Airway smooth muscle phenotype, T helper-1 immunity and associated asthma pathophysiology

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Abstract

Asthma canonically features T helper 2 (Th2) immunopathology and airway structural alterations, referred to as remodelling, which directly associate with airflow limitation and airway hyperresponsiveness. Changes in airway smooth muscle (ASM) properties, including both hyperplasia and increased force generation, are features of airway remodelling and are a main contributor to asthma-associated pathophysiology.

As smooth muscles are generally considered to exist as dichotomized contractile or proliferative/secretory phenotypes, aberrant phenotype switching has been proposed to explain the pathological ASM changes associated with asthma. In the first part of the thesis, we characterized the transcriptional regulatory mechanisms governing the phenotype dichotomy in human ASM. The pro-contractile transcription factor (TF) myocardin (MyoCD), and the mitogenic TF ETS like-1 protein (Elk-1) both associate with the DNA binding element serum response factor (SRF) for their respective transcriptional activities. This results in their competition for SRF binding, and hence a mutually inhibitory relationship that underlies the phenotype dichotomy. In addition to confirming their mutually inhibitory actions in ASM, we also discovered that factors contributing to SRF upregulation eliminate the competition between MyoCD and Elk-1, allowing the unopposed functions of both TF, and therefore permitting a dual contractile-proliferative phenotype of ASM. These results suggest that SRF is a promising therapeutic target for ameliorating asthma-associated smooth muscle alterations.

In the second part of the thesis, we focused on the ASM secretory phenotype, specifically its capacity to produce chemokines responsible for CD4+ T cells recruitment. Existing studies in asthmatic patients and animal models from our group demonstrated that T cells infiltrate ASM

bundles and promote hyperplasia. Using an *in vitro* T cell migration assay, we demonstrated that ASM-derived C-X-C motif chemokine receptor 3 (CXCR3) ligands, namely C-X-C motif chemokine ligand 10 (CXCL10) and CXCL11, are primarily responsible for inducing CD4+ T cell chemotaxis. Furthermore, in response to the T cell-derived interferon- γ (IFN-γ), the production of CXCL10 and CXCL11, which are known interferon-inducible proteins, are upregulated in ASM, thereby forming a positive feedback loop resulting in additional T cell recruitment. These results highlighted the potential contribution of IFN-γ, a Th1 cytokine, to the pathophysiology of asthma.

With these discoveries, we explored the interaction between Th1 response and Th2 immunopathology in the context of asthma, which is the focus of the third part of the thesis. In a mouse model of atopic asthma involving sensitization and intrapulmonary challenges with ovalbumin (OVA) as an allergen, we co-administered poly I:C, a double-stranded RNA and the ligand of many innate pattern recognition receptors (PRRs), which induces interferon production and promotes Th1 immunity. Unexpectedly, we discovered that poly I:C suppressed Th2 immunopathology which was not due to the cross-regulation by Th1 immunity. Rather, poly I:C induced the infiltration of monocyte-derived macrophages with a prominent alternatively activated (M2) phenotype with immunosuppressive functions, which inhibited CD4+ T cell activation *in vitro*. *In vivo* depletion of monocyte-derived macrophages eliminated the poly I:C's protective effect and restored the Th2 immunopathology.

Taken together, our studies advanced the understanding of different aspects of ASM dysfunction and revealed the unexpected immunomodulatory effect of Th1-inducing PRR ligand in the context of asthma.

Résumé

L'asthme est une immunopathologie généralement attribuée à l'action des lymphocytes T auxiliaires de type 2 (Th2) ainsi qu'aux altérations structurelles des voies respiratoires, appelées le remodelage. Ensemble, le remodelage et l'activation excessive des Th2 sont associés à une limitation du débit aérien et à une hyperréactivité des voies respiratoires. La modification des propriétés des muscles lisses des voies respiratoires (MLVRs), notamment l'hyperplasie et l'augmentation de leurs forces contractiles, sont les principales caractéristiques du remodelage qui contribuent à la physiopathologie de l'asthme. Comme les muscles lisses sont communément considérés comme se manifestant en deux phénotypes mutuellement antagoniste; soit un phénotype contractile ou proliférative/sécrétoire, une théorie suggérant le changement aberrant de ce phénotype avait été proposée pour expliquer les modifications pathologiques des MLVRs dans l'asthme. Dans la première partie de cette thèse, nous avons caractérisé les mécanismes de régulation transcriptionnelle qui gouvernent la dichotomie phénotypique chez les MLVR humaines. Le facteur de transcription (FT) pro-contractile myocardine (MyoCD) et le FT mitogénique appelé ETS-like-1 protein (Elk-1), s'associent tous deux au même serum response factor (SRF) pour effectuer leurs activités transcriptionnelles auprès de l'ADN. Cela entraîne leurs compétitions pour la région promotrice du SRF, et explique donc l'inhibition mutuelle de ces deux protéines responsables pour la dichotomie phénotypique susmentionnée. En plus de confirmer leurs actions mutuellement inhibitrices dans les MLVRs, nous avons également découvert que les facteurs qui stimulent la production de SRF éliminent la compétition entre MyoCD et Elk-1, permettant ainsi la fonction simultanée de ces deux FTs, promouvant un phénotype à la fois contractile et prolifératif. Ces résultats suggèrent que le SRF est une cible thérapeutique prometteuse pour améliorer les altérations des MLVRs caractéristiques de l'asthme.

Dans la deuxième partie de cette thèse, nous nous sommes concentrés sur le phénotype sécrétoire des MLVRs, plus précisément à leurs capacités à produire des chimiokines qui recrutent les lymphocytes T $CD4^+$. Un nombre d'études menées sur les humains asthmatiques ainsi que les études de modèle animal de notre laboratoire démontrent que les lymphocytes T infiltrent les MLVRs et favorisent l'hyperplasie. À l'aide d'un test de migration de lymphocytes T *in vitro*, nous avons démontré que les ligands du CXC motif chemokine receptor 3 (CXCR3) provenant des MLVRs, notamment CXC motif chemokine ligand 10 (CXCL10) et CXCL11, sont principalement responsables pour l'induction de la chimiotaxie des lymphocytes T $CD4^+$. De plus, en réponse à l'interféron γ (IFN-γ) sécrété des lymphocytes T, les MLVRs produisent des protéines inductibles par l'interféron, telles que le CXCL10 et CXCL11, qui renforcent doublement le recrutement des lymphocytes T. Ces résultats ont mis en évidence la contribution potentielle de l'IFN-γ, une cytokine de type 1, à la physiopathologie de l'asthme.

