL-22 and IL-23 correspondingly increasing with elevated neutrophil numbers seen in the lamina propria of asthmatic patients (74).

In non-atopic subjects, following contact with an allergen or non-allergen stimulus, $CD4^+$ T cells may be polarized to Th1 and Th17 subtypes accordingly (75). Allergens can stimulate the differentiation of Th1 cells which produce an IFN-γ response that promotes neutrophilia (76). As for non-allergic stimuli, alarmins such as IL-33 and TSLP are released from damaged epithelial cells can enhance Th1 and Th17 polarization. However, both the alarmins cannot function alone to promote polarization. IL-33 has shown to act in synergy with IL-12 and TSLP functions through dendritic cell activation to stimulate their respective $CD4^+$ T cell differentiations (77, 78). Th17 cells produce IL-17 which indirectly attracts neutrophils by acting on endothelial cells to secrete the chemoattractant CXCL1 (79). IL-17A has been shown to stimulate mucus hypersecretion following goblet cell metaplasia (80). IL-6 and TNF-α collectively act to attract neutrophils as well. Blocking the action of IL-6 in a house dust mite (HDM) murine model blocks the recruitment of neutrophils by virtue of reduced levels of CXCL1 and IL-17A (81). Neutralizing TNF-α has been shown to reduce the recruitment of neutrophils following respiratory-syncytial virus (RSV)-mediated exacerbation in an OVA sensitization murine model (82). Macrophages play a supporting role in mediating non-allergic neutrophilic asthma by releasing IL-8 and TNF-α. In addition, macrophages stimulate type 3 innate lymphoid cells (ILC3) which in turn further boosts IL-17A production necessary for neutrophilia, as seen in *ex vivo* co-cultures of ILC3 and macrophages isolated from the sputum

of asthmatics (83). Neutrophilic asthma upregulates NLR family pyrin domain containing 3 (NLRP3) activity and its consequent IL-1β production. IL-1β correlates with IL-8 levels in sputum from asthmatics (84).

The functional response of neutrophils is enhanced in neutrophilic asthma. Activity of neutrophilic proteases such as matrix metalloproteinase 9 (MMP-9), neutrophil elastase (NE), myeloperoxidase is elevated. The neutrophilic proteases have been observed in a HDM murine model and have been shown in biopsies and BAL samples from asthmatic patients (85-88). In addition, inhibitors such as metallopeptidase inhibitor 1 (TIMP-1) and secretory leukocyte peptidase inhibitor (SLPI) are suppressed, hence are not able to counteract the protease activity. Observational studies have shown that both SLPI and the ratio of MMP9/TIMP-1 are reduced in severe asthma and in asthma exacerbations (89, 90). The functionality of neutrophils and its associated proteases in context of asthma is not understood however, excessive influx of neutrophils is hypothesized to cause AHR and airway remodelling. An *in vitro* study has shown neutrophils taken from severe asthmatics in co-culture with epithelial cells causes epithelial-mesenchymal transition (EMT), TGFβ1 expression and epithelial cell morphological changes (91). The link between the neutrophil and AHR has been established *in vivo* following chlorine-induced airway injury in mice (92). Nevertheless, a direct link between neutrophils and AHR or airway remodelling is yet to be established among human asthmatics.

1.4.3 Mixed-granulocytic asthma

Mixed-granulocytic asthma is defined as an elevation of both neutrophils and eosinophils in the sputum. With both cell populations elevated in asthmatics' sputum samples, the extent of air flow obstruction, frequency of exacerbations and greater health care needs is higher are comparison to eosinophilic or neutrophilic form of asthma alone (93). Mixed-granulocytic

asthma has a complex pathophysiology. All three immune responses Th1, Th2 and Th17 are engaged in this form of asthma making the inflammatory milieu much harder to control (9).

1.5 Influenza and asthma

Asthma exacerbations caused by a known trigger or irritant are quite a few in number and can be highly specific to the patient (94). Exposure to one or more of these triggers can stimulate asthma exacerbations in a patient's lifetime.

A well-documented trigger is viral airway infections, most frequently rhinovirus followed by certain strains of influenza (94). Asthma was a considerable risk factor, affecting 10-20% of influenza infected patients worldwide and affecting a third of asthmatic patients in the US, during the 2009 swine flu H1N1 outbreak (95). Currently, seasonal influenza strains continue to be a risk factor for uncontrolled asthma exacerbations. A possible reasoning as posed by H.H. Salter in the 1860s based on his observations was that asthmatic patients have a higher sensitivity to influenza infections (96). Consistent with Salter's view, lowering the dysregulated response of the epithelium and the underlying innate immune response produced in asthmatics during acute influenza infection is important (96).

In vivo experiments in T2-biased BALB/c mice have established that influenza infection stimulates AHR suggestive of an acute asthma attack (97). The onset of AHR is attributed to the release of IL-33 and the IL-33/IL-13 axis during influenza infection in BALB/c mice (97). IL-33 has also been shown to be released in C57BL/6 mice peaking at day 3 post infection coinciding with AHR and IAV viral load (98). The mechanistic details of how IL-33 causes AHR during IAV infection is yet to be fully understood.

Limiting the severity of exacerbations caused by influenza infections remains a top priority for health care professionals; especially when caring for asthmatic patients with a chronic

presentation of lung disease. Further research into how influenza mechanistically stimulates asthma is paramount for better understanding of the human condition.

1.6 Influenza virus

Influenza belongs to the Orthomyxoviridae family that are segmented, single and negative stranded-RNA viruses (99). The influenza virus is composed of 4 genera: A, B, C and D. Influenza virus in general is known to infect mammals and birds; with only genera A and B infecting humans. Influenza virus infections primarily affect the respiratory tract with symptoms ranging from mild to severe. The virus has the potential to cause severe lung conditions such as pneumonia with or without secondary bacterial infections, frequently caused by *Staphylococcus aureus* or *Streptococcus pneumoniae* and/or acute respiratory distress syndrome (ARDS) (100). The influenza A (IAV) virus is of greater importance since it has caused severe epidemics and pandemics, the 1918 Spanish flu pandemic, being a prominent one. The 1918 pandemic claimed an estimated 50 million lives worldwide and infected a third of the world's population (101). The virus continues to be a threat to human and other animals since novel strains of virus evolve yearly leading to continued speculation about another fatal flu pandemic occurring in the future.

1.6.1 Transmission

The influenza virus is transmissible through respiratory droplets; produced during coughing and sneezing as large droplets or smaller aerosols (102). Another common form of transmission is through fomites, touching viral particles surviving on surfaces of inanimate objects for a variable amount of time and bringing them in contact with the eye or nose (103). Besides human transmission, another cause of public health concern is zoonotic transmission. Both animal reservoirs of virus and contact between animals and humans promote antigenic shift and drift leading to mutations creating new strains of virus (104). New strains can have an

enhanced capability to transmit efficiently, promote virulence and evade the action of anti-viral drugs thus, warranting constant worldwide surveillance and the creation of new vaccines. Limiting the extent of both interspecies and intraspecies transmission is critical to fight against not only a worldwide and perpetual viral threat but other serious health conditions such as asthma exacerbations.

1.6.2 Viral structure: Influenza A

A viral particle or virion (80-120 nm) from both genera of influenza include a lipid membrane envelope expressing hemagglutinin (HA) and neuraminidase (NA) transmembrane proteins necessary for viral entry and budding from host cells respectively (105). Supporting the envelope underneath are the M1 matrix proteins that can facilitate viral RNA movement by interacting with the nuclear export protein (NEP). Furthermore, the M2 matrix protein can be seen in the envelope; not as prominently as HA and NA but serving the function of an ion channel for conduction of hydrogen ion to acidify the virus (106). The acidification is required for decapsidation to release the vRNAs from their stably bound viral ribonucleoprotein (vRNP) complexes. The vRNPs are complexes composed of helical hairpins of viral RNA wrapped around multiple copies of nucleoproteins (NP) and viral polymerase subunits PB1, PB2 and PA. An influenza virus consists of 8 vRNP (107). Non-structural proteins like NS1 and NS2 mediate modulating roles in viral replication and transcription along with evading host defence, such as reducing interferon production from host cells, boosting viral pathogenicity and augmenting inflammation (108).

Some accessory proteins are produced only when the virus has infected a host cell. Examples of such proteins include PB1-F2 (a derivative of viral polymerase PB1), PA-X (a derivative of viral polymerase PA) and NEP (nuclear export protein). Accessory proteins are believed to work in conjunction with NS1 to further enhance virulence, elevate the extent of the

inflammatory response and reduce host's antiviral defences (105). Nonetheless, the roles of specific accessory proteins have not been clearly defined.

1.6.3 Influenza trafficking

The trafficking process begins with HA glycoproteins binding to sialic acid (SA) residues on airway epithelial cells. Endocytosis of the virion occurs in a clathrin-dependent manner or by micropinocytosis leading to subsequent fusion to the endosome (107). The fusion event requires exposure of the viral fusion peptide for which virion acidification is conducted by the M2 (matrix-2) proton channel and cleaving of the hemagglutinin (HA) protein by host cell proteases (109). The acidification process inserts the viral ribonucleoproteins (vRNPs) from its capsid into the endosome (110).

The vRNP complexes enter the nucleus using a nuclear localization signal (111). The 8 viral RNAs transcribe in a primer-independent process (using PB1, PB2 and PA) to synthesize corresponding positive strand counterparts (cRNAs) needed for protein synthesis. The cRNAs act as a template for negative strand creation (107). The cRNAs using the polymerase subunits PB1, PB2, PA hijack and cleave host cell's mRNA to transcribe and insert viral mRNA with a poly A tail. Following integration into host mRNA, viral protein synthesis is carried out by endoplasmic-reticulum (ER) associated and cytoplasmic ribosomes (107).

Newly synthesized proteins like PB1, PB2, PA and nucleoprotein (NP) return to the host cell's nucleus to further help in viral vRNA replication and mRNA transcription. Positive feedback results in the synthesis of more proteins further creating positive and negative strands of vRNAs and mRNAs. The new vRNPs are exported from the nucleus and move towards the cell surface to egress the cell. Additionally, HA and neuraminidase (NA) undergo oligomerization of N-linked glycans in the ER aiding in its folding process efficiency and giving their distinctive structure and function (112, 113). Furthermore, newly synthesized HA

is cleaved by enzymes in the Golgi apparatus or at the plasma membrane in order to restore its fusion capability when infecting other host cells (114, 115).

All the protein components of a virion accumulate termed protein or molecular crowding, in an area of host membrane upon which the budding formation is initiated where the membrane starts to curve outwards. HA and NA glycoproteins are believed to be the primary protein members driving the budding process (116). Once the influenza has fully budded, the sialidase activity of NA hydrolyses the glycosidic bonds of the SA residues on the host cell's surface (117). Sialidase also prevents aggregation between viruses and cleaves SA from glycoproteins of airway mucus to allow easier movement of viral particles to surrounding airway epithelial cells (118, 119).

1.6.4 Prevention of infection by influenza

Routine, annual vaccinations are the best interventions against illness and severe complications caused by influenza A/B. Vaccine formulations are determined nine months before the influenza season. Since vaccine formulations are decided before the seasonal wave, the strains that are more commonly circulating in the populations from the previous season are included in the next batch of vaccines hence acting as a prediction tool to know which strain will be the most prevalent (120). Nonetheless, prediction is not definitive due to the large numbers of subtypes circulating and the seasonal antigenic changes taking place each year. Due to the variability in the seasonal vaccine efficacy, seasonal vaccines are not effective against pandemic or antigenic drifted strains (121). Trivalent and quadrivalent vaccines cover both A and B strains that are circulating each year which increases the basis of protection for the public.

Current antiviral drug therapies include NA inhibitors which prevent the exit of viral particles from cells to prevent infections of surrounding cells and adamantanes which function by

inhibiting the M2 ion channel thus preventing the critical acidification step (120). Such drugs only appear to be helpful among high-risk groups such as immunocompromised patients.

While both vaccines and antivirals reduce the risk of symptomatic influenza and spread of infection, vaccines have proven to be uncertain in preventing asthma exacerbations. Likewise, antivirals such as NA inhibitors have a minimal effect in preventing exacerbations (122, 123). Due to a lack in effectiveness of conventional treatment options, further investigation into the inflammatory mediators such as IL-33 and their relationship with the induction of AHR is necessary in order to create targeted therapies.

1.7 IL-33

IL-33 is a cytokine that belongs to the IL-1 family, functioning as an alarmin or as a danger-associated molecular pattern (DAMP) released during states of inflammation and/or stress. A wide range of cells including epithelial, endothelial cells and fibroblasts produce IL-33 (124). IL-33, has effects on a wide range of cell targets including ILC2, macrophages, lymphocytes, neutrophils, natural killer (NK) cells (125). Due to its pleotropic effects, IL-33 has shown to be implicated in numerous health conditions like asthma, inflammatory bowel disease (IBD) and atherosclerosis.

IL-33 has been shown to be expressed in endobronchial biopsies of human asthma subjects mainly localized to bronchial epithelial cells (126). Intranasal instillation of IL-33 following allergic (OVA) sensitization in a murine model augments IL-5 expressing $CD4^+$ T cells, AHR and elevates levels of ILC2s and eosinophils (127, 128). Administration of IL-33 by itself evokes AHR, goblet cell hyperplasia and eosinophilia in the absence of adaptive immunity (129). In addition, IL-33 transgenic mice show substantial airway inflammation marked by increased levels of eosinophils, goblet cell hyperplasia and the presence of inflammatory cytokines such as IL-5, IL-8, IL-13 in the BAL fluid all features indicative of an asthmatic

response (130). Overall, following both observations on human and several murine studies one can conclude that IL-33 has a pivotal role in the development of asthma exacerbations.

1.7.1 IL-33 protein structure

The human IL-33 protein, coded for by the IL-33 gene located on chromosome 9 (chromosome 19 for mice) is composed of 270 amino acids (52% homology with murine protein sequence) and has a molecular weight of approx. 31 kDa (124, 125). The protein is divided into three specific domains: nuclear, central, and IL-1-like cytokine. Unlike its IL-1 family counterparts, the synthesized protein (referred to as full length) has biological activity even though previously thought to require caspase 1 and/or inflammasome-mediated cleavage of full-length IL-33 to acquire biological activity (126).

Furthermore, a number of proteolytic enzymes can cleave IL-33 in the central domain thus preserving the IL-1-like cytokine domain and generating mature protein products with heightened biological activity compared to full-length IL-33. Examples of such enzymes include chymase and tryptase (produced by mast cells) and elastase and cathepsin G (produced by neutrophils) (125). Such enzymes carry out their reaction when full-length IL-33 is released from their corresponding cell sources.

1.7.2 IL-33 release

IL-33 is constitutively expressed in airway epithelial cells (131). IL-33 remains sequestered in the nucleus and associated to the chromatin, specifically, tightly bound to the histone H3 proteins via its chromatin binding domain in the nuclear domain of IL-33 (132). During release, the IL-33 moves out of the cell either in complex with histones or as monomers. The IL-33-histone complex synergizes to activate downstream IL-33 signalling. A comparison of IL-33 in complex with histones reveals an augmented induction of its downstream genes compared to IL-33 alone (132).

Stimuli that cause excessive damage to cells cause necrosis, an unprogrammed form of cell death that can release IL-33. Full-length IL-33 that remains bound to the nucleus is released into the extracellular milieu without undergoing processing and is effective in carrying out its biological activity through its target cells. Caspases 3 and 7 which are produced during apoptosis perform inactivating cleaving reactions thus generating IL-33 mature protein fragments without any biological activity (126). IL-33 can also be actively released without cell damage. For instance, exposure to allergens like fungal Alternaria alternata or HDM causes ATP to be released extracellularly (133, 134). The released ATP acts on purinergic receptors causing intracellular calcium levels to increase in turn leading to IL-33 release. The details of how IL-33 is released without cellular damage remain unclear.

1.7.3 IL-33 signalling pathway

IL-33 carries out its biological effects by binding to its transmembrane receptor heterodimer composed of ST2/IL-1 receptor accessory protein (IL-1RAP) (135). Downstream of the receptor complex, MyD88 is recruited and binds to the intracellular Toll/interleukin-1 receptor (TIR) domain of the receptor complex. MyD88 recruits IL-1R associated kinase 4 (IRAK4) and TNF receptor associated factor 6 (TRAF6) leading to phosphorylation of inhibitor of nuclear factor-κB thus allowing the release of NF-κB. Furthermore, in the absence of TRAF6, IRAK1 can also become activated downstream of MyD88. IRAK1 leads to mitogen activated protein kinase (MAPK) activation and the subsequent activation of AP-1. MAPK can do so by first phosphorylating its downstream protein members like p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) which then collectively activate activator protein 1 (AP-1) (135). Finally, activated AP-1 and NF-κB move into the nucleus and bind to the DNA to induce expression of various Th2 cytokines like IL-4, IL-5, and IL-13 and T2-polarizing transcription factor, GATA3 along with other inflammatory chemokines and markers for cell survival and proliferation.

In terms of regulation, a soluble ST2 (sST2) decoy receptor can bind extracellular IL-33 thus prevent IL-33 from binding to its ST2/IL-1RAP receptor and carrying out its biological effect. The ST2 receptor can by phosphorylated, internalized, and promptly degraded. The Single Ig IL-1R-related molecule (SIGIRR) also acts as a negative regulator by preventing heterodimerization of ST2 and IL-1RAP (124). In addition, IL-33 can undergo cysteine oxidation and the formation of disulphide bridges extensively alter its confirmation which and no longer allows IL-33 to bind to its ST2 receptor complex (136).