Ces découvertes effleurent les interactions potentielles entre les réponses immunitaires de type 1 et l'immunopathologie de type 2 de l'asthme qui fait l'objet de la troisième partie de cette thèse. Dans notre modèle murin d'asthme à sensibilisation atopique, nous avons co-administré une solution intranasale d'ovalbumine (OVA) avec poly I:C , une molécule d'ARN double reconnue par de nombreux récepteurs de reconnaissance de motifs moléculaires (PRRs) et qui induit la production d'interféron et favorise l'immunité de type 1. Nos résultats étaient inattendus, car nous avons découvert que le poly I:C supprimait l'immunopathologie de type 2 indépendamment d'un effet sur l'immunité de type 1. Le poly I:C a induit l'infiltration de macrophages dérivés de monocytes et présentant un phénotype réparateur (M2) et immunosuppresseur, qui ont inhibé l'activation des lymphocytes T $CD4^+$ *in vitro*. La déplétion *in vivo* des macrophages dérivés de monocytes a éliminé l'effet protecteur du poly I:C et a restauré l'immunopathologie de type 2.

Prises ensemble, nos études ont fait progresser la compréhension des différents aspects du dysfonctionnement des MLVRs et ont révélé un effet immunomodulateur inattendu des ligands des PRRs qui induisent une réponse immunitaire de type 1 dans le contexte de l'asthme.

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List of abbreviations

15-HETE: 15-hydroxyeicosatetraenoic acid

α-SMA: α-smooth muscle actin

ADAM: A disintegrin and metalloprotease

AHR: airway hyperresponsiveness

ASM: airway smooth muscle

ASMC: airway smooth muscle cell

BAL: bronchoalveolar lavage

BALF: bronchoalveolar lavage fluid

Bcl-2: B-cell leukemia/lymphoma 2

cAMP: cyclic AMP

CCL: c-c motif ligand

CCR: c-c motif chemokine receptor

CD: cluster of differentiation

CNN1: calponin 1

COPD: chronic obstructive pulmonary disease

COX: cyclooxygenase

CRTh2: chemoattractant receptor-homologous molecule on T helper type 2 cells

CX3CL1: C-X3-C motif ligand 1

CXCL: c-x-c motif ligand

CXCR: c-x-c motif chemokine receptor

CysLT: cysteinyl leukotriene

CysLTR1: cysteinyl leukotriene receptor 1

DC: dendritic cell

ECM: extracellular matrix

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

Elk-1: ETS like-1

FEV_1: forced expiratory volume in 1 second

FeNO: fractional exhaled nitric oxide

FVC: forced vital capacity

GATA3: GATA binding protein 3

GM-CSF: granulocyte-macrophage colony-stimulating factor

HB-EGF: Heparin-binding EGF-like growth factor

HDM: house dust mite

ICAM1: Intercellular adhesion molecule 1

ICS: inhaled corticosteroid

IFN: interferon

Ig: immunoglobulin

IRF: interferon regulatory factor

IL: interleukin

ILC: innate lymphoid cell

LTC_4/LTD_4/ LTE_4: leukotriene C_4/D_4/E_4

MAPK: Mitogen-activated protein kinases

MHC: major histocompatibility complex

MLCK: myosin light chain kinase

MMP: matrix metalloproteinase

MUC5AC: Mucin 5AC

MYH11: myosin heavy chain 11

MyoCD: myocardin

NF-κB: nuclear factor-κB

NK cells: nature killer cell

OVA: ovalbumin

OX40L: OX40 ligand

PAMP: pathogen-associated molecular pattern

PCNA: proliferating cell nuclear antigen

PD-L1: Programmed death-ligand 1

PDGF: platelet-derived growth factor

PGD2: prostaglandin D2

PGE2: prostaglandin E2

PI3K: Phosphoinositide 3-kinases

PIC: Polyinosinic:polycytidylic acid

PKC: protein kinase C

PPARG: peroxisome proliferator-activated receptor gamma

RORα: RAR-related orphan receptor-α

RORγt: RAR-related orphan receptor-γt

SRF: Serum response factor

ST2: suppression of tumorigenicity 2

STAT: Signal transducer and activator of transcription

Th1: T helper

TLR: toll-like receptor

TGF: transforming growth factor

TIMP: tissue inhibitor of metalloproteinase

TNFα: tumor necrosis factor α

TSLP: thymic stromal lymphopoietin

VCAM1: vascular cell adhesion protein 1

VEGF: vascular endothelial growth factor

VSM: vascular smooth muscle

VSMC: vascular smooth muscle cell

Contribution to original scientific knowledge

This thesis is constructed in the manuscript-based format in accordance with the guidelines of the graduate studies of McGill University. Chapter 2, 3 and 4 are three manuscripts that feature important findings concerning pathological changes in airway smooth muscle observed in asthma, as well as a novel immunomodulatory pathway that might contribute to the alleviation of the disease. The former two have been published, and the third is currently prepared for submission.

Chapter 2: In this manuscript published in American Journal of Respiratory Cell and Molecular Biology, we described the ASMC phenotype determination by the interaction between myogenic transcription factors MyoCD and mitogenic transcription factors Elk-1. The key novel findings include:

I. MyoCD over-expression in ASMC conferred a hyper-contractile phenotype.
II. The overexpressed MyoCD was capable of inhibiting Elk-1-mediated gene transcription and cellular proliferation via competition for binding with SRF, a DNA-binding element required for the functions of both transcription factors.
III. Due to the competition for SRF binding, MyoCD and Elk-1 displayed a mutual inhibitory relationship, thereby resulting in a dichotomy of ASMC contractile versus proliferative phenotypes, respectively.
IV. In the cases where SRF availability in the nucleus was upregulated, it allowed unopposed function of both transcription factors, yielding a contractile-proliferative dual phenotype.
V. MyoCD overexpression alone was insufficient to rescue the loss of contractile protein expression in ASMC caused by *in vitro* culturing.