1.7.4 IL-33/IL-13 axis

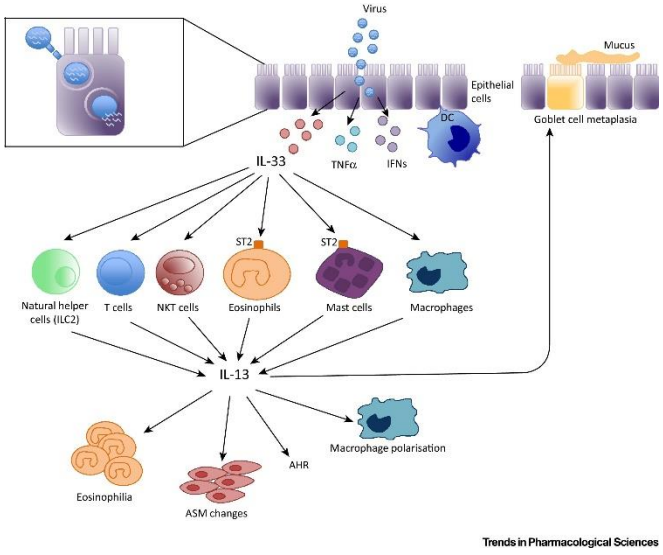


Figure 1.7.4: **Schematic of the IL-33/IL-13 axis**. Viral infection inflicts stress/damage to epithelial cells releases IL-33 which when acting on cells expressing the IL-33 receptor complex produce IL-13. IL-13 is often viewed as a pivotal cytokine stimulating pathophysiological features of asthma such as AHR. From "Targeting the IL-33/IL-13 Axis for Respiratory Viral Infections", Donovan et al., 2016, Trends Pharmacol Sci, 37(4), p.253. Copyright 2016 by Elsevier. Reprinted with permission.

One of the major effector functions of IL-33 is to stimulate both innate and adaptive immune cells to produce IL-13 (137, 138). IL-13 has a strong association with AHR through its actions on ASM and epithelial cells (139, 140). A meta-analysis of clinical studies has shown that anti-IL-13 antibody reduces asthma exacerbations rates and improves lung function (141). There is a positive association between IL-33 and IL-13 levels in nasal aspirates during RSV infections and blocking the action of murine IL-33 with either IL-33 knockouts animals or anti-ST2 treatment abrogates IL-13 levels in the context of RSV infection (142-144). Furthermore, the importance of this axis has been highlighted in IAV infection wherein IAV induces IL-33 and

consequently stimulates the accumulation of an innate lymphoid lineage population responsible for the substantial production of IL-13, irrespective of adaptive immunity in BALB/c mice (97, 145). Therefore, The IL-33/IL-13 axis has been deemed a key pathway to target to prevent acute viral-induced airway exacerbations among asthmatic patients (146).

1.8 IL-33 cell targets

Innate lymphoid cells (ILCs)

ILCs are a unique group of tissue-resident, antigen receptor-lacking, innate cells that are seen as T cell subset equivalents in terms of cellular function. ILCs do not express any lymphoid or myeloid markers. The ILC family comprises six members: NK cells (equivalent to $CD8^+$ T cells), lymphoid tissue inducer (LTi) cells (playing a role in lymphoid tissue development), ILC1, ILC2, ILC3 and ILC regulatory cells (equivalent of T regulatory cells) (147). The ILC3 member is further classified into two groups based on whether there is expression of natural cytotoxicity receptors (NCR) like NKp46 (147). While they can be replenished from bone marrow or lymphoid tissue, ILCs mainly self-renew at their respective tissue-sites.

ILCs in asthma are considered important cell players in mediating asthma pathogenesis in response to alarmins. ILC1 and ILC3 are important in modulating a Th2-low asthmatic milieu whereas ILC2s are essential for a Th2-high milieu and functioning independently of adaptive immunity producing type 2 cytokines (147). Additionally, the ILC2 population responds to cysteinyl leukotrienes C4 and prostaglandin D2 which are two important asthma mediators (148). The ILC2 subset accumulates in number during respiratory infections like rhinovirus and influenza in response to all three alarmins, IL-25, TSLP and IL-33 that are potentially playing an important role in affecting the frequency of asthma exacerbations (148). In the context of influenza, ILC2s, referred to as natural helper cells mediate AHR in BALB/c mice by producing IL-13 (97). Depletion of the natural helper cells attenuates AHR and

reconstituting them in IL-13 deficient BALB/c mice restored IL-13 production and consequential AHR (97). In contrast, ILC2 can contribute to the resolution of lung inflammation and to restoring lung function, as shown by measuring oxygen saturation levels later in influenza infection by producing amphiregulin (149). IAV mediated exacerbations are not exclusively limited to ILC2s. A HDM sensitized murine model infected with IAV shows no induction of ILC2s (150). The proportion of ILC2s did no correlate with IL-33 levels and severity of asthma exacerbation (150). Blocking the action of IL-33 resulted in a reduction in AHR and NCR^+ ILC3 absolute numbers with no changes reported in the ILC2 subpopulation which is contrary to the fact that ILC2s are the only cells expressing the IL-33 receptor and should play a decisive role in this model.

Nonetheless, ILCs in IAV-induced asthma act as a double-edged sword: mediating AHR early in acute infection but adopting a restorative phenotype during later stages of infection. The role of ILCs in context of human asthma has yet to be established since all functional observations are made from murine studies and no ILC targeted therapeutics have been created to ameliorate the asthmatic condition for humans.

Neutrophils

Neutrophils are referred to as polymorphonuclear (PMN) leukocytes and in human are the most abundant innate immune cell in the blood. They are short-lived cells abundantly produced in the bone marrow and can extravasate into any tissue making them one of the first responders against pathogens like IAV infection (151). Neutrophils exert three main functions: phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs) (152). Phagocytosis is the physical engulfment of microorganisms requiring the creation of phagosome and consequent degranulation. Degranulation releases a wide range of antimicrobial proteins (coordinated by NADPH oxidase) to allow effective lysis of the foreign

microorganism. NETs formation relies on the capability of the neutrophils to release its sticky DNA extracellularly to trap pathogens. Anti-microbial proteins such as histones, elastase and cathepsin G can associate with DNA to exert their antimicrobial activity on the trapped pathogens (153). Besides lytic functions, neutrophils release various inflammatory cytokines and can coordinate the inflammatory process with other cells members like macrophages (151).

The IL-33 receptor, ST2 has been shown to be expressed in neutrophils. In certain diseases like rheumatoid arthritis and psoriasis, IL-33 can stimulate neutrophil recruitment via IL-8 production or possibly by downregulation of TLR4-mediated CXCR2 expression (151, 154, 155). Another mechanism of neutrophil recruitment exerted by IL-33 involves the p38-MAPK 2/3 as shown *in vivo* wherein MK2/3 knockouts fails to recruit neutrophils in presence of IL-33 (156). In the context of asthma, IL-33 mediated neutrophilia alters asthmatic pathogenesis. In a viral-induced exacerbation model, neutralizing the effects of IL-33 attenuated neutrophilic inflammation. In addition, IL-33 selectively upregulated a Th2 profile in neutrophils of OVA-challenged C57BL/6 mice. Adoptive transfer of IL-33 neutrophils leads to a further increase in pathogenic severity among OVA-treated mice (157). In context of viral infection, although the association of neutrophils and asthma exacerbations has been established, the mechanistic importance of IL-33 acting on neutrophils hasn’t been shown (158).

Eosinophils

Eosinophils develop in the bone marrow from pluripotent progenitors (159). They have always been associated with parasitic infections but their role in response to other pathogens is still being explored. Like neutrophils, when released from the bone marrow they are phenotypically ready to respond to stimuli such as IL-5 or chemokines CCL11, CCL24. Eosinophils have a wide range of functions ranging from phagocytosis, degranulation, formation of extracellular traps to modulating communication with other leukocytes, epithelial cells and platelets (160).

Many studies have established eosinophils' actions to be pivotal in asthma pathology. *In vivo* experiments involving allergen sensitization and challenge of various species have shown that the establishment of asthma pathology is dependent on eosinophils to a large degree (161-163). Following the same sensitization and challenge approach, eosinophil transfer to IL-5 knockout mice shows restoration of AHR and Th2 cytokines proving eosinophils have a strong association with asthma exacerbations (161). Large observational studies have established that human asthmatic subjects frequently have an elevated level of serum eosinophils (164). Hence, nor surprisingly, targeting the migration and/or the generation of eosinophils has been fruitful as a therapeutic intervention in asthma. Trials into anti-IL-5 in human asthma have revealed both a reduction in eosinophil numbers, reductions in TGF-β in BAL fluid, less histological expression of ECM proteins and reduced asthma exacerbations exclusively in eosinophilic asthma (40, 165, 166).

IL-33 is strongly associated with eosinophilia in asthma. This is so because IL-33, with its ST2 signalling on eosinophils produces IL-5 thus establishing a positive feedback loop of IL-5 and eosinophil migration, survival, activation, and adhesion (167). Furthermore, IL-33 maybe involved in the generation of eosinophils in the bone marrow as ST2-deficient mice failed to develop eosinophils and administration of IL-33 boosted maturation of eosinophils in the bone marrow (168).

In relation to respiratory infections, eosinophils are activated and are involved in bolstering anti-viral defences, epithelial integrity, and viral clearance (169). It is highly possible eosinophils assist in clearance of viral infection while at the same time promoting asthma (170). Nonetheless, the exact role of eosinophils in viral-induced asthma is not fully understood and further studies exploring the definitive role of eosinophils in viral infections is imperative.

Macrophages

Macrophages are a prominent member of the innate immune system possessing a wide range of functions. These functions include being first responders in recognizing and destroying invading pathogens, in antigen presentation, and in maintaining tissue homeostasis. They communicate with other immune cells both innate and adaptive to mount an inflammatory response (171).

The lung consists of distinct populations of macrophages that can be divided into tissue-resident and monocyte-derived macrophages. Tissue-resident macrophages include alveolar macrophages (AM) in the airway lumen and interstitial macrophages (IM) in the parenchyma (171). Both these populations have their origins from the yolk-sac developed during the pre-natal phase of life and later into adulthood are maintained *in situ*, without seeding of cells from the bone marrow under homeostatic conditions. Alveolar macrophages are the first subset of macrophages to encounter inhaled foreign particles and hence carry out host defensive roles achieving recognition of pathogens, their clearance and eventual regulation of tissue homeostasis (172). IMs have a regulatory role in coordination with adaptive immunity by secreting the immunomodulatory cytokine IL-10 (171). Monocyte-derived macrophages are recruited from the bone marrow during infection or other inflammatory conditions differentiate into macrophages to support tissue-resident populations.

Phenotypically, macrophages are viewed along a spectrum with the classical, pro-inflammatory M1 at one end and the alternative, anti-inflammatory M2 at the other. In asthma, both the M1 and M2 macrophage phenotypes are increased. The M1 phenotype is important to drive a non-Th2 endotype of asthma and the M2 population promotes a Th2 environment. In addition, the AM and IM's regulatory functions are essential to prevent an immune response to inhaled allergens and their depletion worsens inflammation in allergen-mediated asthma (173, 174). Other macrophage dysfunctions include impaired phagocytosis which has shown in sputum analyses of non-eosinophilic asthma patients (175). The pro-inflammatory cytokine profile

becomes elevated further aggravating airway inflammation. Leukotriene signalling is associated with asthma and macrophages are important sites where lipid mediator synthesis takes place in severe asthma (176).

Macrophages express the ST2 receptor thus revealing the capacity to be influenced by IL-33 signalling. IL-33 polarizes macrophages towards a M2 phenotype (177). The polarization towards M2 macrophages requires mitochondrial rewiring involving uncoupling of the respiratory chain leading to GATA3 transcription factor activation (178). The result of M2 polarization is production of Th2-cyotkines, IL-5 and IL-13. Activation of macrophages is enhanced in the presence of IL-33 by TLR4 signalling (179). Following infection with IAV, AMs produce substantial amounts of IL-33 using the NLRP3 mechanism, further promoting IL-13 production and in consequence, airway hyperresponsiveness (97).

Considering the role macrophages play in IAV infection, their importance in IAV-induced asthma hasn’t been explored and in doing so will reveal whether macrophages are simultaneously controlling IAV infection and limiting induction of asthma or not.

Rationale

The mechanism by which influenza (IAV) infection causes AHR is not well understood, with the exception of the T2-biased BALB/c mouse. Experimental work has shown IAV infection stimulates AHR by releasing IL-33 in BALB/c mice. IAV-induced AHR in BALB/c mice is based on IL-33 release predominantly from alveolar macrophages activating a subset of innate lymphoid cells, natural helper cells now referred to as innate lymphoid type 2 (ILC2) cells that produce IL-13. IL-13 has been shown to be the principal cytokine responsible for enhanced airway smooth muscle contraction leading to AHR. The dual relation between IL-33 and IL-13 release is referred to as the IL-33/IL-13 axis.

These observations have been made in the BALB/c mice, which as a T2-biased murine model may not provide insights into the role of IL-33 in a less T2-biased mouse strain that may also be more representative of the asthmatic situation among humans. The objective of this thesis was to determine whether the IL-33/IL-13 axis was responsible for the induction of AHR in a less-T2 biased model, C57BL/6 with IAV infection.

Aims of the study

The study addressed the following aims:

1. To determine the effect of blocking the action of IL-33 on IAV-induced AHR, bronchoalveolar lavage cell count, IL-33, IL-13, CCL2 and cysteinyl leukotrienes levels and gene expression patterns of IAV non-structural (NS1) protein, inflammatory cytokines and chemokines
2. To determine the effect of blocking the action of IL-33 on weight loss and viral load following IAV infection
3. To evaluate the effects of depleting neutrophils on IAV-induced AHR and inflammation
4. To characterize the ILCs and M1/M2 macrophages during IAV infection and to assess the effects of blocking the action of IL-33
5. To assess the effects of depleting monocyte-derived macrophages on IAV-induced AHR

Chapter 2: Materials and methods

2.1 Mice

C57BL/6 female mice (8-12 weeks) from The Jackson Laboratory and in-house bred mice with the same background were housed in a conventional animal facility at the Research Institute, McGill University Health Centre (MUHC). All mice were treated according to the guidelines of the Canadian Council on Animal Care (CCAC). The protocols for *in vivo* experiments were approved by the Animal Care Committee of McGill University.

2.2 Viral stock preparation

Mouse-adapted Influenza A virus (A/Puerto Rico/8/1934(H1N1)) stock was passaged at a MOI of 0.01 from a pre-existing stock using Madin-Darby canine kidney (MDCK) cells (ATCC). The viral inoculum is prepared in Dulbecco's modified eagle medium (DMEM) with 0.1% BSA (referred to as infection medium). After 1 hour incubation at 37 °C, the viral inoculum was washed off and replaced with infection medium with tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (2 µg/mL) and further incubated for 72 hours at 37 °C. After 72 hours, the MDCK cells were decanted and centrifuged at 1500 rpm for 10 minutes. The supernatant was aliquoted and frozen at -80 °C for long-term storage and future use. The newly-prepared viral stock was diluted 10^{-1} to 10^{-12} in order to perform a plaque assay to determine its viral concentration in plaque forming units/mL (PFU/mL).

2.3 Sub lethal viral dose determination

Mice were intranasally infected, following light anaesthesia (isoflurane) administration of varying doses of IAV inocula (25, 50, 100, 150 and 200 PFU). The weights of the mice were recorded one day pre-infection and their weights monitored daily until they reached a humane endpoint. The endpoint was defined as a loss of 25% of initial weight, meeting the criteria for euthanasia. Beginning at 6 dpi and onwards, Kaplan-Meir curve analysis revealed 100, 150 and

200 PFUs had reduced probability of survival greater than 25% weight loss compared to their respective initial weights. The highest possible dose without an affect on probability of survival was 50 PFU which was determined to be the sub lethal dose.

2.4 Experimental protocol: antibody treatments

Anti-ST2 and rat IgG_{2B} isotype control antibodies (R&D Systems), 10 µg each in sterile PBS, were delivered twice intranasally a day before and a day following sub lethal IAV infection under light anaesthesia (142). Control mice were intranasally instilled with the same volume of sterile PBS as the IAV inocula. All groups of mice were weighed on the day of the first intranasal antibody exposure and at the endpoint (3 dpi) followed by assessments of respiratory mechanics, profiling of BAL fluid inflammation and lung harvesting for RT-qPCR, flow cytometry and MDCK plaque assay.

For IL-13 neutralization, 100 µg of anti-IL-13 and its IgG1 isotype both in sterile PBS were delivered intraperitoneally 2 hours prior to IAV infection. Respiratory mechanics and viral burden were assessed on 3 dpi.

For neutrophil depletion, mice were injected intraperitoneally (IP) with 100 µg/mouse of anti-Ly6G and rat IgG2a isotype control antibodies (Bio X Cell) in sterile PBS 1 hour prior to IAV infection and on 2 dpi. The endpoint for determination of the effect of neutrophil depletion was 3 dpi at which time respiratory mechanics, BAL fluid inflammation and lungs harvested for RT-qPCR and MDCK plaque assay were assessed.

For monocyte-derived macrophage depletion, mice were injected IP with 400 µg/mice of anti-Ly6C and rat IgG2a isotype control antibodies (Bio X Cell) in sterile PBS 1 hour prior to IAV infection and with 200 µg on 2 dpi. The endpoint for determination of the effect of monocyte-derived macrophage depletion was 3 dpi. Respiratory mechanics was assessed on 3 dpi.

2.5 Measurement of airway hyperresponsiveness to methacholine challenge

At 3 dpi, respiratory system mechanics were assessed using a small animal ventilator (FlexiVent, Scireq). The mice were sedated with xylazine hydrochloride (10 mg/kg IP) and anesthetized with pentobarbital sodium (50 mg/kg IP). Once the mice did not show the pedal withdrawal reflex upon stimulation, they were tracheotomized and cannulated with an 18-guage cannula and promptly connected to the ventilator. Muscle paralysis was induced using rocuronium bromide (20 mg/kg IP). The mice were ventilated with the following settings: a tidal volume of 10 ml/kg, maximal inflation pressure of 30 cm H_2O, a positive end-expiratory pressure of 3 cm H_2O and a frequency of 150 breaths/min. The FlexiWare^{TM} software provided a real-time output of total airway resistance and elastance using the single compartment model.

Baseline respiratory resistance and elastance were measured following an aerosolization of sterile PBS, followed by measurements of responses to aerosolized methacholine doses of 6.25, 12.5, 25 and 50 mg/ml. The methacholine was aerosolized using an in-line nebulizer attached to the ventilator. Each dose of methacholine was administered to the mouse for 10 seconds. At each dose of methacholine, 6 measurements of total respiratory resistance and elastance were made with the highest value (subject to a coefficient of determination >0.90) considered for the construction of concentration-response curves.

2.6 Bronchoalveolar lavage (BAL)

Following measurements of responses to methacholine, the lungs were lavaged with 1 mL of sterile PBS and the fluid recovered by gentle suction was placed in 1.5 mL microcentrifuge tubes kept on ice. Following BAL collection, the samples were centrifuged at 5000 rpm for 5 mins. The supernatant was collected and bubbled with nitrogen for 10 seconds for long-term preservation at -80°C. The pellet was re-suspended in 1 mL of PBS for total cell determination using a hemacytometer with trypan blue. Cytospins of 100,000 and 500,000 cells were prepared on microscope slides (1000 rpm for 5 minutes) and stained for a differential cell count using

Diff-Quik solution (Fisher Scientific). The differential cell count was based on a count of 300 cells.

2.7 Evaluation of cytokines, chemokine and inflammatory lipid mediator in BAL

To assess the effect of anti-ST2 antibody treatment, BAL fluid from IgG_{2B} IAV and anti-ST2 IAV groups from 3 dpi were compared by enzyme-linked immunosorbent assay (ELISA) of supernatant using mouse DuoSet ELISA kits for IL-33, IL-13, CCL2 (R&D Systems) and Express EIA kit for cysteinyl leukotrienes (Cayman Chemical). All protocol steps for analyses were followed according to the manufacturer's instructions.

2.8 Tissue processing: lung single cell suspensions

Mice were euthanized with an overdose of pentobarbital sodium. The chest cavity was opened, the blood from the heart was exsanguinated and the lungs perfused with 10 mL of cold sterile PBS. All lobes of the lungs were surgically removed and placed in 2 mL of sterile RPMI medium and kept on ice. Following extraction, the lungs were cut into small pieces using a scalpel, suspended in 2 mL RPMI. Subsequently, 2 mL of digestion solution comprising 300 units/mL collagenase (Sigma Aldrich) and 20 µg/mL DNase I (Sigma) were added to the lung pieces in RPMI and were incubated for 45 minutes. The products of lung digestion were passed through an 18-gauge needle and filtered through a 70 µm strainer. Red blood cells were lysed by incubation on ice for 5 minutes in RBC lysis buffer (Biolegend). Live single cells were suspended in cold sterile PBS and counted using a hemacytometer using trypan blue exclusion.

2.9 Flow cytometry

Following every staining step, the cells are washed with FACS buffer (1% BSA in PBS) and centrifuged at 500xg for 5 minutes. After single cell suspension acquisition, cells were stained with eBioscience Fixable Viability Dye efluor 780 (Invitrogen) (1:1000) incubated in the dark at 4°C for 30 minutes. Fc receptors were blocked with Tru Stain FcX (anti-mouse CD16/32)

(101320, BioLegend) (1:100) in the dark at 4°C for 15 minutes. For surface staining, 1:100 dilutions were used for each of the anti-mouse fluorochrome-conjugated antibodies. The surface antibodies were as follows: Brilliant violet 510-conjugated anti-CD45 (103137, BioLegend), Fluorescein isothiocyanate-conjugated anti-CD3ε (100305, BioLegend), anti-CD5 (100605, BioLegend), anti-CD11b (101205, Biolegend), anti-CD11c (117305, BioLegend), anti-Ly6G/Ly6C (Gr-1) (108405, BioLegend), anti-Ter-119/Erythroid cells (116205, BioLegend), anti-CD45R/B220 (103205, BioLegend), anti-TCRγ/δ (118105, BioLegend), anti-TCRβ chain (109205, BioLegend), Allophycocyanin-conjugated antibody anti-CD127 (IL-7Rα) (135011, BioLegend), Brilliant violet 605-conjugated anti-CD90.2 (Thy 1.2) (140317, BioLegend), Brilliant ultra violet 395-conjugated anti-CD11b (563553, BD Biosciences), Brilliant violet 786-conjugated anti-CD11c (563735, BD Biosciences), Brilliant violet 421-conjugated Hamster anti-mouse CD11c (562782, BD Biosciences), Alexa Fluor 700-conjugated anti-CD170 (SiglecF) (Invitrogen), Brilliant violet 786-conjugated Rat anti-mouse siglecF (740956, BD biosciences), Alexa Fluor 488-conjugated anti-CD80 (104715, BioLegend), Brilliant violet 711-conjugated anti-CD64 (FcγRI) (139311, BioLegend), Brilliant violet 421-conjugated anti-CD206 (MMR) (141717, Biolegend), phycoerythrin–Texas red–conjugated anti-F4/80 (Invitrogen), Allophycocyanin-conjugated anti-Ly6C (Invitrogen), Alexa Fluor 700-conjugated Ly-6G (127621, Biolegend) and Brilliant violet 605-conjugated anti-IL33Rα (IL1RL1, ST2) (145323, BioLegend).

For intracellular staining, 1:50 dilutions were used for each of the anti-mouse fluorochrome-conjugated antibodies. For intracellular staining the antibodies used were phycoerythrin-Cyanine7-conjugated anti-human/mouse arginase 1 (Invitrogen) and phycoerythrin-conjugated iNOS (Invitrogen). Cells were incubated for an hour at room temperature in the dark for intracellular staining.

Cells were fixed using fixation reagent prepared from Fixation/Permeabilization concentrate and diluent (eBioscience) and were incubated in the dark for 20 minutes. Extra sample cells were pooled to create appropriate CD80, CD206, Ly6C and ST2 fluorescence minus ones (FMOs) and unstained control. Compensations were done using compensation beads (UltraComp eBeads, Invitrogen) and each antibody with a particular fluorophore to account for fluorescence signal spillover. Cells were suspended in FACS buffer for data acquisition using a BD LSRFortessa X-20 Cell Analyzer with FlowJo v10 software.

2.10 RNA isolation

Murine lungs were flash frozen in liquid nitrogen and homogenized in 0.1% BSA DMEM medium. 200 µL of the supernatant was transferred to 500 µL of Trizol reagent (Invitrogen). Following phase separation and isopropyl-glycogen precipitation, RNA was washed with 70% ethanol to remove residual salt precipitations. RNA concentration was measured using a spectrophotometer.

2.11 Reverse transcription and quantitative polymerase chain reaction (qPCR)

RNA (500 µg) was reverse transcribed to complementary DNA (cDNA) using the LunaScript RT SuperMix Kit (New England BioLabs) according to the manufacturer's instructions.

Following reverse transcription, PCR samples were prepared consisting of 6 µL of PCR mix (SYBR Select Master Mix (Thermo Fisher), forward and reverse primers and water) and 4 µL of cDNA. Per 4 µL sample, 5 µL of SYBR Select Master mix, 0.3 µL of each forward and reverse 10-fold diluted primer and 0.4 µL of water were used. The forward and reverse Mus musculus primer sequences used were as follows:

Gene	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
TNF-α	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG

IL-12a	ACGAGAGTTGCCTGGCTACTAG	CCTCATAGATGCTACCAAGGCAC
IL-6	TACCACTTCACAAGTCGGAGGC	CTGCAAGTGCATCATCGTTGTTC
IL-1β	TGCCACCTTTTGACAGTGATG	ACGGGAAAGACACAGGTAGC
CXCL1	CTGCACCCAAACCGAAGTCA	CCGTTACTTGGGGACACCTTTTA
NS1	AGAAAGTGGCAGGCCCTCTTTGTA	TGTCCTGGAAGAGAAGGCAATGGT
CCL2	GCTACAAGAGGATCACCAGCAG	GTCTGGACCCATTCCTTCTTGG
CCL3	ACTGCCTGCTGCTTCTCCTACA	AGGAAAATGACACCTGGCTGG
CCL5	CCAATCTTGCAGTCGTGTTTGT	CCCATTTTCCCAGGACCGAG
CCL7	CCCTGGGAAGCTGTTATCTTCAA	CTCGACCCACTTCTGATGGG
CCL22	GGACTACATCCGTCACCCTC	GACGGTTATCAAAACAACGCC
C-MAF	AGAGGCGGACCCTGAAAAAC	GTTTTCTCGGAAGCCGTTGC
CSF1	GACCAGGAACAGCTGGATGAT	CTCTCGGTGGCGTTAGCATT
GAPDH	GCAGACTTCAGGAATGTG	GGTCCTCAGTGTAGCCCAAG

The qPCR was conducted using SYBR Select master mix using a BioRad CFX96 thermal cycler. GAPDH was used as a reference gene. The corresponding Biorad CFX software was used for qPCR analysis. All Ct values from genes of interest were normalized to their respective GAPDH values and results were plotted as delta Ct values between IgG_{2B} isotype-treated IAV and anti-ST2 treated IAV groups.

2.12 Plaque assay

MDCK cells were seeded at a density of 750,000 cells per well in 6-well plates. Supernatant from lung homogenate or viral stock were diluted in DMEM ranging in concentrations from 10^{-1} to 10^{-12} and 100 µL aliquots were added to the monolayer of MDCK cells and incubated for an hour. Following a one-hour incubation, a 1:1 ratio of 2x minimum essential medium

(MEM) and 1% agarose were prepared. 2 µg/mL of TPCK-treated trypsin was added to the 1:1 solution. The viral inocula were removed and the 1:1 solution with TPCK-treated trypsin was added to the MDCK monolayer and allowed to solidify for an hour. The plates are returned to the incubator for 48 hours. After 48 hours, a fixative solution of a 3:1 ratio of methanol to acetic acid was added to the wells for 4 hours at room temperature. Subsequently, the agarose plugs were carefully removed and allowed to dry. The plaques were counted and viral concentration was determined. For viral concentration from lung homogenates, the total viral load was expressed as PFU/lung.

2.13 Statistical analysis

Data were analysed using non-parametric t tests, one-way and two-way repeated measures ANOVA with Bonferroni's correction post-hoc as appropriate using GraphPad Prism version 9 software. Data were expressed as mean ± SEM. Statistical significance was considered following $p<0.05$.

Chapter 3: Results

3.1 Effect of anti-ST2 on responsiveness to methacholine among H1N1-infected mice at 3 dpi

H1N1 IAV infection induced significantly greater responses to methacholine among C57BL/6 mice. Statistical significance was achieved when comparing both resistance and elastance values between IgG_{2B} isotype-treated IAV and IgG_{2B} PBS groups at 12.5, 25 and 50 mg/mL of methacholine. Similar trends of higher resistance and elastance between IgG_{2B} IAV and anti-ST2 PBS groups were observed (comparison not indicated on graph). Anti-ST2 treated IAV mice showed significantly lower total respiratory resistance and elastance when compared to the IgG_{2B} IAV group. Statistical significance was achieved at methacholine challenge concentrations of 25, 50 mg/mL and 12.5, 25 and 50 mg/mL for total respiratory resistance and elastance, respectively. Anti-ST2 PBS group showed a significantly higher resistance value at 50 mg/mL of aerosolized methacholine challenge compared to IgG_{2B} isotype PBS group (comparison not indicated on graph).

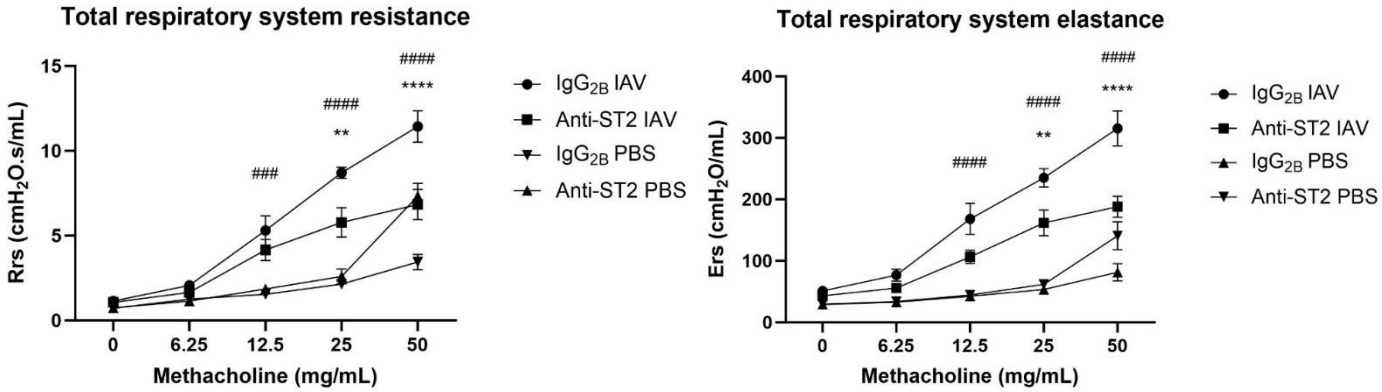


Figure 3.1: **Respiratory mechanics of anti-ST2 treated IAV mice**. Dose response of total respiratory resistance (Rrs) and elastance (Ers) for IgG_{2B} isotype and anti-ST2 infected groups and their respective PBS vehicle subjects at 3 dpi. Corresponding Rrs and Ers values are plotted for inhaled methacholine concentrations ranging from 0 to 50 mg/mL. Values at each dose are represented as mean ± SEM for IAV infected groups (n=8) and for PBS groups (n=5). ###p=0.006 and ####p<0.001 represent statistical significance between IgG_{2B} IAV and IgG_{2B} PBS groups. *p<0.05, **p<0.01 and ****p< 0.0001 represent statistical significance between

IgG_{2B} IAV and Anti-ST2 IAV groups. Two-way ANOVA with Bonferroni's post hoc test was used for both plots.

3.2 Effect of anti-ST2 on IAV infection mediated weight changes at 3 dpi

IAV infection caused significant weight loss in mice. Weight change is depicted as final weight as compared to initial weight prior to infection. Both IAV groups experienced weight loss compared to their respective PBS control groups. Anti-ST2 antibody administration did not significantly affect weight loss caused by IAV infection.

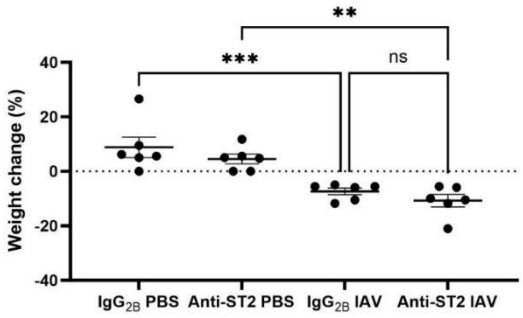


Figure 3.2: **Weight change of anti-ST2 treated IAV mice**. Weight change (presented as %) compared between IgG_{2B} isotype and anti-ST2 PBS groups (n=6 per group), isotype and anti-ST2 IAV groups (n=6 per group) groups. *p<0.05 and **p<0.01 represent statistical significance between anti-ST2, isotype vehicle groups and isotype infected group, respectively. One-way ANOVA with Bonferroni's post hoc test was used to assess statistical significance. Weight change values are represented as mean ± SEM.

3.3 Effect of anti-ST2 on IAV NS1 expression and viral load at 3 dpi

qPCR delta Ct values indicate no difference in expression of viral NS1 between IgG_{2B} isotype and anti-ST2 IAV groups. Plaque assay show no difference in the number of plaque forming units (PFU) per lung between isotype and anti-ST2 IAV groups.

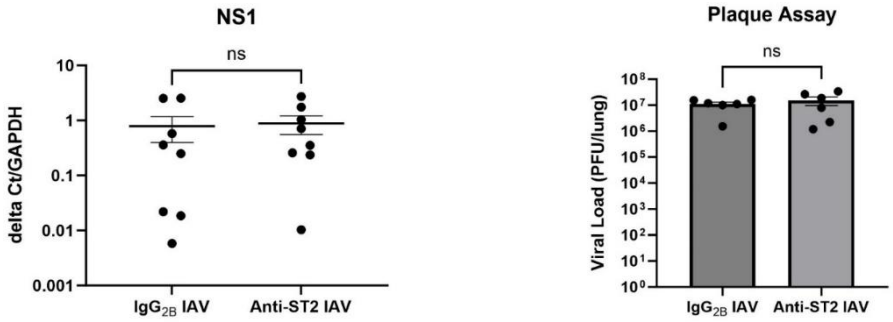


Figure 3.3: **NS1 expression and viral load assessment in anti-ST2 treated IAV mice**. qPCR analysis of NS1 expression (n=8 per group) and viral load assessed by plaque assay (n=6 per group) for IgG_{2B} isotype and anti-ST2 IAV groups. Delta Ct values are relative to GAPDH. Values for both graphs are represented on a $\log_{10}$ scale. Mann Whitney U test was used for analyses for both graphs. Delta Ct and viral load values are represented as mean ± SEM.

3.4 Evaluation of differential cell count of inflammatory cells in BAL fluid at 3 dpi affected by anti-ST2 administration

H1N1 IAV infection induced an influx of inflammatory cells into the BAL fluid. Both IgG_{2B} isotype and anti-ST2 PBS groups showed a low number of total inflammatory cell counts with no significant difference between their BAL cell populations. The primary innate cell for both control groups are macrophages with no substantial presence of either neutrophils or lymphocytes. There was a significant increase in the total cell count and number of neutrophils between IgG_{2B} PBS and IAV infected groups. There was no statistically significant difference in the number of macrophages and lymphocytes among both isotype and anti-ST2 antibody and PBS-treated mice compared to isotype antibody-treated IAV infected mice. The anti-ST2 antibody treatment did not alter the total inflammatory cell count, neutrophils, macrophages or lymphocytes.

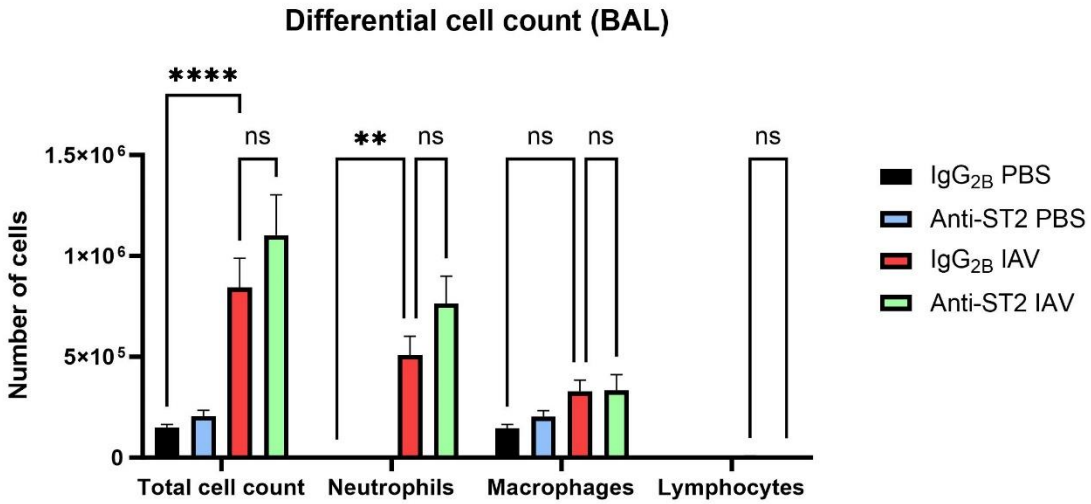


Figure 3.4: Differential cell count on BAL cells of anti-ST2 treated IAV mice. Total cell count and differential cell count for neutrophils, macrophages and lymphocytes in BAL fluid

3 dpi for IgG_{2B} isotype (n=5), anti-ST2 (n=5) control groups and IgG_{2B} isotype (n=9) and anti-ST2 (n=9) IAV groups. ****p<0.0001 and **p<0.01 indicate statistical significance between isotype PBS and isotype IAV groups for total cell count and neutrophils respectively. Cell count numbers are presented as mean ± SEM. Two-way ANOVA with Bonferroni's post hoc test was used to assess statistical significance.