Chapter 3: In this manuscript published in the FASEB Journal, we explored the chemokine pathway responsible for ASMC-mediated CD4+ T cell recruitment, as the infiltration of T cells in ASM bundles has been observed *in vivo*. The key novel findings include:

I. Cultured ASMCs secreted CXCR3 ligands in the basal state and upon exposure to CD4+ T cells, which induced CD4+ T cell chemotaxis. ASMC-derived CCR3 or CCR5 ligands were not responsible for mediating T cell chemotaxis.

II. The CXCR3 receptor prominently underwent internalization upon exposure to its cognate ligands in CD4+ T cells.

III. CD4+ T cell-derived IFN-γ was responsible for upregulating the secretion of CXCR3 ligands by ASMC, resulting in a positive feedback loop between ASMC chemokine secretion and T cell recruitment.

IV. ASMCs derived from non-asthmatic and asthmatic lung donors demonstrated similar CXCR3 ligand secretion capacity in response to CD4+ T cells or recombinant IFN-γ.

Chapter 4: In this manuscript, we co-administered synthetic double-stranded RNA, PIC: Poly I:C , a viral-like PAMP, during allergen challenges in a mouse asthma model to explore the interaction between responses to virus and asthma-associated immunopathology. The key novel findings include:

I. PIC suppressed asthma-associated Th2 immunopathology and AHR.

II. The induction of interferons, interferon-inducible genes and inflammatory cytokines was deficient in allergen-challenged mice, indicating that the suppression of Th2 immunopathology was not due to the cross-inhibitory effects of Th1 immunity upregulated by Poly I:C exposure.

III. Poly I:C co-administration with allergen led to an enrichment in monocyte-derived macrophages with a prominent immunoregulatory phenotype, which were capable of suppressing CD4+ T cell activation *in vitro*.

IV. Poly I:CIC did not suppress Th2 immunopathology in the absence of monocyte-derived macrophages.

Co-author contributions

1. Jang, J. H. *et al*. Induction of a memory-like CD4(+) T-cell phenotype by airway smooth muscle cells. *Eur J Immunol* **54**, e2249800 (2024).
2. Zhou, M. *et al*. CD4(+) T cell-derived IFN-gamma and LIGHT synergistically upregulate chemokine production from airway smooth muscle cells. *FASEB J* **38**, e23405 (2024).
3. Nakada, E. M., Sun, R., Fujii, U. & Martin, J. G. The Impact of Endoplasmic Reticulum-Associated Protein Modifications, Folding and Degradation on Lung Structure and Function. *Front Physiol* **12**, 665622 (2021).

Contribution of authors

All research work included in this thesis was supervised by Dr. James G. Martin. The contributions by other co-authors (represented by the initials) are listed below:

Chapter 1: The comprehensive literature review was written by RS. JGM made edits and provided feedback.

Chapter 2: RS and JGM designed the experiments; RS performed all experiments with the exception of the following: experiments shown in Figure E2 and E4 were performed by XP under RS's supervision; experiments shown in Figure 3E, 3F and E1 were performed by EW under RS's supervision; experiments shown in Figure 6C-E was performed by AM under RS's supervision; experiments shown in Figure 7 were performed with technical assistance of RI; RS, XP, EW and AM performed data analysis for their respective experiments as specified above; RS and JGM wrote and edited the manuscript; AML was responsible for tissue procurement and ethics approval and edited the manuscript.

Chapter 3: RS and JGM designed the study; RS performed all experiments with technical assistance of JHJ; RS analyzed the data; RS and JGM wrote and edited the manuscript; AML was responsible for tissue procurement and ethics approval and edited the manuscript.

Chapter 4: RS and JGM designed the study; RS performed all experiments with the exception of the following: experiments shown in Figure 2A-C and Figure 3D-H were performed by EW under RS's supervision; experiments shown in Figure 2D and 2E were performed by DP under RS's supervision; RS performed data analysis; RS and JGM wrote and edited the manuscript.

Chapter 5: The discussion was written by RS. JGM made edits and provided feedback.

Chapter 1: Introduction & Literature Review

Asthma remains one of the most common respiratory diseases worldwide, the treatment and management of which represent in significant public health burdens. The ongoing research reveals the complexity and heterogeneity in the pathogenic mechanisms of asthma. Therefore, future improvements in asthma care rely on an in-depth understanding of the disease biology. Our investigation focused on two main aspects of the disease. Regarding airway structural changes in relation to asthma, we specifically explored the roles of alteration in the characteristics of ASM, the principal actor of bronchoconstriction. In terms of the asthma-associated immunity, we aimed to examine the role of concomitant T_H1 immunopathology, which is poorly understood in the context of asthma. This section provides a general review of asthma, followed by the background on the pathophysiology and immunobiology relevant to the rationales of our investigation.

1.1 Overview of asthma

1.1.1 Epidemiology and risk factors

Asthma epidemiology

Asthma is one of the most prevalent chronic non-communicable diseases affecting more than 300 million people worldwide[1,2]. The disease can develop at any point throughout life, but it begins more commonly during the childhood and disproportionately affects children more than adults. In terms of its sex disparities, prepubescent males (younger than 13) and adult females are more affected than their counterparts[3], representing more than 60% of their respective demographics, suggesting the role of sex hormones in asthma etiology[3]. Its prevalence also varies among geographical locations, ranging from 1% to 18% among countries and regions[4]. As

a disease known to be the most common in developed countries, the rise in its prevalence has been reported since 1960s in parallel with other atopic diseases[2]. With improved treatment and awareness, the incident rate has seen a decrease in high-income countries in the 21st century, though it is still on the rise in developing countries[5]. Socioeconomic disparities are shown to be clearly associated with asthma prevalence, with both adults and children of racial minorities disproportionately affected[2,6].