3.5 Effect of anti-ST2 on IL-33, IL-13 and cysteinyl leukotriene levels in BAL fluid at 3 dpi

Both IgG_{2B} isotype-treated and anti-ST2-treated PBS groups showed a similar low concentration value for IL-33 (figure 3.5A). Comparing isotype-treated IAV infected and PBS vehicle groups, there was an increase in the levels of IL-33 among infected mice. There was no significant difference in IL-33 levels between isotype and anti-ST2 IAV infected groups.

Assessment of IL-13 levels between both isotype and anti-ST2 treated PBS and IAV groups showed no significant differences in IL-13 in BAL fluid (figure 3.5C). During IAV infection, anti-ST2 did not affect IL-13 levels as seen in the comparison between isotype and anti-ST2 infected groups showed no difference.

Both isotype antibody and anti-ST2 antibody-treated PBS groups showed similarly low values for total CysLTs (figure 3.5D). Comparison of either isotype or anti-ST2 PBS control groups to isotype IAV infected mice showed a significant elevation in the levels of total CysLTs in BAL fluid. IAV infected mice treated with anti-ST2 show a further significant augmentation of CysLTs when compared to its control groups. Furthermore, treatment with anti-ST2 augmented the BAL fluid levels of CysLTs in the presence of IAV infection. There was a significant increase in the CysLTs detected in the anti-ST2 IAV group compared to its isotype-treated IAV group.

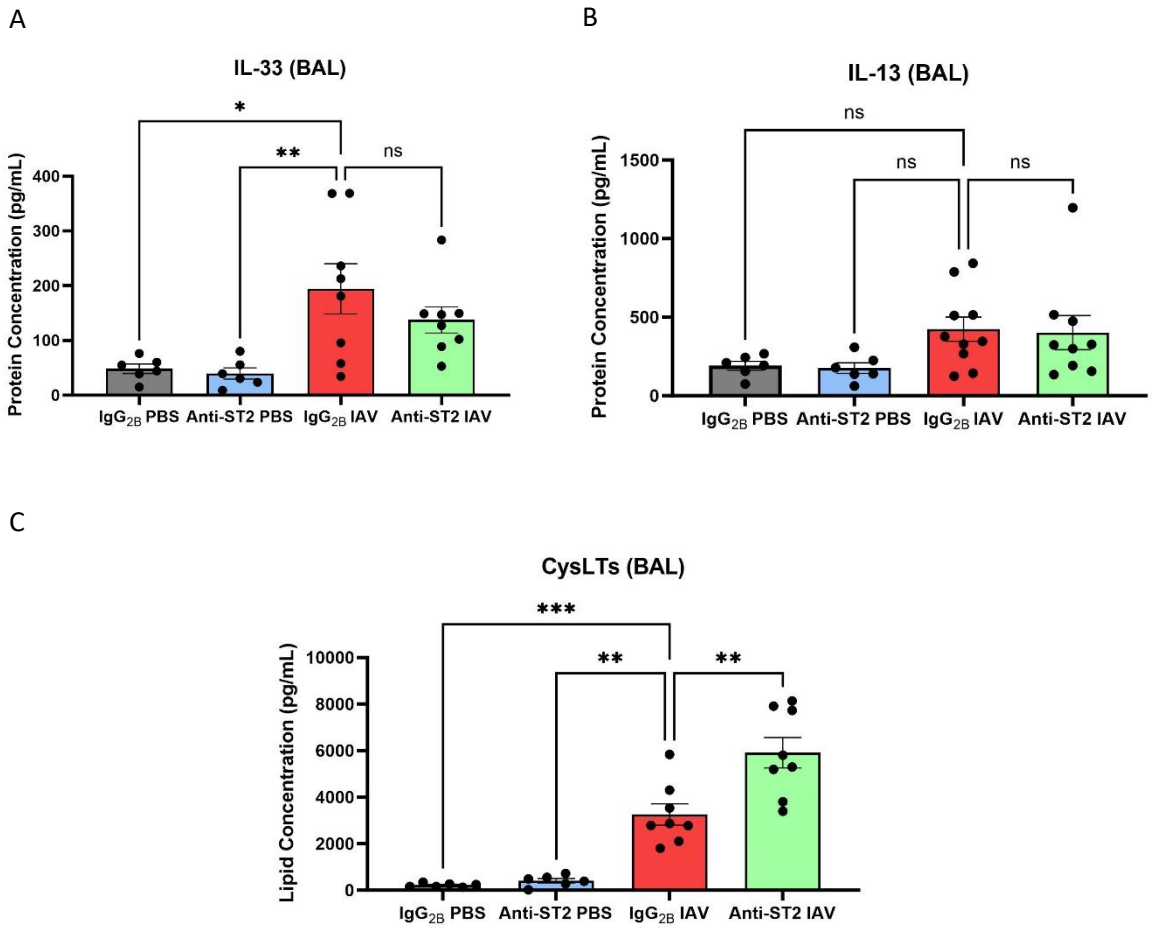


Figure 3.5: **Quantification of IL-33, IL-13 and CysLTs in BAL fluid of anti-ST2 treated IAV mice**. A. IL-33, B. IL-13 and C. CysLTs for IgG_{2B} isotype (n=6), anti-ST2 (n=6) PBS groups and isotype-treated (n=8-10) and anti-ST2-treated (n=8-9) IAV groups from BAL fluid supernatant at 3 dpi. *p<0.05 and **p<0.01 indicate statistical significance between isotype-treated PBS and IAV groups and anti-ST2-treated PBS and IAV groups accordingly for IL-33. **p<0.01 indicates statistical significance between anti-ST2-treated PBS, isotype-treated IAV groups and isotype-treated IAV and anti-ST2-treated IAV groups. ***p<0.001 indicates statistical significance between isotype PBS and IAV groups. Repeated measures ANOVA with Bonferroni's post hoc test was used to assess statistical significance. Data are shown as mean ± SEM.

3.6 Outcome of anti-IL-13 treatment on IAV-induced responsiveness to methacholine and viral load at 3 dpi

Treatment with anti-IL-13 during IAV infection did not affect total respiratory system resistance induced by methacholine but further increased total respiratory system elastance. There was no significant difference in resistance values for any of the methacholine doses between isotype IgG1 and anti-IL-13 (αIL-13) IAV-infected groups (figure 3.6A). No

statistical significance was observed in elastance values between both isotype and αIL-13 infected groups from 0 to 25 mg/mL (inclusive). At 50 mg/mL of methacholine, αIL-13 treatment further elevated total respiratory elastance significantly compared to its isotype group. There was no significant difference in viral load (PFU/lung) between isotype and anti-IL-13 infected groups (figure 3.6B).

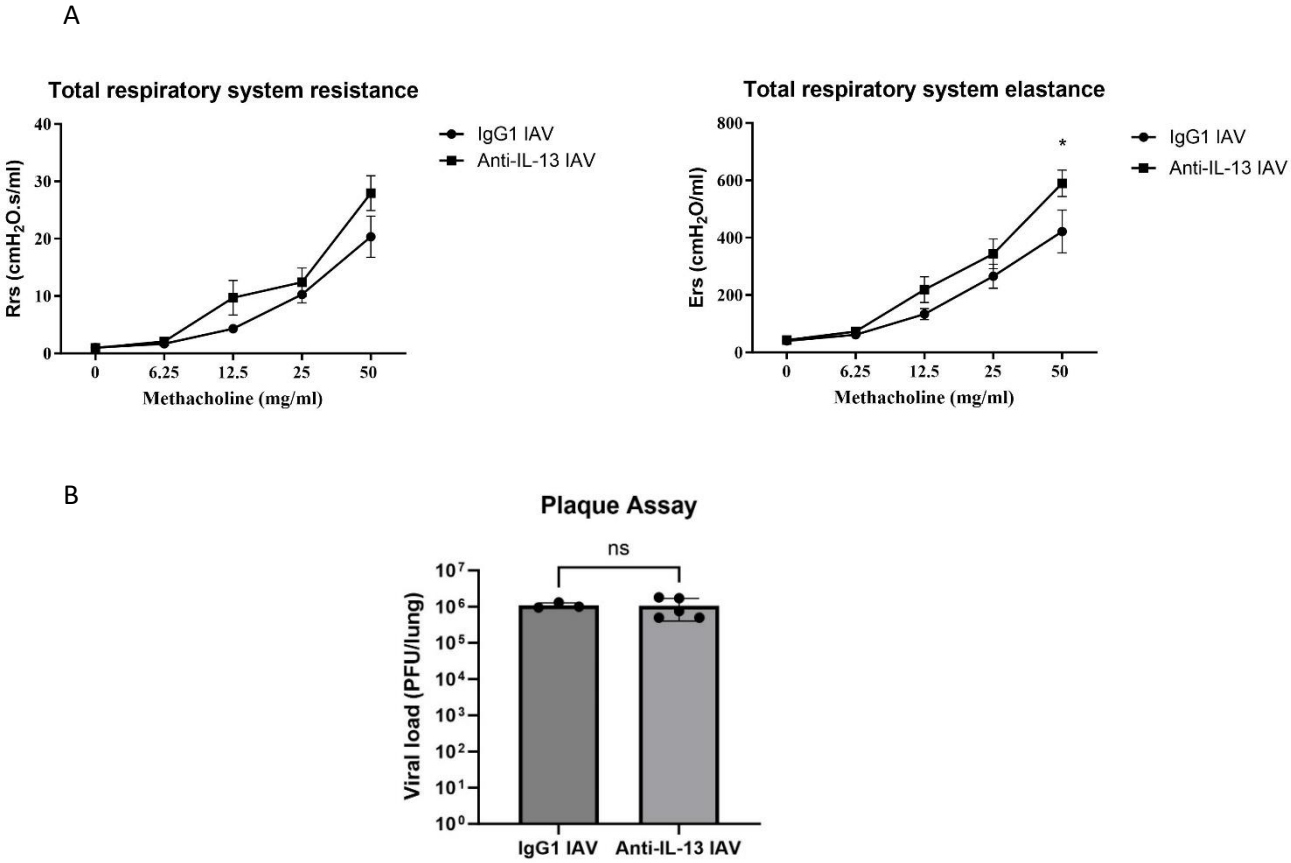
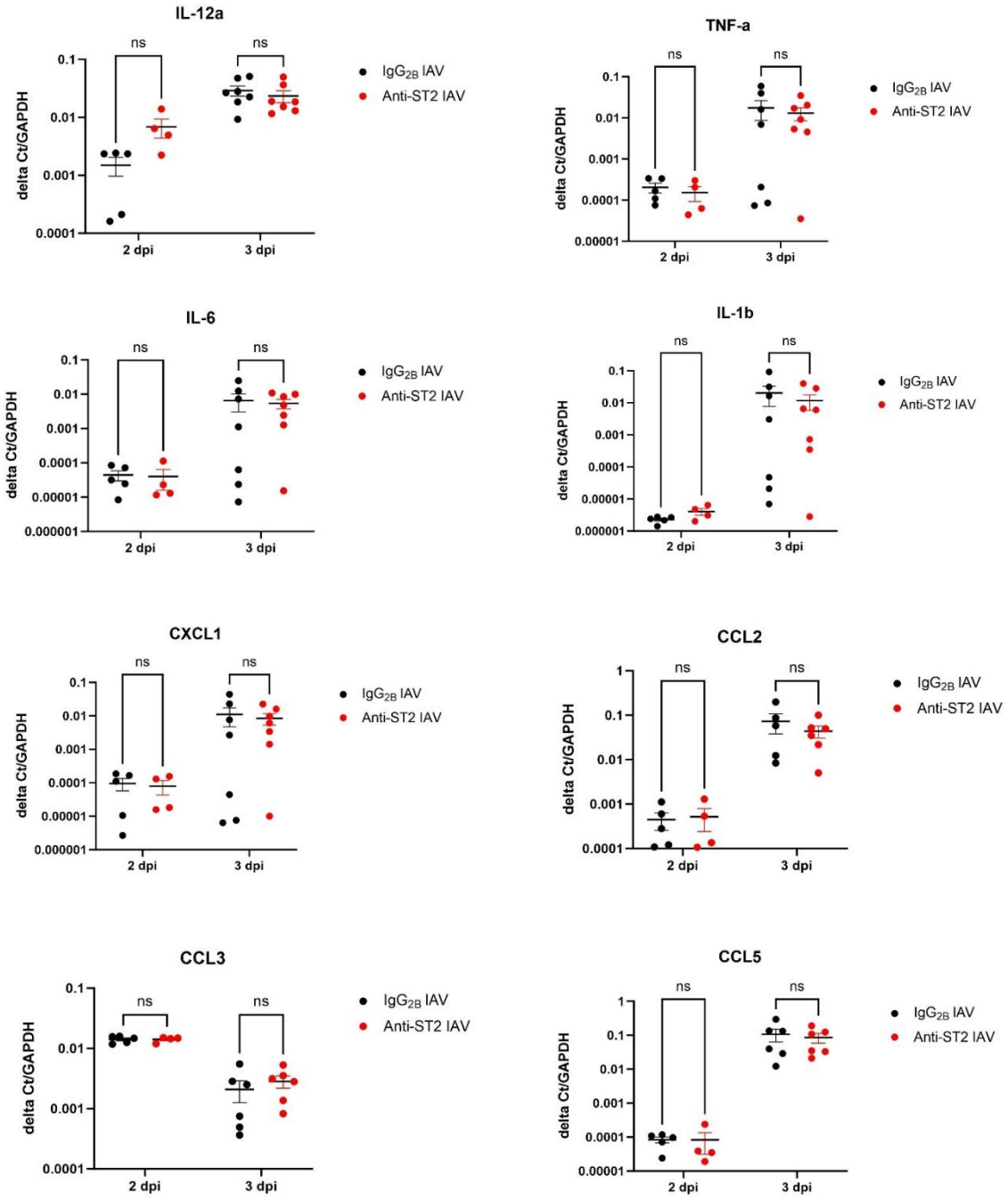


Figure 3.6: **Respiratory mechanics and viral load examination of anti-IL-13 IAV mice**. A. Concentration-response curves of total respiratory resistance (Rrs) and elastance (Ers) for IgG1 isotype and anti-IL-13 IAV infected groups at 3 dpi. Rrs and Ers values are plotted for methacholine challenges from 0 to 50 mg/mL. Values at each dose are represented as mean ± SEM for each group (n=5). *$p<0.05$ represents statistical significance between IgG1 control antibody-treated and anti-IL-13 groups. Two-way ANOVA with Bonferroni's post hoc test was used for both plots. B. Viral load (PFU/lung) assessed by plaque assay for IgG1 isotype (n=3) and anti-IL-13 (n=5) infected groups are represented. Mann-Whitney U test was used to assess statistical significance in viral load between both groups.

3.7 Effect of anti-ST2 treatment on expression of pro-inflammatory cytokines, inflammatory cell-mediating chemokines in whole lung homogenates at 2 and 3 dpi

IAV stimulates the synthesis of pro-inflammatory cytokines and chemokines during the acute phase of the infection. IAV IgG_{2B} isotype-treated groups showed considerably greater expression of cytokines (IL-12a, TNFα, IL-6, IL-1β) and chemokines (mouse ortholog CXCL1, CCL2, CCL5, CCL7 and CCL22) at 3 dpi compared to 2 dpi, with the exception of CCL3 whose expression levels were higher on 2 dpi. Treatment with anti-ST2 did not significantly change the level of expression of any of the aforementioned cytokines or chemokines at either 2 or 3 dpi of IAV infection.



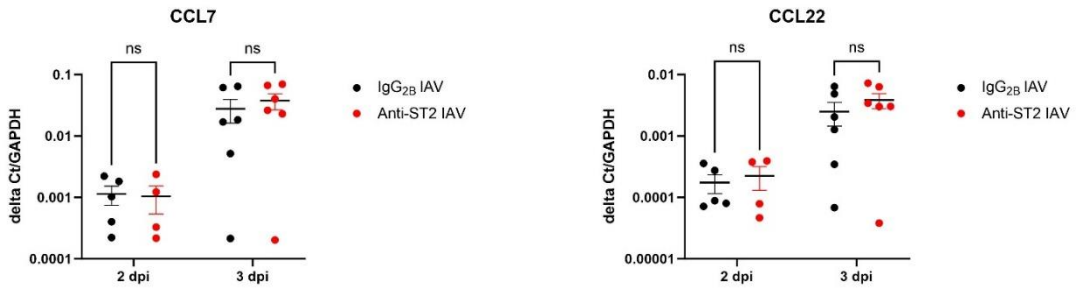


Figure 3.7: **Evaluation of anti-ST2 on gene expression of IAV-induced inflammatory mediators**. mRNA quantification represented as delta Ct values on a $\log_{10}$ scale (normalized to GAPDH) for inflammatory cytokines (IL-12a to IL-1β inclusive) and chemokines (CXCL1 to CCL22 inclusive) in response to IAV infection compared between IgG_{2B} and anti-ST2 groups on 2 and 3 dpi. Sample sizes of n=5 and n=4 for isotype and anti-ST2 respectively are compared on 2 dpi and sample sizes of n=5-7 and n=6-7 for isotype and anti-ST2 accordingly are compared. Two-way ANOVA with Bonferroni's post hoc test was used to assess statistical significance. Delta Ct values are represented as mean ± SEM.

3.8 Outcome of anti-Ly6G antibody administration on differential cell count of BAL fluid at 3 dpi

Neutrophils are the dominant innate cells present in the BAL fluid during acute IAV infection and the effects of IL-33 on neutrophils in the context of IAV-induced AHR hasn't previously been described. Therefore, the contribution of neutrophils to total airway resistance and elastance during IAV infection was examined.

Administration of anti-Ly6G antibody significantly depleted neutrophils during IAV infection. Both IgG2a isotype-treated and anti-Ly6G PBS groups showed a lower total cell count with the primary immune cell, macrophages not being different between the PBS groups. Significant increases in total cell count and neutrophils were observed in the isotype-treated IAV-infected mice compared to the isotype-treated PBS vehicle group. No significant increases in macrophages or lymphocytes were present with IAV infection. Compared to its IgG2a IAV control, anti-Ly6G reduced the number of mature neutrophils in the BAL fluid during IAV infection thereby also reducing the total cell count of inflammatory cells in the BAL fluid.

Examination of the BAL fluid further revealed that anti-Ly6G did not affect the numbers of macrophages or lymphocytes during IAV infection.

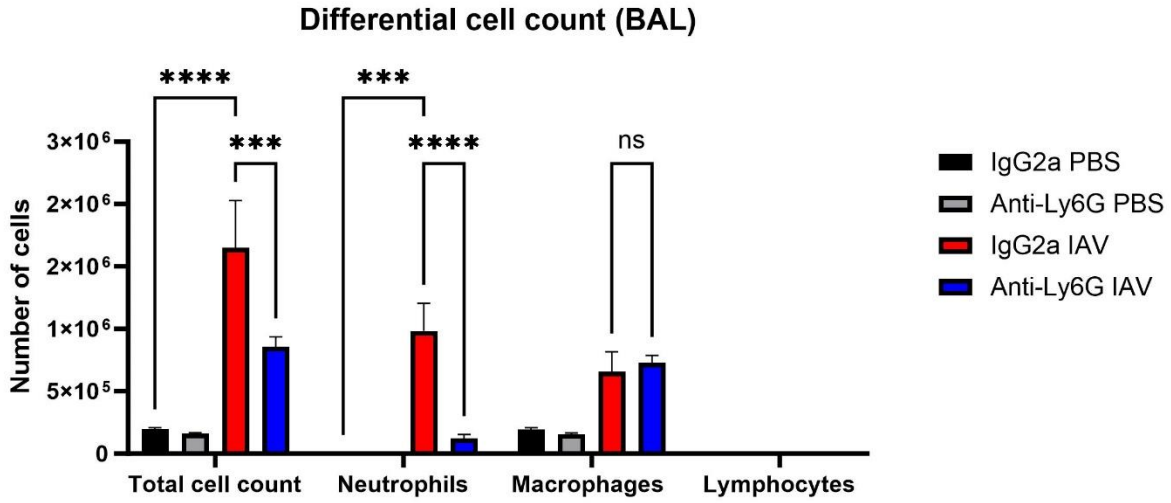


Figure 3.8: **Differential cell count on BAL cells of anti-Ly6G treated IAV mice**. Total cell count and differential cell count for neutrophils, macrophages and lymphocytes in BAL fluid 3 dpi for IgG2a isotype-treated (n=3), anti-ST2-treated (n=3) PBS groups and isotype-treated (n=5) and anti-ST2-treated (n=5) IAV groups. ****p<0.0001 and ***p=0.0001 indicates statistical significance between isotype PBS and IAV groups for total cell count and neutrophils respectively. ***p=0.0001 and ****p<0.0001 indicates statistical significance between isotype IAV and anti-Ly6G IAV groups for total cell count and neutrophils accordingly. Two-way ANOVA with Bonferroni's post hoc test was used to assess statistical significance. Cell count numbers are shown as mean ± SEM.

3.9 Effect of anti-Ly6G antibody on IAV NS1 expression and viral load at 3 dpi

Assessment of viral NS1 expression between isotype and anti-Ly6G infected groups indicated a lack of statistical significance in the difference in mRNA expression between both groups. In addition, anti-Ly6G did not affect the viral load. Determination of viral concentration, by plaque assay showed no significant difference in PFUs per lung between isotype and anti-Ly6G infected groups.

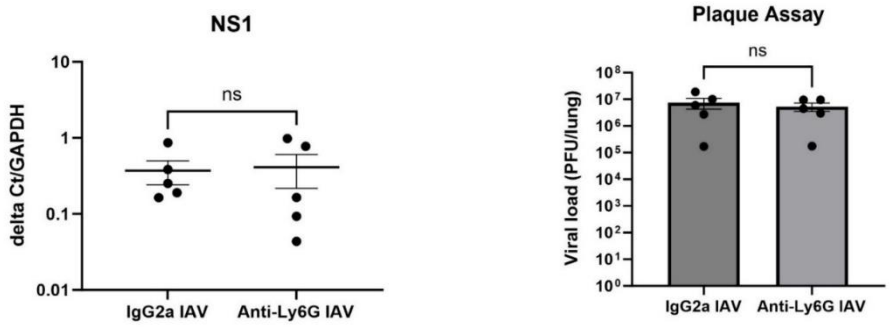


Figure 3.9: **NS1 expression and viral load assessment in anti-Ly6G treated IAV mice**. qPCR analysis of NS1 expression (n=5 per group) and viral load assessed by plaque assay (n=5 per group) for isotype and anti-Ly6G infected groups represented on a $\log_{10}$ scale. Non-parametric t tests were used to assess for statistical significance. Delta Ct and viral load values are shown as mean ± SEM.

3.10 Determination of effect of anti-Ly6G on IAV-induced responsiveness to methacholine at 3 dpi

A significant increase in the total respiratory system resistance and elastance was measured at 50 mg/mL, and 25, 50 mg/mL of methacholine respectively between isotype PBS-treated and IAV-infected groups. No differences in respiratory mechanics were observed when comparing both isotype-treated and anti-Ly6G-treated PBS groups. Following anti-Ly6G treatment, the AHR reflected in total respiratory resistance and elastance did not change significantly in presence of IAV infection at any of the concentrations of aerosolized methacholine.

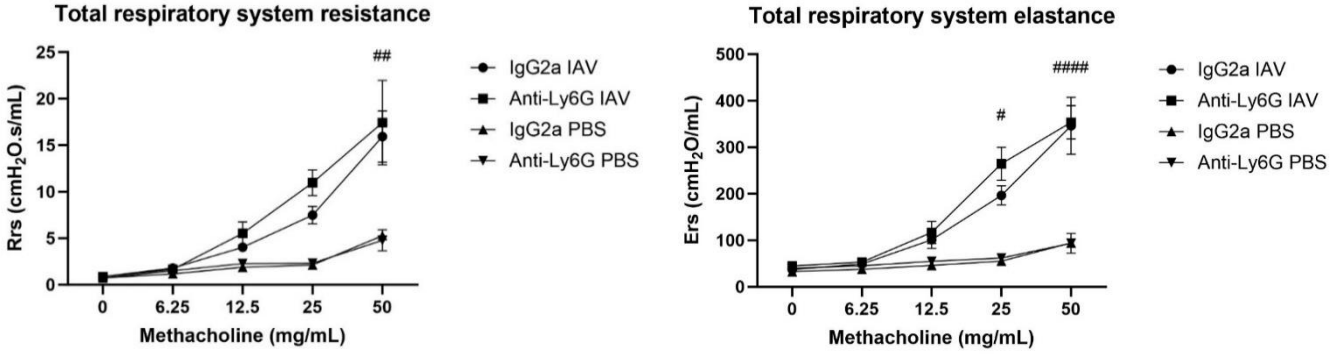


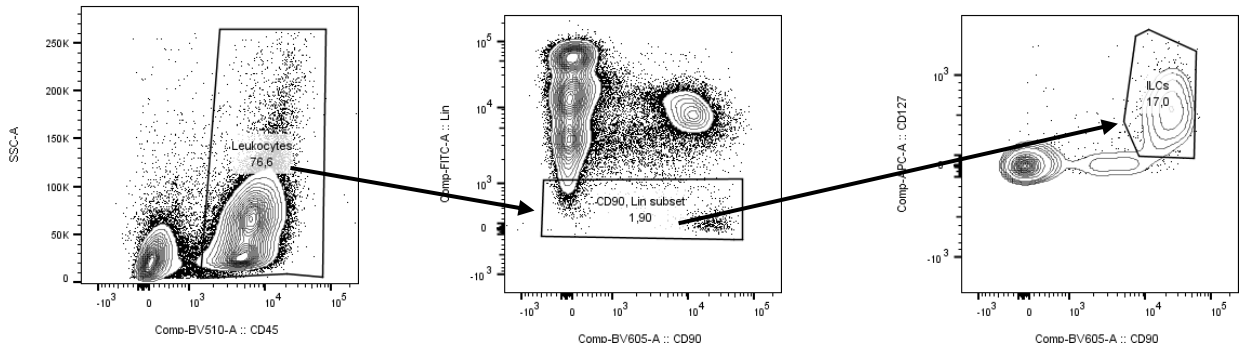
Figure 3.10: **Respiratory mechanics of anti-Ly6G treated IAV mice**. Concentration-response curves of total respiratory resistance (Rrs) and elastance (Ers) for IgG2a isotype-treated and anti-Ly6G-treated IAV groups and their respective PBS groups at 3 dpi.

Corresponding Rrs and Ers values are plotted for methacholine challenges from 0 to 50 mg/mL. Values at each concentration are represented as mean ± SEM for IAV infected (n=5) and for PBS groups (n=3). #p<0.05, ##p<0.01 and ####p<0.0001 represent statistical significance between IgG2a IAV and IgG2a PBS groups. Two-way ANOVA with Bonferroni's post hoc test was used to assess for differences.

3.11 Quantification of total lung ILCs following anti-ST2 treatment at 3 dpi

The gating strategy for isolating ILCs from IAV infected lungs was as follows: $CD45^+$ lineage-negative ($CD3\varepsilon^-$, $CD5^-$, $CD11b^-$, $CD11c^-$, Ly6G/Ly6C (Gr-1)$^-$, Ter-119$^-$, CD45R/B220$^-$, $TCR\gamma/\delta^-$, $TCR\beta^-$), $CD90.2^+$, $CD127^+$ leukocytes. Both vehicle controls showed no difference in the presence of ILCs in the percentage of cells (standardized to live cells) and in absolute number of cells. IAV infection did not produce an increment in the percentage or absolute number of ILCs. Furthermore, both the percentage of cells and absolute numbers were significantly reduced following IAV infection. Comparison of both percentage and absolute number of cells of ILCs between isotype and anti-ST2 infected groups indicated no significant difference.

A



B

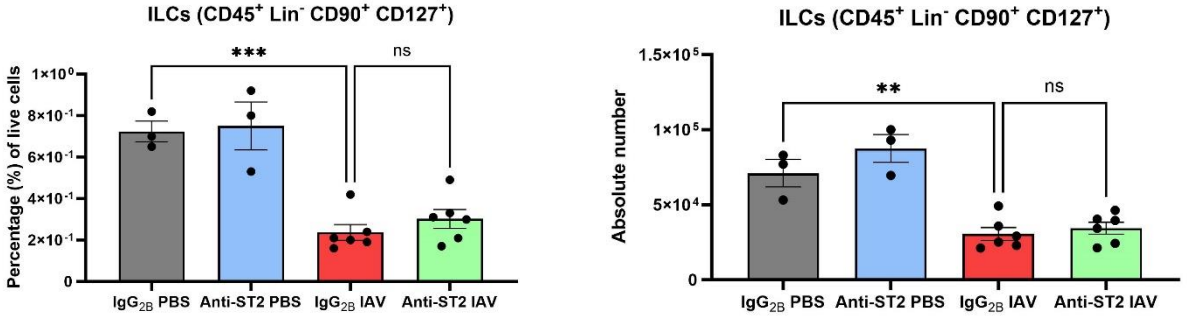


Figure 3.11: **Assessment of lung ILCs in anti-ST2 treated IAV mice**. A. Gating strategy for isolating lung ILCs. ILCs are gated following isolation of leukocytes followed by gating on cells that were lineage$^-$, CD90$^+$ and CD127$^+$. The percentage of cells within each gate is shown. SSC-A, side scatter area. B. ILC population, both as a percentage normalized to live cells (SSC-A$^+$ Viability$^-$ gating not shown) and absolute numbers for IgG_{2B}-treated and anti-ST2-treated both as PBS controls (n=3 per group) and IAV infected (n=6 per group). One-way ANOVA with Bonferroni's post hoc test was utilized for statistical analyses. **p<0.01 and ***p<0.001 indicate statistical significance in percentage and absolute number respectively, comparing isotype-treated PBS and IAV groups. Percentage and absolute number of cells are represented as mean ± SEM.

3.12 Effects of anti-ST2 on alveolar, interstitial and monocyte-derived macrophages populations at 3 dpi

The gating strategy for isolating alveolar macrophages from lungs was as follows: gating on CD45$^+$ CD11c$^+$, CD11b$^-$, CD64$^+$, F4/80$^+$ leukocytes. CD45$^+$, CD11b$^+$, SiglecF$^-$, F4/80$^+$ and Ly6C$^-$ and Ly6C$^+$ for interstitial and monocyte-derived macrophages based on Ly6C FMO gating (figure 3.12A).

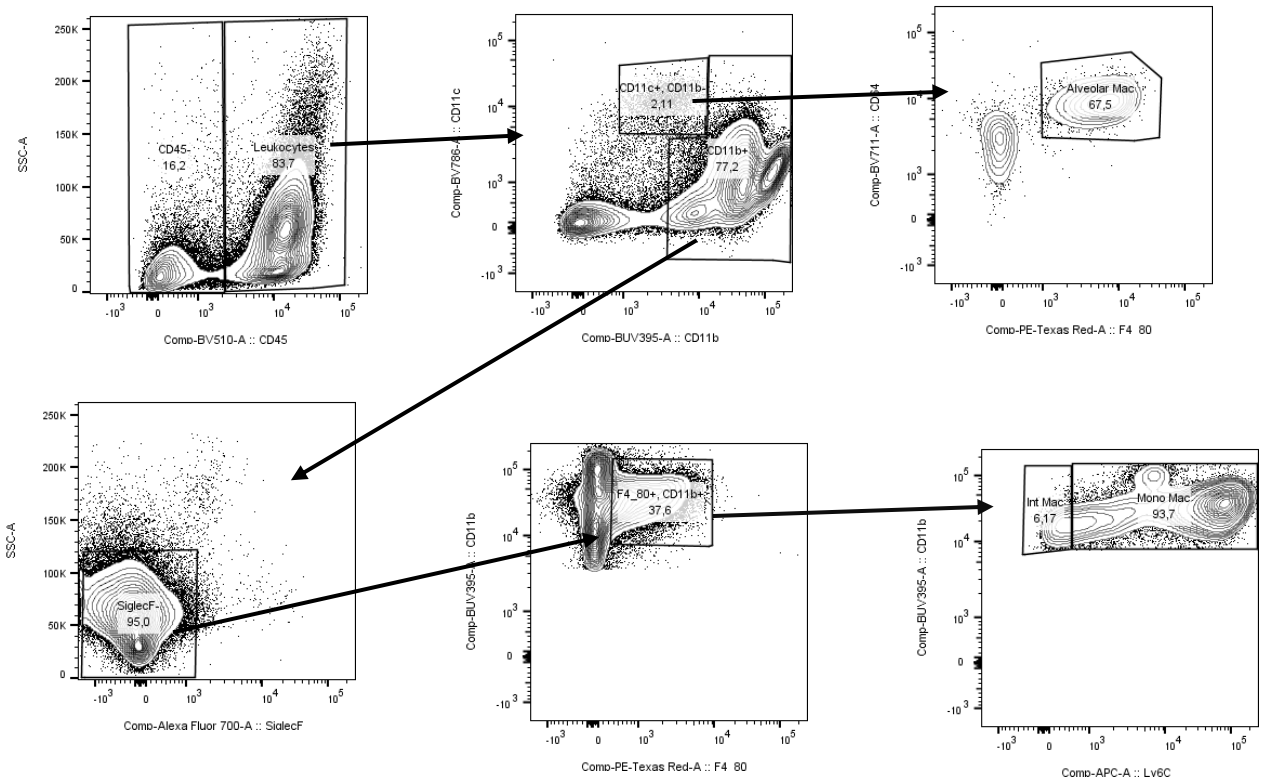
Based on this gating strategy, there was no difference in the alveolar macrophage populations in either IgG_{2B} isotype-treated and anti-ST2-treated PBS groups. Absolute numbers of alveolar macrophages in the isotype-treated IAV group were significantly lower when compared to its isotype-treated PBS group. There was no significant difference comparing alveolar macrophage population numbers between isotype-treated and anti-ST2-treated IAV groups (figure 3.12B).

Isotype-treated and anti-ST2-treated PBS groups showed no difference in the interstitial macrophage population in the lung. Absolute numbers of interstitial macrophages in the isotype IAV group showed a significant reduction when compared to its isotype-treated PBS group. The interstitial macrophage population was not affected by anti-ST2 treatment during IAV infection. Both isotype and anti-ST2-treated PBS groups showed no substantial change in interstitial macrophage M1 and M2 subpopulations. IAV infection only significantly decreased

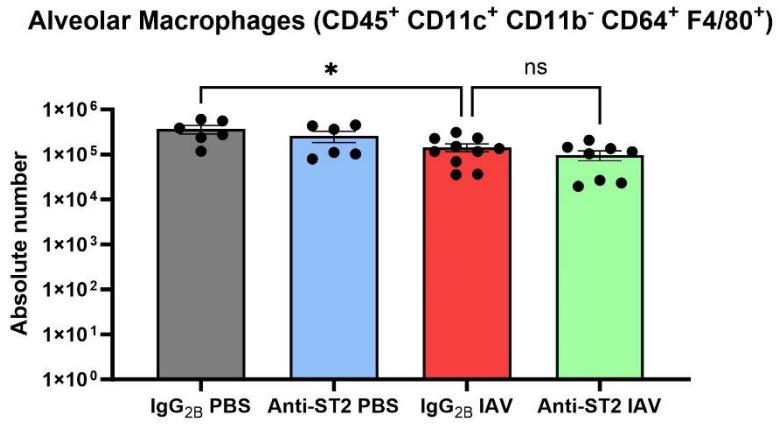
CD206 expressing interstitial macrophages without affecting on other populations, namely CD80, iNOS and arginase 1 expressing interstitial macrophages. There was a significant decrease in the number of CD206 interstitial macrophages in isotype-treated IAV-infected mice compared to isotype-treated vehicle control. However, there was no statistically significant change in the number of any surface and intracellular M1 and M2 markers between isotype- and anti-ST2-treated IAV groups (figure 3.12C).