Risk factors – current perspectives on the hygiene hypothesis

Some well-defined risk factors of asthma include exposure to environmental pollution, smoking, obesity, altered gut and lung microbiome, history and family history of allergic diseases, and psychosocial stress[7]. In addition, work-place exposure to animal and plant-derived enzymes and protein allergens, as wells as a variety of metals and synthetic chemicals contribute to occupational asthma[7].

The hygiene hypothesis has been a popular idea that is believed to explain the increased prevalence of asthma since the mid-20th century. The hypothesis originated from epidemiological studies showing an inverse correlation between the number of siblings and household size and the prevalence of allergic diseases[8]. The evidence led to postulates that improved hygiene, use of antibiotics and urbanization correlated with higher incidence of atopic diseases dominated by type 2 immunopathology[9]. In terms of the mechanism associated with the hygiene hypothesis, it has been proposed that infection and exposure to microbes that promotes Th1 response early in life would drive immune response away from a Th2-dominant "default setting" of the young immune system, thereby conferring tolerance to allergic diseases[9]. Indeed, studies today have demonstrated that increased exposure to microbes, via attendance at daycare[10,11] or exposure to

farm environment[12,13], is associated with a lower prevalence of asthma and allergy among children and also later in life. On the other hand, the investigations into the links between various childhood viral or bacterial infections and atopy yield mixed results[9]. Particularly, viral lower respiratory track infections have been linked to the development of childhood asthma, as the associated inflammation potentially triggers host factors predisposing to the disease[14].

The original hygiene hypothesis has obvious flaws such that it is not supported by today's understanding of atopic disease. First of all, epidemiological studies of certain demographics, such as inner-city urban population with exposures to higher endotoxin levels, South American countries with high infection rates yield opposite conclusions[15]. The current decrease of asthma prevalence in high-income countries is also clearly not a result of increased microbe exposure[15]. With better understanding of the complexity of immune responses, the simple Th1/Th2 balance paradigm as an explanation of hygiene hypothesis is clearly outdated. In addition, it has been shown that early microbe exposure offers protection against not only atopic asthma, but also non-atopic asthma, which falls outside the proposed mechanism[15]. Therefore, it is possible that the concept of the hygiene hypothesis holds true in that exposure to microbes overall reduces the risks of atopic diseases, but further investigations are required to define the underlying mechanism[15]. Many factors associated with Westernization since the 1950s are likely responsible for the increase in asthma prevalence.

1.1.2 Clinical diagnosis and treatment

<u>The diagnosis of asthma – the Global Initiative for Asthma guidelines</u>

The Global Initiative for Asthma (GINA) guideline is the most widely used, evidence-based standard for asthma diagnosis and management in both adults and children[16]. According to

which, the two defining features of asthma are respiratory symptoms including cough, wheezing, shortness of breath and chest tightness, and variable expiratory airflow limitation[18]. The latter is determined via spirometry, which measures a key parameter named the forced expiratory volume in 1 second (FEV_1) to forced vital capacity (FVC) ratio (FEV_1/FVC). An FEV_1/FVC below 0.9 in children, or below 0.7 in adult, or lower than the lower limit (lowest 5%) of their respective reference populations indicates the presence of airflow obstruction[17,18]. In addition, GINA recommends against using FEV_1/FVC as a hard criterion for diagnosis as the ratio naturally decreases with age[18]. It is also important to note that the airflow limitation might not be present at the time of the test, therefore, repeated testing and supplementary diagnostic testing is often necessary[18]. As variability is a main feature in the airflow obstruction associated with asthma, other tests based on spirometry are used to demonstrate the variability, including peak-flow meter measurement throughout the day, testing the responsiveness to a short acting bronchodilator or improvement of lung function following 4 weeks of treatment[18]. Bronchial provocation test by exercising or with pharmacological reagents (only in adults) are used to determine the risk of excessive bronchoconstriction[18]. A 20% or greater reduction in FEV_1 following inhalation of standard doses of methacholine or histamine, or 15% or greater reduction following inhalation of hypertonic saline or mannitol indicates the presence of airway hyperresponsiveness[18]. Persistent airflow limitation may be present during an exacerbation or in cases with coexisting COPD[18]. Lastly, a history or a family history of atopic diseases, and evidence of elevated type 2 inflammation might be used to support the diagnosis of asthma, but caution should be taken as asthma may be non-allergic, and these characteristics are not specific to asthma[18].

Standard treatments of asthma

If diagnosed with asthma, the management regimen is determined based on disease severity. The main medication used for controlling asthma is a combination of inhaled corticosteroid (ICS) and $beta_2$ agonist, commonly referred to as "reliever" therapy. The former usually includes beclomethasone dipropionate, fluticasone, budesonide and mometasone, which act on the immune system to reduce inflammation[19]. The latter are classified as long-acting beta-agonists (LABA), including formoterol (the preferred reliever according to GINA, because it is a full agonist and relatively rapid in onset of its action) and salmeterol, which are sometimes formulated in combination with ICS in the inhalers, and short acting beta-agonists (SABA, the alternative reliever according to GINA) including salbutamol and terbutaline[20]. These medications act primarily on airway smooth muscle to trigger its relaxation, thereby resulting in bronchodilation[20]. In recent years, GINA has recommended against SABA monotherapy for asthma, as it is associated with greater risk of severe exacerbation and asthma-associated death, as well as the probability of underestimating the disease severity by patients themselves[18]. Studies also have shown that the use of SABA contributes to increased allergen-induced airway responses and inflammation[21]. In addition, the use of ICS has also been proven to reduce the need for oral corticosteroid, which is associated with greater side effects[18]. The combination of ICS and bronchodilator is taken as needed in mild cases and raised to daily dosing and higher doses with increased severity[18]. Other supplementary therapies, such as a leukotriene receptor antagonist, a long-acting muscarinic antagonist, house dust mite sublingual immunotherapy could also be considered with increasing severity and as appropriate[18]. In the most severe cases, oral corticosteroid and biologics including anti-IL-4R, anti-IgE, anti-IL-5 and anti-TSLP monoclonal antibodies are used, based on the assessment of asthma phenotype[18]. Lastly, the

effectiveness of the treatment should be routinely assessed and adjusted based on improvement of lung function, patient satisfaction, side effects of medications and comorbidities[18].