Both IgG_{2B} and anti-ST2-treated PBS groups showed no difference in numbers of monocyte-derived macrophages. A significantly higher number of monocyte-derived macrophages was observed when comparing IgG_{2B} isotype-treated and IAV groups. Following anti-ST2 treatment during IAV infection, there was a significant decrease in the number of monocyte-derived macrophages when comparing between isotype-treated and anti-ST2-treated IAV groups. Both IgG_{2B} and anti-ST2-treated PBS groups showed no difference in monocyte-derived macrophage M1 and M2 subpopulation numbers. With IAV infection, monocyte-derived macrophages expressing CD80 were increased without any changes to CD206, iNOS and arginase 1 expressing populations. There was a significant increase in $CD80^+$ cells when comparing isotype vehicle and IAV groups. Anti-ST2 treatment during IAV infection showed no significant change to CD80, iNOS and arginase 1 expressing monocyte-derived macrophages. However, a statistically significant decrease in number of $CD206^+$ monocyte-derived macrophages was observed when comparing isotype and anti-ST2-treated IAV groups (figure 3.12D).

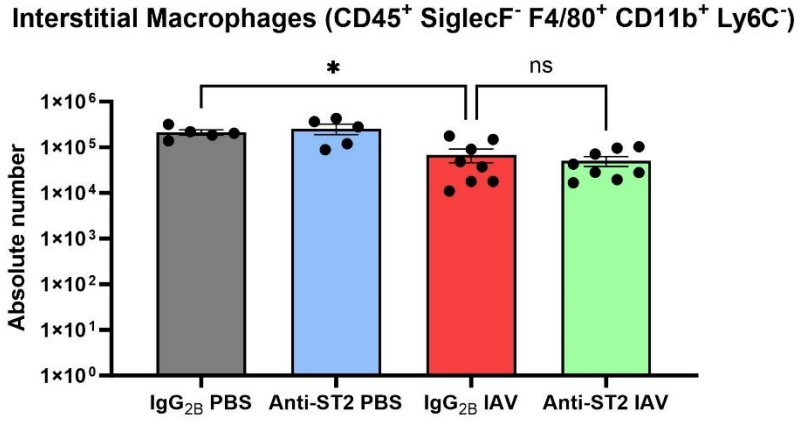
A

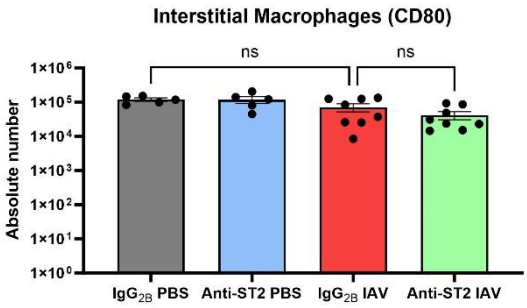

SSC-A
CD45-
16,2
Leukocytes
83,7
Comp-BV510-A :: CD45
Comp-BV786-A :: CD11c
CD11c+, CD11b-
2,11
CD11b+
77,2
Comp-BUV395-A :: CD11b
Comp-BV711-A :: CD64
Alveolar Mac
67,5
Comp-PE-Texas Red-A :: F4 80
SSC-A
SiglecF-
95,0
Comp-Alexa Fluor 700-A :: SiglecF
Comp-BUV395-A :: CD11b
F4_80+, CD11b+
37,6
Comp-PE-Texas Red-A :: F4 80
Comp-BUV395-A :: CD11b
Int Mac
6,17
Mono Mac
93,7
Comp-APC-A :: Ly6C

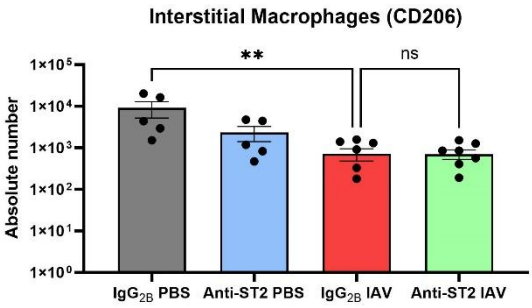
B


Alveolar Macrophages (CD45+ CD11c+ CD11b- CD64+ F4/80+)
*
ns
Absolute number
1×10⁶
1×10⁵
1×10⁴
1×10³
1×10²
1×10¹
1×10⁰
IgG2B PBS
Anti-ST2 PBS
IgG2B IAV
Anti-ST2 IAV

C


Interstitial Macrophages (CD45+ SiglecF- F4/80+ CD11b+ Ly6C-)
*
ns
Absolute number
1×10⁶
1×10⁵
1×10⁴
1×10³
1×10²
1×10¹
1×10⁰
IgG2B PBS
Anti-ST2 PBS
IgG2B IAV
Anti-ST2 IAV


Interstitial Macrophages (CD80)
ns
ns
Absolute number
1×10⁶
1×10⁵
1×10⁴
1×10³
1×10²
1×10¹
1×10⁰
IgG2B PBS
Anti-ST2 PBS
IgG2B IAV
Anti-ST2 IAV


Interstitial Macrophages (CD206)
**
ns
Absolute number
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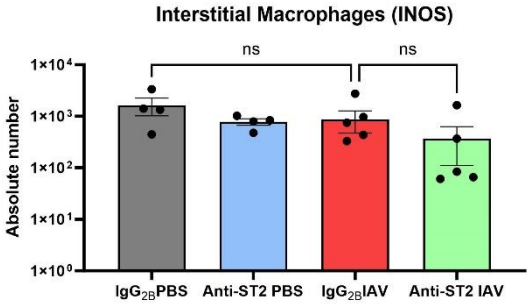
1×10⁴
1×10³
1×10²
1×10¹
1×10⁰
IgG2B PBS
Anti-ST2 PBS
IgG2B IAV
Anti-ST2 IAV


Interstitial Macrophages (INOS)
ns
ns
Absolute number
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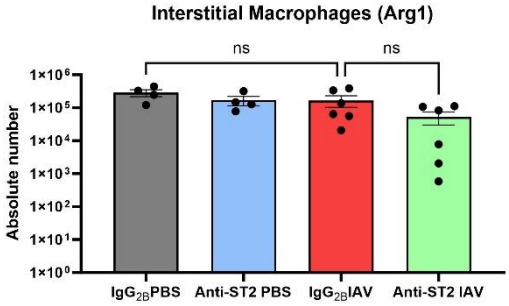
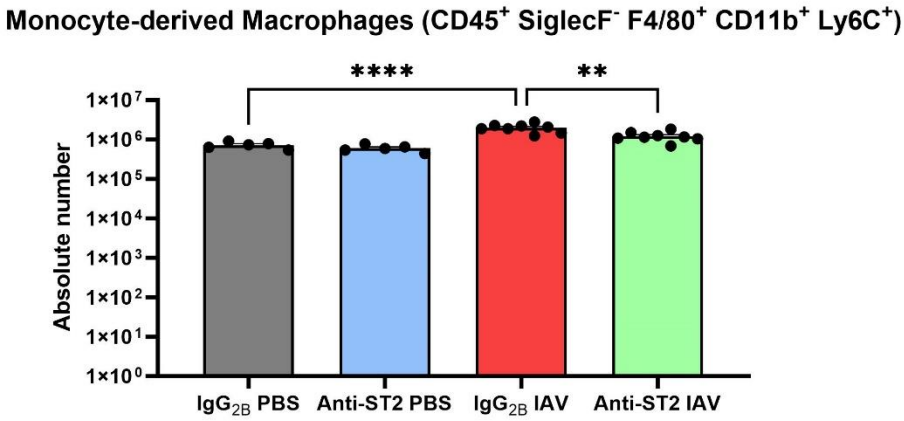
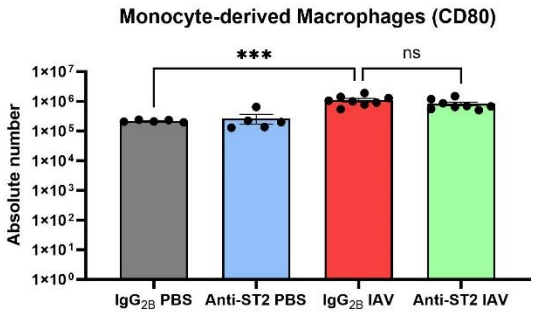
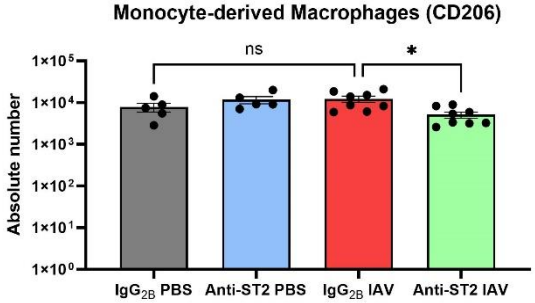
1×10³
1×10²
1×10¹
1×10⁰
IgG2BPBS
Anti-ST2 PBS
IgG2BIAV
Anti-ST2 IAV


Interstitial Macrophages (Arg1)
ns
ns
Absolute number
1×10⁶
1×10⁵
1×10⁴
1×10³
1×10²
1×10¹
1×10⁰
IgG2BPBS
Anti-ST2 PBS
IgG2BIAV
Anti-ST2 IAV

D


Monocyte-derived Macrophages (CD45+ SiglecF- F4/80+ CD11b+ Ly6C+)
****
**
Absolute number
1×10⁷
1×10⁶
1×10⁵
1×10⁴
1×10³
1×10²
1×10¹
1×10⁰
IgG2B PBS
Anti-ST2 PBS
IgG2B IAV
Anti-ST2 IAV


Monocyte-derived Macrophages (CD80)
***
ns
Absolute number
1×10⁷
1×10⁶
1×10⁵
1×10⁴
1×10³
1×10²
1×10¹
1×10⁰
IgG2B PBS
Anti-ST2 PBS
IgG2B IAV
Anti-ST2 IAV


Monocyte-derived Macrophages (CD206)
ns
*
Absolute number
1×10⁵
1×10⁴
1×10³
1×10²
1×10¹
1×10⁰
IgG2B PBS
Anti-ST2 PBS
IgG2B IAV
Anti-ST2 IAV

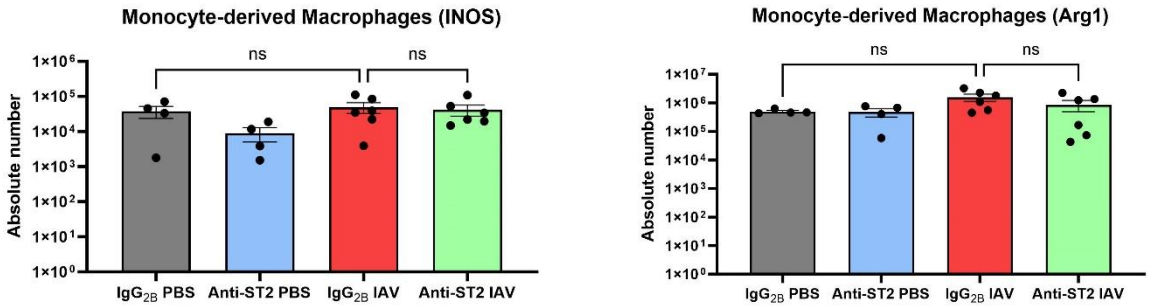


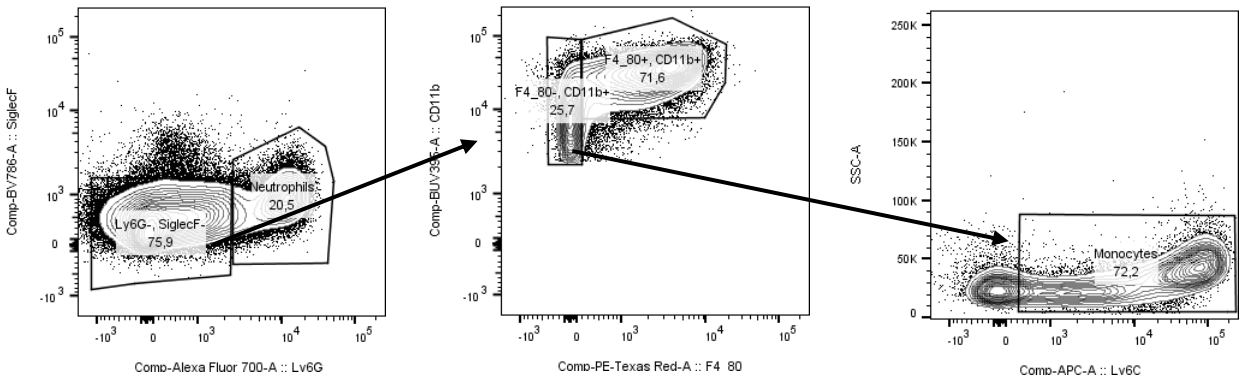
Figure 3.12: **Assessment of lung macrophage populations in anti-ST2 treated IAV mice**. A. The gating strategy for isolating lung macrophage populations. Macrophages were gated following isolation of leukocytes followed by gating on cells that are $CD11c^+$, $CD11b^-$ and double-positive for CD64 and F4/80 for alveolar macrophages ('Alveolar Mac'). $CD11b^+$ only leukocytes which were negative for SiglecF and double-positive for F4/80 and CD11b were isolated. The $CD11b^+$ $SiglecF^-$ $F4/80^+$ leukocytes were assessed for Ly6C expression. The lack of expression of Ly6C was deemed to represent interstitial macrophages ('Int Mac') and $Ly6C^+$ cells were defined as monocyte-derived macrophages ('Mono Mac'). B. Alveolar macrophage absolute numbers for IgG_{2B} isotype-treated (n=6), anti-ST2-treated (n=6) PBS and isotype-treated (n=10), anti-ST2-treated (n=8) IAV groups. *p<0.05 shows statistical significance between IgG_{2B}-treated PBS and IAV groups. C. Interstitial macrophage absolute numbers, CD80, CD206, iNOS and arginase 1 (Arg1) for isotype-treated (n=4-5), anti-ST2-treated (n=4-5) PBS and isotype-treated (n=6-8), anti-ST2-treated (n=6-8) IAV groups. **p<0.01 indicates statistical significance between IgG_{2B}-treated PBS and IgG_{2B}-treated IAV groups for CD206 expression. D. Monocyte-derived macrophage absolute numbers, CD80, CD206, iNOS and arginase 1 (Arg1) for isotype-treated (n=4-5), anti-ST2-treated (n=4-5) PBS and isotype-treated (n=6-8), anti-ST2-treated (n=6-8) IAV groups. ***p<0.001 and ****p<0.0001 indicates statistical significance between IgG_{2B}-treated PBS and IAV groups for CD80 expression and between IgG_{2B}-treated IAV and anti-ST2-treated IAV groups for monocyte-derived macrophage numbers, respectively. *p<0.05 and **p<0.01 indicates statistical significance between IgG_{2B}-treated PBS and IgG_{2B}-treated IAV groups for CD206 expression and IgG_{2B}-treated IAV and anti-ST2-treated IAV groups for monocyte-derived macrophage numbers, respectively. Statistical significance was assessed by one-way ANOVA with Bonferroni's post hoc test. Absolute number of cells are represented as mean ± SEM.

3.13 Effect of anti-ST2 on recruited $F4/80^-$ $Ly6C^+$ lung monocyte population

The gating strategy for $F4/80^-$ $Ly6C^+$ monocytes isolated from the lungs was as follows: following isolation of $CD45^+$ $CD11b^+$ leukocytes, $Ly6G^+$ neutrophils were gated out and cells that were SiglecF- were isolated. $CD45^+$ $CD11b^+$ $SiglecF^-$ cells negative for F4/80 and positive for Ly6C (based on Ly6C FMO gating) were quantified.

Both IgG_{2B} isotype-treated and anti-ST2-treated PBS groups showed no significant difference in absolute number of monocytes. Comparison of isotype-treated PBS and IAV groups showed a significant increase. F4/80$^-$ Ly6C$^+$ monocyte population was not significantly different between isotype-treated and anti-ST2-treated IAV groups (figure 3.13B).

A



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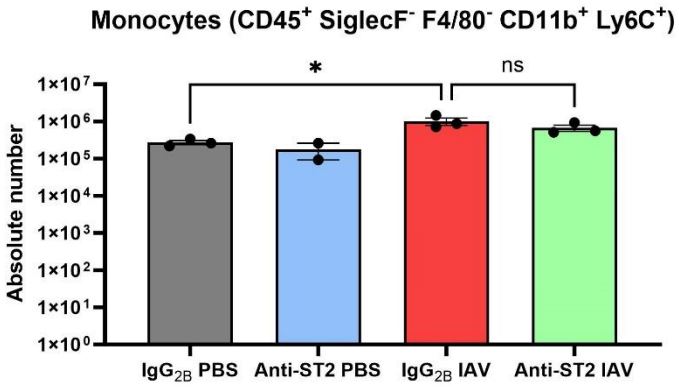


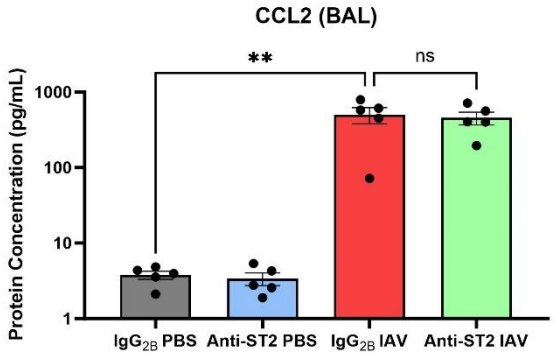
Figure 3.13: **Assessment of lung-recruited monocytes in anti-ST2 treated IAV mice**. Gating strategy for isolating F4/80$^-$ Ly6C$^+$ monocytes. Following isolation of CD45$^+$ CD11b$^+$ leukocytes, cells were assessed for SiglecF and Ly6G expression. Ly6G$^-$ SiglecF$^-$ cells were isolated which were further isolated based on negative expression for F4/80. CD45$^+$ CD11b$^+$ SiglecF$^-$ F4/80$^-$ leukocytes positive for Ly6C were deemed to be recruited monocytes from the circulation. B. F4/80$^-$ Ly6C$^+$ monocytes absolute numbers for isotype-treated (n=3), anti-ST2-treated (n=2) PBS and isotype-treated (n=3), anti-ST2-treated (n=3) IAV groups at 3 dpi are compared. One-way ANOVA with Bonferroni's correction post hoc test was utilized for statistical analysis. *$p<0.05$ and indicates statistical significance between IgG_{2B} isotype-treated PBS and IAV groups. Absolute number of cells are represented as mean ± SEM.