1.1.3 Asthma phenotypes & endotypes

For a prolonged period of time, asthma has only been considered to be caused by Th2 immunopathology and antigen-specific IgE responses. However, approximately 50% of severe asthma cases, which make up 5-10% of all cases, are non-Th2-high. With the increasing understanding of its pathogenesis in the recent years, the notion that asthma is a heterogeneous disease with diverse phenotypes and endotypes is now widely accepted. Some also consider asthma to be an umbrella term referring to several distinct airway diseases. As patients with different features of clinical presentation or cellular and molecular signatures clearly respond differently to therapies, it is therefore important to employ the rationale of precision medicine, and better categorize the disease phenotype and endotypes, as well as to study the distinctions in pathophysiology corresponding to them for better treatment outcomes. Such approach would also facilitate the research into targeted therapies. Overall, the terms phenotype and endotype are both used for the categorization of asthma presentation, while the former refers to the classification based on observable clinical presentation, and the latter relies on confirmed biological pathways[22].

Atopic asthma endotypes

Asthma has long been categorized into atopic (extrinsic) and non-atopic (intrinsic) phenotypes, with the former being generally early onset and seen among children and the latter observed in older age groups. Atopic, early onset asthma features typical Th2-high biomarkers

including increased blood and sputum eosinophils, total and allergen-specific IgE and fractional expired nitric oxide (FeNO). It is commonly steroid sensitive and unlikely to compromise lung function in the long term. Late onset atopic asthma, however, features similar cellular and molecular signatures but is more likely to be steroid resistant and result in more severe disease and frequent exacerbations. In addition, chronic rhinosinusitis with nasal polyps (CRSwNP) is present in some patients of this asthma endotype[22]. Furthermore, non-steroidal anti-inflammatory drugs (NSAID)-exacerbated respiratory disease (NERD) also occurs among atopic individuals while more often among non-atopic individuals. Due to the reduced PGE_2 synthesis, an immunomodulatory factor, following inhibition of cyclooxygenase by NSAIDs, arachidonic acid metabolism shifts towards the 5-lipoxygenase pathway and results in increased CysLT production, which contributes to bronchoconstriction and exacerbates Th2 inflammation[22-24]. NERD is characterized as severe, late-onset with frequent exacerbations, with some developing CRSwNP[22,24].

Non-atopic asthma endotypes

It is important to note that Th2 inflammation can still be present without atopy, in which the innate immune compartment, especially alarmins, leukotrienes and ILC2, is thought to be the predominant driver of associated immunopathology in response to microbial and irritant triggers. Non-atopic eosinophilic asthma is a prominent example, which usually features late onset and poor response to steroid[25]. The Th2-high phenotype represents only half of the total cases of asthma[26] and 37% of severe asthma[27], while on the other hand, Th2-low, non-atopic asthma appears to correlate with more severe disease, yet the mechanism is relatively under-studied[22]. The Th2-low asthma endotype regularly features steroid insensitivity, neutrophilic or

paucigranulocytic inflammation and elevated Th1/Th17 biomarkers, potentially caused by chronic inflammation and activation of NLRP3 and IL-1β pathways[28]. An elevated Th1 signature marked by increased IFN-γ is observed in 50% of severe asthmatic patients and is proposed to contribute to AHR by downregulating SLP1[29]. Th17 and its associated cytokines IL-17 and IL-22 promotes neutrophilic inflammation by driving the secretion of IL-8, while IL-17 promotes ASM proliferation and collagen synthesis[30]. Smoking-associated asthma that is likely accompanied by neutrophil and macrophage activation by oxidative stress also presents as a Th2-low phenotype, with characteristics including frequent exacerbation, steroid resistance and neutrophilia[31]. Notably, smoking increases the risk of allergy development[26]. Many smokers demonstrate characteristics of both asthma and COPD, referred to as Asthma-COPD overlap syndrome (ACOS) [26]. Obesity associated asthma, another Th2-low, steroid-refractory endotype more commonly found among middle-aged women, has been associated with Th1 and Th17 signatures, ILC3 activation and IL-6 production[32]. Lastly, very late onset asthma has been described among individuals over 50-60 years old and features steroid refractory, Th1/Th17-associated, eosinophilic inflammation[33]. Immunosenescence has been proposed to contribute to this endotype[33].

1.2 The pathophysiology of asthma

1.2.1 Airway hyperresponsiveness and allergen-induced airway response

Measuring airway hyperresponsiveness

As a key diagnostic feature of asthma that aids in the classification and management of the disease, airway hyperresponsiveness is defined as a predisposition to excessive airway narrowing in response to bronchoconstriction-inducing stimuli[34,35]. The presence of AHR is linked to

increased disease severity[36], lung function decline[37], risk of exacerbation and treatment required for disease control[38]. Additionally, it assists in the diagnosis of individuals with no impairment of baseline lung function[34]. It's important to note that within individual patients, AHR changes over the course of the disease, and has been found to be exacerbated by factors such as seasonal allergy and infection, and reduced by treatment with corticosteroid or biologics[34].

Clinically, AHR is quantified as the dose of bronchoconstrictive stimulus inhaled that provokes a 20% or more reduction of FEV_1 (PC20)[34]. A variety of stimuli have been used and there are differences in the mechanisms of action among them. Histamine and methacholine directly induce smooth muscle contraction (though histamine has been found to also induce infiltration of immune cells including T cells[39], eosinophils[40] and mast cells[41]), while other agents such as mannitol, monophosphate AMP, hypertonic saline and exercise activate pathways leading to bronchoconstrictive mediator release and therefore these agents assess the releasability of mediators and the ability of ASM to respond[34]. These latter stimuli are considered to be indirect challenges[34]. It is important to understand the difference among these stimuli as they could reflect separate mechanisms resulting in AHR. For example, AHR induced by mannitol better correlates with eosinophilia and airway inflammation in general, while methacholine is more sensitive for AHR detection[42]. As an alternative to measuring FEV_1, which is highly dependent on patient cooperation, the forced oscillation technique is used during normal tidal breathing to measure respiratory resistance, a reflection of airway caliber, and reactance, as a measure of respiratory elastance[43]. A dose-response curve of a patient with AHR shows not only increased sensitivity to stimuli (*i.e.* reduced PC20), but also an increase in maximum response[34]. The resultant changes in FEV_1 not only yield information about airway narrowing, but also about closure of peripheral airways, characterized by simultaneous excessive decline of FVC[34]. There

is a great heterogeneity among patients in terms of whether airway narrowing or closure dominates the response, and the significance of such distinction requires further investigation[34].