3.14 Evaluation of anti-ST2 treatment on CCL2 BAL levels and expression of whole lung monocytic differentiation factors at 3 dpi

To support flow cytometric analysis of recruited monocytes (figure 3.13), a key monocyte recruiting CCL2 chemokine was assessed. Both IgG_{2B} isotype-treated and anti-ST2-treated PBS groups showed no significant difference in CCL2 levels in BAL fluid. There is a statistically significant increase in CCL2 protein levels comparing isotype-treated PBS and IAV groups. Anti-ST2 treatment had no effect on CCL2 protein levels as evidenced by the comparison of CCL2 levels between isotype-treated and anti-ST2-treated IAV groups that showed no significant difference (figure 3.14A).

Anti-ST2 treatment during IAV infection increased expression of the monocytic differentiation factor, c-maf and m-CSF (Csf1). IgG_{2B} isotype-treated groups showed considerably greater expression of c-maf and Csf1 when comparing between 2 and 3 dpi. At 3 dpi, there was a significant increase in expression of both genes between isotype-treated and anti-ST2-treated IAV groups (figure 3.14B).

A



B

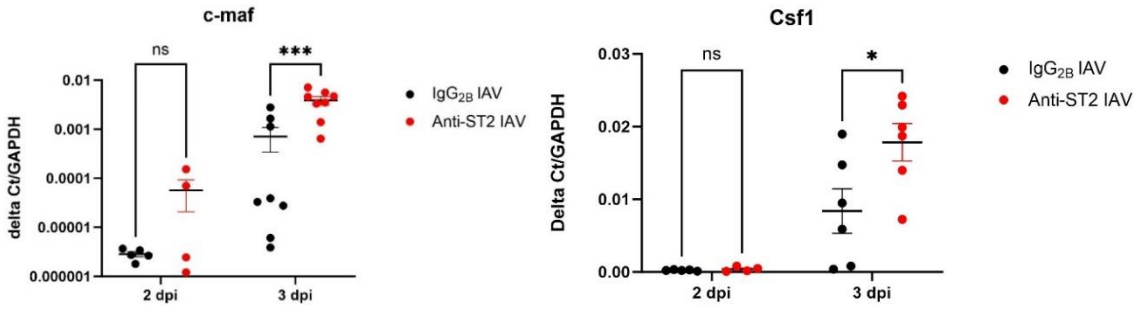


Figure 3.14: **BAL fluid CCL2 levels and expression of monocytic differentiation factors in anti-ST2 treated IAV mice**. A. ELISA for CCL2 for isotype-treated (n=5), anti-ST2-treated (n=5) PBS groups and isotype-treated (n=5) and anti-ST2-treated (n=5) IAV groups from BAL fluid supernatant at 3 dpi on a $\log_{10}$ scale. One-way ANOVA with Bonferroni's correction post hoc test was utilized for statistical analysis. **$p<0.01$ and indicates statistical significance between isotype-treated PBS and IAV groups. ELISA values are presented as mean ± SEM. B. mRNA quantification represented as delta Ct values on a $\log_{10}$ scale (normalized to GAPDH) for c-maf and Csf1 in response to IAV infection compared between IgG_{2B} isotype-treated (n=5-8) and anti-ST2-treated (n=4-8) groups on 2 and 3 dpi. Two-way ANOVA with Bonferroni's post hoc test was used to assess statistical significance. *$p<0.05$ and ***$p<0.001$ indicates statistical significance between isotype-treated IAV and anti-ST2-treated IAV groups. Delta Ct values are represented as mean ± SEM.

3.15 Quantification of ST2 expression between macrophage populations and $CD45^-$ cells during IAV infection at 3 dpi

Expression of the ST2 receptor was assessed on alveolar, interstitial, monocyte-derived macrophages and $CD45^-$ cells (including the airway epithelium) based on gating on ST2 FMO from whole lung digests for isotype IgG_{2B} IAV with isotype PBS for non-infected comparison at 3 dpi.

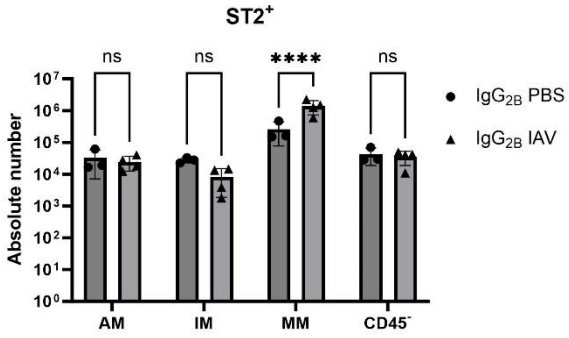
During IAV infection, monocyte-derived macrophages showed an increased level of ST2 expression which was not observed with other macrophage or $CD45^-$ populations. A statistically significant increase in $ST2^+$ cells for monocyte-derived macrophages was observed comparing isotype-treated PBS and IAV groups (figure 3.15A).

To further support expression of the ST2 receptor among macrophage populations and $CD45^-$ cells, histograms were also compared between IgG_{2B} isotype-treated PBS and IAV groups from two randomly chosen examples from each group. Both alveolar macrophages and interstitial macrophages isotype-treated PBS and IAV histograms showed an appreciable shift to the right compared to ST2 FMO. However, the isotype PBS and IAV groups' histograms could not be differentiated. For $CD45^-$ cells, both subject histograms did not shift relative to its ST2 FMO. Monocyte-derived macrophage isotype-treated PBS and IAV histograms show a shift to the

right compared to its ST2 FMO with isotype IAV further shifting to the right relative to the isotype sham group. With evidence from the comparison of histograms, the monocyte-derived macrophages increase expression for the ST2 receptor that is not observed with other macrophage and CD45$^-$ populations (figure 3.15B).

Furthermore, the absolute numbers of ST2$^+$ cells for each macrophage population and CD45$^-$ cells were compared to their respective total cell counts during IAV infection to better highlight the proportion of cells expressing the ST2 receptor (figure 3.15C). More than 50% of monocyte-derived macrophages showed expression of the ST2 receptor followed by interstitial macrophages, alveolar macrophages and lastly, the CD45$^-$ cells (Table 3.15).

A



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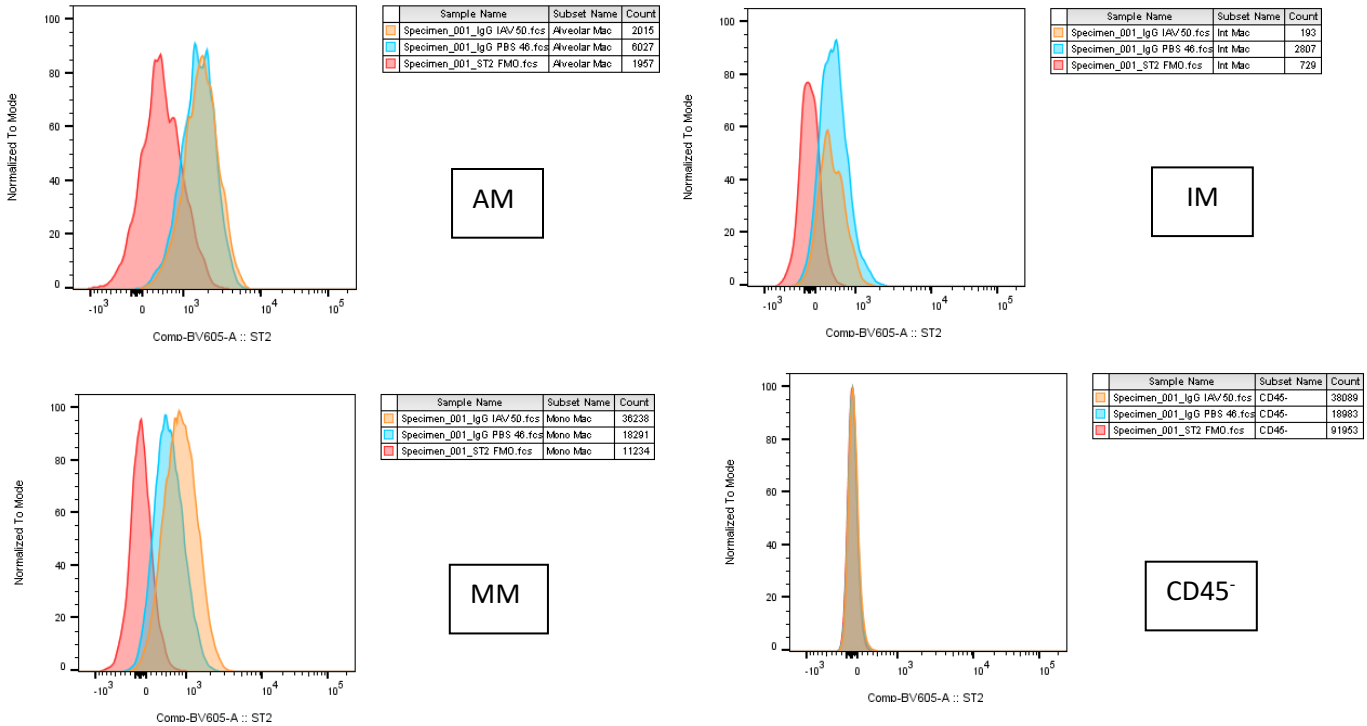
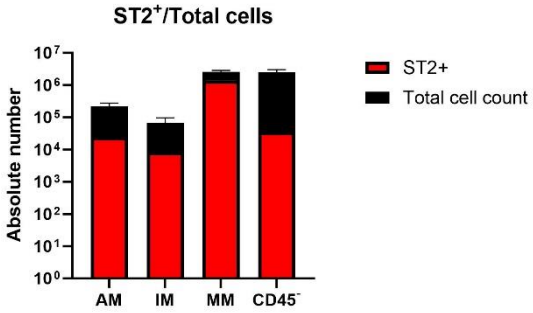


C



Cell type	ST2$^+$/Total cells (%)
AM	11.06
IM	12.30
MM	54.62
CD45$^-$	1.41

Figure 3.15: **Analysis of ST2 expression among macrophages and non-immune cells during IAV infection**. A. Absolute number of cells expressing ST2 receptor from alveolar (AM), interstitial (IM), monocyte-derived (MM) macrophages and CD45$^-$ cells for IgG_{2B} isotype-treated PBS (n=3) and IAV (n=4) groups on a log_{10} scale. ****p<0.0001 indicates statistical significance between isotype-treated PBS and IAV groups for MM. Two-way ANOVA with Bonferroni's correction post hoc test was utilized for statistical analysis. Absolute number of cells are represented as mean ± SEM. B. Modal histograms of IgG_{2B} isotype-treated PBS and IAV (relative to ST2 FMO) from two examples from each group compared for AM, IM, MM macrophages and CD45$^-$ cells. C. Proportion of ST2$^+$ cells to respective total cell counts represented for each macrophage population and CD45$^-$ cells on a log_{10} scale. **Table 3.15**: **Comparison of ST2$^+$ proportions among macrophages and non-immune cells**. Table indicating quantification of ST2$^+$ cells/total cell count (as a percentage) for each macrophage population and CD45$^-$ cells.

3.16 Effect of anti-Ly6C depletion on IAV-induced responsiveness to methacholine at 3 dpi

IgG2a isotype-treated IAV group's total respiratory system resistance and elastance showed trends towards higher values compared to the anti-Ly6C-treated PBS control group. Total

respiratory system resistance and elastance showed a trend towards lower values for anti-Ly6C-treated IAV group in comparison to its isotype-treated IAV group. Furthermore, a significant decrease in resistance at 12.5 mg/mL and 12.5, 50 mg/mL of methacholine for elastance is observed when comparing values between isotype-treated and anti-Ly6C-treated IAV groups.

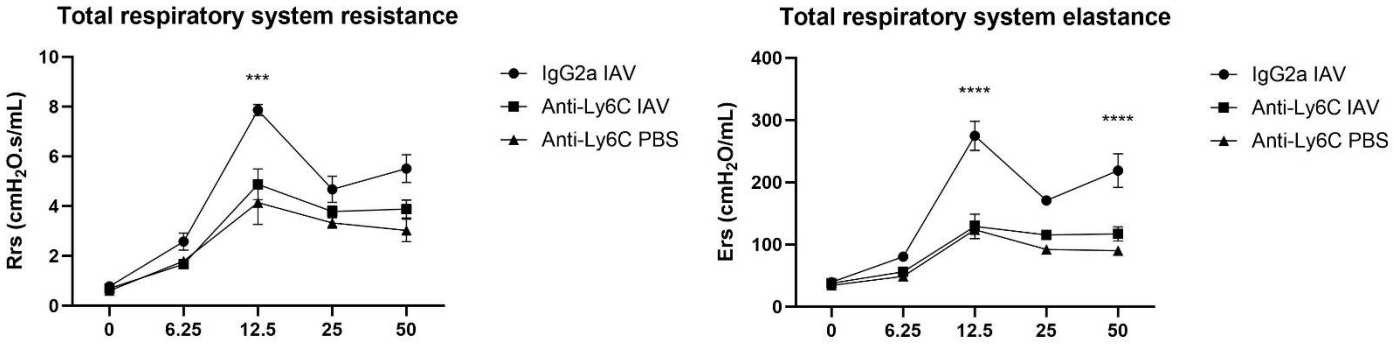


Figure 3.16: **Respiratory mechanics of anti-Ly6C treated IAV mice**. Concentration-response curves of total respiratory resistance (Rrs) and elastance (Ers) for anti-Ly6C-treated PBS, IgG2a isotype-treated and anti-Ly6C-treated IAV groups at 3 dpi. Corresponding Rrs and Ers values are plotted for methacholine concentrations from 0 to 50 mg/mL. Values at each concentration are represented as mean ± SEM. ***$p<0.001$ and ****$p<0.0001$ indicates statistical significance between isotype and anti-Ly6C IAV groups. Repeated measures ANOVA with Bonferroni's post hoc test was used to assess statistical significance.

Chapter 4: Discussion

The mechanism by which IAV infection causes AHR in the mouse is not well elucidated. In published work the IL-33/IL-13 axis is proposed as the principal pathway in the induction of AHR (97). However, the validity of the IL-33/IL-13 axis as the primary driver of IAV-induced AHR has only been established based on the study of the T2-biased BALB/c model and thus whether the findings can be generalized to less T2-biased strains are uncertain. Thus, the objective of this thesis was to evaluate whether the IL-33/IL-13 axis was applicable in a non-T2 biased C57BL/6 model in explaining IL-33 induced AHR.

Our findings revealed that targeting the action of IL-33 with an anti-ST2 antibody to block the activation of its receptor attenuated AHR in C57BL/6 mice. Administration of anti-ST2 antibody reduced both total respiratory system resistance and elastance following inhalation of aerosolized methacholine indicating a role for IL-33 in the development of AHR caused by acute IAV infection in C57BL/6 mouse. Chang et al. (2011) had reported similar outcomes with anti-ST2 administration to IAV-infected BALB/c mice (97). Similarity in the effectiveness of anti-ST2 in attenuating IAV-induced AHR between T2-biased and non-T2 biased models further highlight the importance of IL-33/ST2 signalling as a key requirement in the development of AHR during acute IAV infection.

Given the wide range of effects of IL-33 on immune cells, we assessed whether an altered immune response following inhibition of its signalling might have affected viral replication and provided a possible explanation for the altered AHR (180). However, anti-ST2 treatment caused no difference in the expression of viral NS1 or the viral load by plaque assay resulting from IAV infection, indicating that the reduction in AHR by anti-ST2 treatment was not mediated by an altered immune response through the extent of viral burden. Anti-ST2 also did not alter the viral burden during RSV infection as reported by Zeng et al. (2015) (142).

Contrary to expectations and despite the increase in IL-33 following IAV infection, we observed that the activation of innate immune responses with acute IAV infection was not affected by anti-ST2 treatment. The expression of various inflammatory cytokines and chemokines from whole lung homogenates showed no changes in expression following anti-ST2 treatment, assessed both at 2 and 3 dpi. All of the cytokines and chemokines chosen for assessment were gene targets of IL-33 signalling which plausibly would have been affected in their expression levels following anti-ST2 treatment (181). Assessment of such inflammatory markers with acute IAV infection in the absence of IL-33 signalling hasn't been reported previously, to our knowledge. None of the inflammatory markers explored in this study induced by IAV infection provide mechanistic explanations for the attenuation of AHR by anti-ST2 treatment in C57BL/6 mice.

In contrast to the reported findings on BALB/c mice, IAV infection in C57BL/6 showed no substantial induction of lung ILCs. Anti-ST2 treatment paradoxically further elevated cysteinyl leukotriene release. Neutrophil depletion had no affect on the changes in total respiratory system resistance and elastance induced by inhaled methacholine during IAV infection. However, anti-ST2 treatment reduced the number of lung monocyte-derived macrophages. We reasoned that the reduction in the recruited macrophage population was achieved through reduced influx of monocytes into the lung from the bloodstream. However, in characterizing $F4/80^{-}Ly6C^{+}$ lung monocytes we found no alteration in numbers during anti-ST2 treatment. Furthermore, anti-ST2 also reduced the $CD206^{+}$ M2 subset of monocyte-derived macrophages. More than half of monocyte-derived macrophages expressed the ST2 receptor during acute IAV infection. Monocyte-derived macrophage depletion showed promising trends for reduction in total respiratory system resistance and elastance following methacholine challenge during IAV infection.