Mechanisms of airway hyperresponsiveness

The detection of AHR as a diagnostic tool was popularized in the 1960s, but despite decades of research, the mechanisms contributing to airway hyperresponsiveness are still yet to be fully elucidated[34]. Airway inflammation and structural changes are thought to be the principal contributors to AHR[34]. AHR has been divided into persistent and variable components, with the former considered to originate from the structural changes and is identified preferentially by challenge with direct stimuli, while the latter is determined by the extent of inflammation at the time of testing and best reflected by challenge with indirect stimuli[44].

In terms of inflammation, a positive correlation between eosinophilia, or its associated biomarker FeNO and AHR has been observed[45,46]. Additionally, studies of patients undergoing allergen challenges have demonstrated a correlation between increased eosinophilia and increased AHR.[47] However, a link between neutrophilic inflammation and AHR seems to be lacking, though there is evidence indicating neutrophilia might contribute to airway closure[47]. Additionally, inflammation in general is thought to result in impairment of the epithelial barrier, tissue damage and release of other mediators that contribute to an elevated response to challenge with bronchoconstrictive stimuli[48]. Additionally, mediators released during the inflammatory processes act on the structural cells, resulting in remodelling and longer-lasting effects on AHR[48].

Thickening of the airway walls and alterations of ASM are the two aspects of remodelling that contribute to AHR[49]. In terms of gross pathology, the remodelling of asthmatic

airways is reflected by the overall thicker airway wall due to factors including thickened epithelium and basal membrane, subepithelial fibrosis and inflammation-associated edema, which results in the reduction of airway calibre[50]. Though this might not directly contribute to obstruction at the baseline level as the diameter of the lumen is not reduced to a degree that greatly impacts airway resistance, it does have a significant impact during broncoconstriction[50]. As resistance is inversely related to airway diameter, a similar degree of airway narrowing would result in a drastically higher increase of resistance (proposed to be to the 4^{th} power) in a narrower airway than in a wider airway[50]. Indeed, airway calibre and airway wall thickness demonstrate a positive correlation with AHR among asthmatic patients[51,52]. ASM has long been considered a main contributor to airway narrowing in asthma. Asthma-associated alterations of ASM is described in diverse aspects, including phenotype alteration, proliferation, force generation, migration, ECM and inflammatory mediator production [53]. However, a consensus is yet to be reached in terms of what changes it has undergone that is the most relevant to asthma pathophysiology. A detailed review on asthma-associated ASM alteration can be found in "Airway remodelling" sections below. Briefly, increased force generation due to extrinsic (changed induced by inflammatory mediators and ECM) and/or intrinsic (changes in contractile protein expression and signaling) factors impacting ASM, as well as impaired relaxation[54], promotes airway narrowing or closure. In addition, the distance between ASM and epithelium is decreased, especially in severe asthma[55], causing reduced load against which ASM contracts, which would also promote airway narrowing[56]. Similarly, thickened airway, due to particularly excessive ECM deposition and edema, has an effect of decoupling ASM from the tethering of the parenchyma, thereby reducing the relaxation effect caused by cyclical stretching from breathing movements and potentiating airway narrowing caused by ASM contraction[56,57]. The

inflammatory mediators also act on ASM. ASM is able to adapt to a basal tone, which enhances its capability of further force generation, a phenomenon referred to as force adaptation. An increased presence of endogenous bronchoconstrictive mediators such as CysLTs and histamine could contribute to increased basal ASM tone, which exacerbates further contraction[58].

Finally, genetic and epigenetic factors are proven to contribute to AHR, as it is observed to be heritable, and linked to a large number of genes determined by genomic-wide association studiesy[59,60]. Studying the genetics of AHR could benefit the effort of better phenotyping the disease and advancing personalized medicine[61].

Allergen-induced airway response

In close relation to AHR measurements described above, allergen-induced airway response is studied among atopic asthmatic patients and in animal models of asthma, in which subjects are challenged with inhaled allergens and monitored for a prolonged period of time (6 hours to several days) for the assessment of lung function and inflammation[62-65]. The challenge triggers immediate bronchoconstriction (termed the early airway response, or EAR) occurring within 30 minutes of challenge reflected by declines in FEV_1 or increased airway resistance. However, such response quickly dissipates by 3 hours but recurs among some subjects approximately 6-12 hours after the challenge (termed the late airway response, or LAR). This is accompanied by increased methacholine-provoked AHR and sputum eosinophilia that persist for several days after the challenge[66,67]. It has since been established that EAR and LAR are mediated by partially overlapping molecular pathways, with potential differences in their cellular sources. Antigen-specific IgE cross-linking upon binding to allergen is primarily responsible for EAR, which activates FcεR1 on mast cells and triggers the release of bronchoconstrictive agonists including

histamine, CysLTs, PGD_2 as well as cytokines including IL-3, IL-4, IL-5 and IL-6 that initiate inflammatory cell recruitment[68]. The LAR is also generally initiated by IgE-dependent events and corresponding histamine and CysLT release, as antihistamines, CysLTR1 antagonists and CysLT synthesis inhibitors are shown to effectively inhibit both EAR and LAR[69-71]. However, as the release of these mediators is also accompanied by the infiltration of basophils, eosinophils, and additional mast cells, the source of these bronchoconstrictive mediators is less certain[72]. It is important to note that not all subjects demonstrate LAR upon allergen challenge, and its occurrence is dependent on the type of allergens[72,73]. In addition, the development of LAR following allergen challenge correlates with increased severity of the subsequent AHR and airway inflammation[67,74]. Inhaled corticosteroid and anti-IgE biologics also effectively inhibit the LAR and associated inflammation while having no significant impact on the EAR[75,76]. These observations collectively demonstrate that the LAR is mediated by allergen-induced cellular infiltration and inflammation[72].

1.2.2 Asthma-associated airway remodelling

Asthma-associated airway remodelling refers to pathological, irreversible changes in almost all structural components of the airways, which favour increased susceptibility to airway narrowing and promote inflammation[77]. These alterations are proven to be clinically significant and contribute to disease severity and decline in lung function, partly via its contribution to AHR as discussed above[77].

Airway epithelial shedding and barrier dysfunction

Being the primary site of exposure to pathogens and insults, the airway epithelium serves not only as a physical barrier, but also as a source of soluble factors that signal immune responses or trigger alterations of other structural elements. Epithelial damage and shedding of ciliated cells have long been observed in asthma, primarily among the post-mortem studies[78]. Increases in sloughed epithelial cells, some known as Creola bodies, have also been reported in bronchoalveolar lavage (BAL) fluid and in the sputum of asthmatic patients[79]. However, these conclusions remain somewhat controversial, as the observations are not found in other similar studies[80]. It has also been suggested that the finding of epithelial damage and sloughing merely reflects the artifacts originated from the sampling techniques[81]. The increased expression of molecular markers such as HSP70, EGFR and CD44[82,83] and activation of inflammatory signaling pathways such as NF-κB, STATs[82] in the asthmatic airways further indicate the presence of airway epithelial stress and injury.

One of the widely accepted causes of epithelial shedding is the cytotoxic factors released during eosinophil degranulation[84]. A reduction in the tight junction protein claudin-18 has been reported in asthmatic airway epithelium compared to healthy controls and is proposed to contribute to barrier dysfunction[85]. Evidence also suggests the overall pro-inflammatory milieu in asthmatic airways is also detrimental to the barrier function. *In vitro* studies have demonstrated that both Th1 cytokines (IFN-γ and TNFα) and Th2 cytokines (IL-4 and IL-13) disrupt the airway epithelial barrier integrity, as reflected by reduced trans-epithelial electrical resistance and junctional protein disassembly[86,87]. A reduction in cell survival signals such as Rb and Bcl-2 in the epithelium, and elevated presence of apoptotic cells measured by TUNEL assay are observed only in severe asthma[80]. Overall, the injury and shedding of epithelial cells in

asthmatic airways confer loss of barrier function, which predisposes the underlying tissues to increased exposure to allergens and irritants.

Epithelial proliferation

Epithelial hyperplasia and metaplasia are commonly observed following airway inflammation. In asthma, epithelial shedding is quickly followed by restitution processes featuring increased proliferation, indicating overall increased epithelial turnover[82]. Increased thickness of asthmatic airway epithelium and Ki-67+/PCNA+ cells are also evidence of epithelial hyperplasia[80,88]. The activation of the EGFR, which in the physiological context acts as an injury/repair pathway, is largely responsible for epithelial hyperplasia in asthma[89]. EGFR activates downstream pathways including MAPK, PI3K/Akt and PKC, which subsequently drive cellular proliferation and survival[89]. Increased expression of EGFR as well as its ligands EGF, HB-EGF and amphiregulin have been reported in asthmatic airway epithelium compared to a control group[90,91]. Histamine has been proven to play a role in upregulating the expression of HB-EGF and amphiregulin in cultured airway epithelial cells, which subsequently lead to increased proliferation and goblet cell differentiation[90]. *In vitro* studies have demonstrated that IL-13 promotes ADAM17-dependent release of one of the EGFR ligands, TGFα, which activates the EGFR in paracrine manner to induce proliferation of airway epithelial cells[92,93]. In a rat model of asthma induced by OVA sensitization and challenges, cysteinyl leukotrienes are responsible for HB-EGF upregulation in airway epithelium and subsequent increase in proliferating epithelial cells, which was effectively inhibited by both EGFR and CysLTR1 inhibitors[94]. Overall, the dysfunctional repair mechanisms of asthmatic airway cause epithelial hyperplasia with altered barrier characteristics that contribute to pathophysiology[89].

Mucus gland and goblet cell hyperplasia

Mucus is secreted by goblet cells and submucosal glands found in the larger airways, which serves as an essential component of mucociliary clearance to facilitate the elimination of pathogens and foreign particles[95]. Increased numbers of goblet cells, submucosal glands as well as increased quantity of mucin stored in intracellular vesicles have been observed in asthmatic patients and various animal models of asthma, which contributes to increased sputum production especially during exacerbations, airway obstruction and decline of the FEV_1[96]. MUC5AC and MUC5B are the most abundant gel-forming mucins expressed in the airways.

Similar to the induction of airway epithelial proliferation, EGFR activation also plays an important role in directing the differentiation of airway epithelium, mainly ciliated cell and club cells, into goblet cells. Intratracheal instillation of TGFα in rats induced goblet cell hyperplasia, which was potentiated by TNFα due to its effect on upregulating EGFR in the epithelium[97]. An EGFR inhibitor effectively reduced the quantity of goblet cells in OVA-sensitized and challenged rats[97]. IL-13 also induces goblet cell metaplasia in rat, which is dependent on EGFR activation and neutrophil-derived TNFα[98].