IL-33 is known to induce its own expression by autocrine/paracrine signalling therefore, attenuating IL-33 signalling was anticipated to reduce IAV-induced IL-33 production as a means of explaining reduced AHR with anti-ST2 treatment (181). The reduction of IL-33 by anti-ST2 has been reported by Ravanetti et al. (2019) in a chronic C57BL/6 model of HDM sensitization followed by IAV infection wherein anti-ST2 reduced AHR and acutely decreased IL-33 production (150). However, in our experiments, BAL fluid assessment following anti-ST2 treatment showed no change in the levels of IL-33 protein during IAV infection. Moreover, differential cell counts in acute IAV-infected C57BL/6 mice revealed no changes to the total cell count, neutrophils, macrophages or lymphocytes following anti-ST2 treatment. Acute IAV infection in C57BL/6 mice did not stimulate the appearance of a considerable eosinophil population in the BAL fluid. In contrast, anti-ST2 treatment to HDM-sensitized IAV infected mice showed a decrease in total cell count and innate immune cells comprising neutrophils, macrophages and eosinophils (150). The context in which anti-ST2 is used differs between models which may explain the differences observed in differential cell count of innate immune cells and the level of IL-33 in BAL fluid.

IL-13 has been shown to be a critical mediator in animal models of allergic asthma and it induces hypercontractility of smooth muscle cells to potentially explain AHR (139, 182). In addition, considering the link between IL-33 and IL-13 through the expansion and differentiation of ILC2s, IL-13 was a strong candidate to examine in our IAV-induced AHR model (70). IL-13 was not affected by anti-ST2 treatment in its levels in the BAL fluid, consistent with a lack of effect on ILCs. Furthermore, blocking the action of IL-13 in our model proved to not lower IAV-induced AHR but unexpectedly further elevated total respiratory system resistance and elastance. In contrast, IL-13-deficient mice on the BALB/c background showed reduced IAV-induced AHR (97). The observations from BALB/c mice and the strong association of IL-13 and AHR are in strong contrast to C57BL/6 mice in which IL-13 is not

the principal mediator in the development of IAV-induced AHR and may actually have a modest protective role.

Another category of inflammatory mediators to explain the observed reduction in AHR following anti-ST2 treatment during IAV infection was the cysteinyl leukotrienes. CysLTs are pro-inflammatory lipid mediators that have strong associations with asthma due to their potent bronchoconstrictive properties on airways (183). With medications like montelukast (CysLT receptor 1 antagonist) preventing asthma attacks and certain members of the cysteinyl leukotriene family having potential roles in altering immunopathogenesis of IAV infection, these lipid-derived mediators are major causes of AHR following allergen sensitization and challenge (184, 185). To explore this possible explanation for reduction of IAV-induced AHR by anti-ST2 treatment we assessed CysLTs in BAL fluid. Contrary to expectations, despite reduction in AHR, anti-ST2 further increased CysLTs levels in BAL fluid during IAV infection. IL-33 has been shown to upregulate the CysLT receptor 1 expression in peripheral blood $CD4^+$ T cells in humans (186). The direct association of IL-33 signalling with CysLT production is novel, but these compounds are not responsible for the observed reduction in AHR by anti-ST2 in this model.

Next, we assessed the role of neutrophils in IL-33 induced AHR during IAV infection. Neutrophils have been shown to account for AHR following the oxidative airway injury from inhaled chlorine and after inhalation of organic dust (92, 187). Depletion of neutrophils was accomplished by the administration of anti-Ly6G antibody. However, the depletion did not alter total lung resistance or elastance induced by inhaled methacholine, leading us to conclude that neutrophils had no obvious role in shaping the acute development of AHR. Ravanetti et al. (2019) had shown that anti-ST2 lowered luminal NETosis but, no direct association of neutrophilia with AHR was made (150). Foong et al. (2010) have shown that neutrophil elastase knockout does not affect IAV-induced AHR which supported our observations (188).

ILCs, particularly natural helper cells (now commonly referred to as ILC2s) were shown to be responsible for producing IL-13 in BALB/c mice as an important cell member of the IL-33/IL-13 axis for induction of AHR (97, 189). Characterization of total lung ILCs in C57BL/6 mice with IAV infection showed no appreciable induction of ILCs with the total cell population approximately comprising 0.2% of all lung cells post lung-enzymatic digestion for flow cytometric analysis. In instances with IAV infection in BALB/c but also following a glycolipid stimulus, ILC2s are increased in cell numbers which are corelated with their capacity to produce IL-13 (97, 190). In C57BL/6, total ILCs were not induced which makes ILC2s unlikely cellular mediators of AHR in addition to the lack of elevation in IL-13 production during IAV infection. Furthermore, anti-ST2 antibody did not alter the total lung ILC population arguing that ILCs, in general don't have an obvious role in contributing to IAV-induced AHR and are not affected by endogenously synthesized IL-33 from IAV infection in C57BL/6 mice.

The next innate cell that we considered was the macrophage. Based on experiments in our model, anti-ST2 did not alter cell population numbers of either alveolar or interstitial macrophages. The interstitial macrophage M1 and M2 subsets were not altered in numbers. IL-33's effects on interstitial macrophages are not well elucidated, however alveolar macrophages and their role in IL-33 mediated asthma pathology have been described. Kurowska-Stolarska et al. (2009) and Bunting et al. (2013) have shown IL-33 to activate alveolar macrophages by virtue of expression of proinflammatory cytokines such as TNFα, IL-1β, IL-6 and CXCL1 and polarization of alveolar macrophages to its M2 subtype (191, 192). Alveolar macrophages stimulated by IL-33 have been shown to contribute to airway inflammation in models of exacerbation of allergic asthma (192, 193). However, alveolar macrophages in the context of IAV infection show no clear categorization of its M1 and M2 subpopulations, which we confirmed with flow cytometric analysis (not shown). No alteration of the expression of the abovementioned cytokines from qPCR conducted on whole lung homogenates occurred

following anti-ST2 treatment. Alveolar macrophages were reduced in number following IAV infection, consistent with previous reports in BALB/c mice (194). Anti-ST2 treatment during IAV infection caused no alterations in alveolar macrophage numbers. The lack of effect of IL-33 on alveolar macrophages in this context suggest that they do not contribute to AHR. However, they may be a cellular source of IL-33 during IAV infection. Stimulation of the TLR7 and NLRP3 inflammasome pathways in IAV-infected alveolar macrophages produces IL-33 (97, 195).

The effects of anti-ST2 on monocyte-derived macrophages were more profound. Anti-ST2 treatment reduced the number of monocyte-derived macrophages and their $CD206^{+}$ M2 subset during IAV infection. A possible reason for a reduction in the number of monocyte-derived macrophages could be anti-ST2 interfering with its cell cycle progression. Absence in IL-33 signalling has proven to prevent expression of various transcription factors involved in controlling cell cycle progression *in vitro* among murine bone marrow derived macrophages (BMDMs) (196). Additionally, IL-33 is known to stimulate a M2 phenotype among macrophages by promoting GATA3 activation (178). M2 macrophages have restorative and repair functions; however, their presence has proven to be strongly associated with allergic asthma (197). The reduction in the M2 subpopulation in the current study was restricted to CD206-expressing cells and not other markers such as arginase 1 or the chemokine CCL22. Both of these latter markers were expected to be reduced in number and degree of expression. IL-33 can also polarize macrophages to the M1 phenotype. Dagher et al. (2020) have shown IL-33 to induce the prototypical M1 marker, NOS2 transcription in cultured bone marrow-derived macrophages (196). *In vitro* culture of BMDMs is considered equivalent of monocyte-derived macrophages since they are differentiated from bone marrow monocytes. However, following anti-ST2 treatment, there was no reduction in either CD80 or iNOS M1 subsets of monocyte-derived macrophages in our observations. Nonetheless, our observations have

indicated anti-ST2 treatment altered both cell numbers and M2 polarization of monocyte-derived macrophages. Based on these findings we reasoned that the monocyte-derived macrophages may play a role in controlling IAV-induced AHR in our model.

We hypothesized that the reduction in monocyte-derived macrophages was a result of anti-ST2 antibody affecting the differentiation process of recruited monocytes from the blood during IAV infection. Flow cytometric determination of F4/80$^-$ Ly6C$^+$ monocytes which differentiate into monocyte-derived macrophages did not reveal an alteration in their absolute numbers by anti-ST2 treatment. The lack of a reduction in monocyte numbers suggested that anti-ST2 had an effect on the monocyte-derived macrophage function. In addition, the principal chemokine for monocyte/macrophage recruitment CCL2 in BAL fluid showed no change with anti-ST2 treatment. Similarly, there was also no change in CCL7 and CCL22. All of these chemokines are stimulated by IL-33 to drive monocyte recruitment into the lung (198). We assessed the expression of two factors known to drive monocytic differentiation, c-maf and m-CSF (199, 200). Anti-ST2 treatment led to an elevation in the expression of c-maf and m-CSF in whole lung homogenates. While anti-ST2 did not alter the recruitment of monocytes, the antibody may have permitted greater differentiation of monocytes, possibly to compensate for the reduction in monocyte-derived macrophages during IAV infection.

In order to address the importance of monocyte-derived macrophages in IL-33 induced AHR in this model, the expression of the ST2 receptor during IAV infection was assessed among all macrophage populations and CD45$^-$ cells, which included the airway epithelium. Only the monocyte-derived macrophages showed an increase in ST2$^+$ cells with IAV infection, suggesting that it was the population of cells most likely to be affected by IL-33 signalling. More than 50% of the cells categorized as monocyte-derived macrophages expressed the ST2 receptor, the highest expression compared to alveolar, interstitial macrophages and CD45$^-$

cells. Determination of ST2 expression during IAV infection in C57BL/6 mice among subsets of innate immune cells does not appear to have been previously described.

Following the observed effects of anti-ST2 on the monocyte-derived macrophage population and the quantification of ST2 expression, the effect of depletion of monocyte-derived macrophages was next addressed. Lee et al. (2004) and Kim et al. (2008) established macrophages as the cells responsible for inducing AHR and chronic asthma during acute parainfluenza infection and Sendai virus infection, respectively (201, 202). Depletion of monocyte-derived macrophages showed trends for lower airway resistance and elastance during methacholine challenge, indicating a lower IAV-induced AHR. The findings suggest that the monocyte-derived macrophages are important cells in contributing to IAV-induced AHR. Considering the fact that monocyte-derived macrophages numbers are lowered by anti-ST2, it is a cell of interest that may be essential in IL-33 induced AHR during IAV infection in the C57BL/6 mouse.

The IL-33/IL-13 axis has been proposed to be the principal pathway involved in mediating AHR both in allergic and influenza-induced asthma. With experiments conducted so far, new insights into the relationship between IL-33 and AHR have been gleaned from our non-T2 biased model, indicating a lack of involvement of the IL-33/IL-13 axis. Additionally, the experimental approach has aimed to narrow the potential innate cell members and mediators involved in IL-33 mediated AHR in an effort to identify an alternate mechanism to the IL-33/IL-13 axis in our model. The experiments have built on existing evidence of macrophages' importance in controlling respiratory viral-induced AHR, but also into the mechanisms by which anti-ST2 lowers IAV-induced AHR. Astegolimab, an anti-ST2 biologic currently undergoing clinical trials has proven to be effective in preventing in asthma exacerbations, even among T2-low asthmatics (58). Hence, understanding the mechanism of AHR caused by an alarmin like IL-33 provides insight into its pathogenic mechanisms which could potentially

be useful in elucidating other respiratory related conditions besides acute AHR caused by IAV infection.

The proposed replacement pathway for the IL-33/IL-13 axis in this model is promising. Nonetheless, there are limitations to the experimental work which need to be addressed. Even though no induction of lung ILCs was observed in our experiments, ILCs cannot be entirely ruled out since the evaluation of their absence and consequent effect on AHR hasn't been explored. Furthermore, observing no change in IL-13 levels after anti-ST2 treatment and no significant increase in IL-13 during IAV infection, addressing the absence of IL-13 signalling needs to be addressed. This is so because no AHR measurements among knockout mice lacking constitutive IL-13 signalling has been conducted to definitively show the role of IL-13 in affecting AHR in our model. While anti-ST2 has proven to reduce IAV-induced AHR, other approaches to neutralizing IL-33 could be used to complement these findings. In addition, due to the lack of data confirming the blocking actions of both anti-ST2 and anti-IL-13, we cannot be absolutely confident that the antibodies are maximally pharmacodynamically effective. The association of transcription factors c-maf and the colony stimulating factor m-CSF with monocytic differentiation remains speculative since currently their increase in expression by anti-ST2 treatment is at the level of whole lung homogenates and not the recruited monocytes specifically. Lastly, the generalizability of the monocyte-derived macrophage depletion on IAV-induced AHR is limited due to a small sample size for comparison between isotype and anti-Ly6C IAV groups at present.

Avenues for future studies include addressing ILCs and IL-13 more completely in our experimental model. Administration of anti-CD90 to RAG deficient mice to deplete ILCs and the use of a STAT6 knockout mouse to eliminate downstream IL-13 signalling would be useful tools to further dissect these candidates in affecting IAV-induced AHR in C57BL/6 mice. The reduction in IAV-induced AHR by anti-ST2 treatment can further be supported by other

methods of blocking IL-33 signalling such as the administration of an anti-IL-33 antibody or constitutive absence in IL-33 signalling in ST2 knockout mice. Furthermore, cell sorting following flow cytometric analysis may be conducted both on monocytes and monocyte-derived macrophages. Following sorting of monocytes, expression of c-maf and m-CSF could be verified to see whether expression levels still increase following anti-ST2 treatment. If the trends follow what has been observed with qPCR from whole lung homogenates, the association of c-maf and m-CSF to monocytic differentiation during anti-ST2 administration will be consolidated. Additionally with cell sorting, profiling gene expression patterns of monocytic macrophages will inform about potential factors involved in controlling cell proliferation to explain the reduction in their numbers as observed with flow cytometric analysis of whole lung digestion following anti-ST2 treatment.

The observed effects of depletion of monocytic macrophages with anti-Ly6C on IAV-induced AHR must be validated by increasing the sample size. Furthermore, utilization of constitutive gene models such as CCR2 knockout mice can be considered. In CCR2 knockout mice, there is no recruitment of $CCR2^{+}$ monocytes. Therefore, there is a substantial absence in the number of monocytes undergoing differentiation to support the monocytic macrophage population during IAV infection. Henceforth, it will allow us to evaluate whether the $CCR2^{+}$ lineage monocytic macrophages is important in controlling IAV-induced AHR in addition to our anti-Ly6C depletion experiments. To further support the importance of monocytic macrophages as key innate immune cells in the mechanism of IAV-induced AHR, a selective adoptive transfer of sorted monocytic macrophages from IAV-infected wild type mice to naïve CCR2 knockouts may be conducted. Considering CCR2 knockout mice don't develop AHR, restoration of monocytic macrophages from wild type mice is predicted to show induction of AHR. Lastly, if the monocytic macrophages are proven to be crucial in the development of AHR during IAV infection through depletion and adoptive transfer experiments, exploration of the effect of IL-

33 on monocytic macrophages functionality may be pursued; namely to assess presence of inflammatory mediators other that the CysLTs and IL-13 affected by IL-33 signalling. To address this objective, gene expression profiling of *in vitro* treated BMDMs with IAV infection with administration of IL-33 may be conducted. We speculate that the experiment will yield changes in expression of possible mediator(s) affected by IL-33 signalling therefore, explaining how the anti-ST2 antibody lowers IAV-induced AHR on a molecular level.

Taking into consideration the future experimental directions proposed, a different pathophysiological mechanism to replace the IL-33/IL-13 axis may be identified in order to explain how IAV-induced IL-33 release causes AHR in non-T2 biased C57BL/6 mice.

Conclusions

The current research undertaken was to understand the generalizability of the IL-33/IL-13 axis as the principal pathway in the induction of AHR during acute IAV infection in a non-T2 biased C57BL/6 mouse model. If the IL-33/IL-13 axis was not the key pathway, the objective was to elucidate what the alternative pathway could be in the C57BL/6 mice responsible for AHR in response to anti-ST2, a blocking antibody to prevent the action of IL-33.

Following the observation of anti-ST2 lowering IAV-induced AHR, assessment of ILCs cell numbers and IL-13 levels showed no increments with IAV infection with anti-ST2 having no substantial changes in either. Blocking the action of IL-13 did not decrease IAV-induced AHR. Combining both results from ILCs and IL-13 experiments confirmed IL-33/IL-13 to not be the mechanism of AHR in C57BL/6 mice.

The examination of macrophage populations showed the monocyte-derived macrophages and its $CD206^{+}$ M2 subset cell population numbers were both reduced in presence of anti-ST2 treatment. The monocyte-derived macrophages highly expressed the ST2 receptor during acute IAV infection among all macrophage populations and $CD45^{-}$ cells making it as an innate cell

of interest to consider in the mechanism of IL-33-mediated AHR in C57BL/6 mice. Preliminary experiments of anti-Ly6C monocyte-derived macrophage depletion showed lower trends of total airway resistance and elastance indicating a reduction in AHR during IAV infection. The monocyte-derived macrophages hence are suggested to be key innate cells in IAV-induced AHR and are functionally affected by anti-ST2 to reduce AHR.

In addition to the completion of anti-Ly6C depletion experiments, AHR observations of IAV-infected CCR2 knockout mice acts as a constitutive method to observe the effects of CCR2-dervied monocyte-derived macrophages in the mechanism of IAV-induced AHR. Furthermore, the consequent effect of induction of AHR by adoptive transfer of sorted monocyte-derived macrophages will further validate the role of these cells in controlling IAV-induced AHR. Isolating the responsible population of monocyte-derived macrophages will then allow us to better understand the IL-33 induced changes in cellular functionality of monocyte-derived macrophages in an effort to link our findings to strategies to control airway hyperresponsiveness.

Overall, contrary to our initial hypothesis, the mechanism by which IL-33 induces AHR in C57BL/6 is different to the IL-33/IL-13 axis in BALB/c mice. With C57BL/6 mice having a genetically determined a less T2-biased immunity, the mechanism of AHR induction is proposed to be different. Clearly elucidating a different pathway responsible will serve to better understand how astegolimab functions to reduce asthma exacerbations in patients with more or less T2 asthma. Subsequently, our experimental work may reveal how the mechanism of alarmin induced-exacerbation can mechanistically be different dependent on the endotypic nature of asthma.

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