ASM changes

The causes of changes in ASM have been categorized into intrinsic and extrinsic factors. The former refer to the functional and biomechanical alterations that impact its force generation, while the latter is considered to be a result of the inflammatory milieu of the asthmatic airways[34]. Research on the innate biomechanical properties of airway smooth muscle did not yield a unifying observation generalizable to all studies. This is possibly due to the heterogeneity of

asthma as a disease, the limited availability of human bronchial tissue from organ donors who are impossible to be fully characterized at the time of harvest[99]. Some investigators have reported increased force generation by asthmatic ASM[100,101], and that smooth muscle contractile protein expression is inversely correlated to PC20 of methacholine[102]. However, these observations were not replicated by other studies[103,104]. It seems AHR in asthma cannot be simply explained by increased force generation of ASM, at least not in all subjects. Rather, some have proposed that asthmatic airway smooth muscles feature an increase of shortening velocity, likely as a result of MLCK upregulation, yielding faster myosin phosphorylation and actin-myosin cross-bridge cycling[105]. This observation is reported more prevalently among studies of human tissues and animal models[106-108], with some exceptions[104]. If asthmatic ASM indeed possesses higher shortening velocity, it is particularly detrimental as faster shortening could counteract tidal breath-mediated airway stretching and calibre increases, causing heightened response to bronchoconstrictors[99,109]. Indeed, in a study of *in vitro* rat ASM tissue with oscillating force mimicking tidal breath- and deep breath-mediated stretching, a higher shortening velocity corresponded to increased rate and amount of shortening[110].

Cytokines such as IL-13 and TNFα increase ASM force generation by enhancing agonist-induced intracellular calcium signals[111]. An increase of ASM mass, potentially due to both hypertrophy and hyperplasia has been observed in asthmatic airways [112,113] and it is positively correlated to disease severity[112,113]. With these observations, it is also widely held that increased overall quantity of ASM results in elevated force generation[112,113]. Growth factors upregulated in asthma, including PDGF, TGF-β and EGF have all been demonstrated to promote ASMC proliferation, with TFG-β being shown to also induce ASMC hypertrophy[94,114,115]. Beyond ASMC proliferation, other cellular sources contributing to the increase of ASM mass are also of

interest[116].The recruitment of circulating pericytes to the airway has been described in a mouse model of airway remodelling, which may contribute to ASM mass[117]. Increased circulating and airway fibrocytes, which are derived from mesenchymal progenitor cells, have been observed among asthmatics, and are present in the ASM layers[118]. It also have been suggested that airway epithelial cells could undergo epithelial-mesenchymal transition, forming fibroblast-like cells and contribute to increased ASM mass in a similar manner, but direct evidence proving the occurrence of such phenomenon is lacking[116].

Interestingly, direct ASMC-immune cell interactions have been reported in the context of asthma, which potentially contribute to ASM changes and reactivity. Mast cells are localized within ASM bundles, with a significantly higher number in asthmatic airways[119]. ASM-derived CXCL10 and CX3CL1 are shown to be responsible for the recruitment of mast cells[120,121]. In addition, they were described to produce IL-4 and IL-13[122], induce ASMC proliferation through a PAR-2-dependent pathway[123] and potentiate contraction via the secretion of tryptase[124]. Similarly, increased T cell infiltration in ASM bundles has been reported in bronchial biopsies of asthmatic patients[113]. These T cells frequently are in juxtaposition to proliferating ASMCs, and their quantity is correlated with increased ASM mass and disease severity[113]. Similar observations have been reported in an antigen-specific model of asthma in rats[125]. Additionally, the latter study has shown the migration of adoptively transferred, antigen-specific CD4+ T cells towards ASM bundles, and demonstrated the pro-proliferative effect of the T cells to ASM *in vitro*[125]. Co-culture studies of activated human CD4+ T cells and ASMCs demonstrated that T cells promote ASMC proliferation via ICAM-1-, VCAM-1 and CD44-mediated direct adhesion[126], as well as activation of the EGFR pathway[127].

Subepithelial fibrosis

Subepithelial fibrosis is characterized as an excessive deposition of ECM proteins such as collagen types I, III, IV, fibronectin, proteoglycan and tenascin in the lamina reticularis[128]. The presence of subepithelial fibrosis is correlated to FEV_1 decline and increased disease severity and AHR[129,130]. Airway fibroblast activation and differentiation into myofibroblasts, accompanied by excessive secretion of ECM protein is primarily responsible for such changes[131]. Studies show that TGF-β derived from epithelial cells or eosinophils might play a central role in mediating related pathological changes of the fibroblasts[132-134]. Furthermore, an imbalance between the activity of protease and its inhibitor, namely a decrease in the ratio of the former to the latter, also contribute to excessive subepithelial ECM deposition. For example, IL-4 results in a reduction of MMP-2 expression while causing an increase in its inhibitor TIMP-2 expression by bronchial fibroblasts[135]. Furthermore, an increase in the level of both MMP-9 and its inhibitor TIMP-1, yet a decrease in MMP-9/TIMP-1 ratio is found in the sputum of asthmatic patients, with the ratio also positively correlated with FEV_1[136]. It is proposed that subepithelial fibrosis contributes to AHR by increasing airway wall thickness and potentially influencing the mechanics changes related to airway stiffness[137]. However, with the observation that AHR alone could result in subepithelial fibrosis, it is debatable if it indeed contributes to AHR though being a key feature of airway remodeling[138]. Rather, it might simply be a consequence of AHR instead with no association to airflow obstruction[138].

Angiogenesis and vascular remodelling

Angiogenesis refers to the formation of new blood vessels[139]. Changes of the airway vasculature, including not only angiogenesis, but features like vasodilation[140], increased vasculature size and permeability[141] are well documented in both mild[142] and severe asthma[143]. These changes are proposed to be mainly caused by VEGF derived from immune cells and the airway epithelium[144]. VEGF primarily activates vascular endothelium to form new blood vessels while increasing its permeability[139]. Other angiogenic factors, including basic fibroblast growth factor, angiogenin, flt-1 and flk-1 are also associated with increased angiogenesis in the airway[144,145]. Increased neovasculature, accompanied with their increased permeability promotes airway edema as well as immune cell infiltration[146]. Indeed, the VEGF level in sputum and BALF is correlated with increased disease severity[144]. Increased vascularity observed in airway biopsies is also linked to increased AHR[147]. In rodent models of asthma, inhibition of the VEGF pathway resulted in a reduction of airway inflammation, AHR[148] and features of airway remodelling[149]. Overall, therapeutics targeting angiogenic factors upregulated in asthma have promising potential in alleviating the airway structural changes associated with the disease, although the side-effects likely would outweigh the benefit derived.

1.3 The immunology of asthma

1.3.1 Atopic response and Th2 immunity

Th2 cells and Th2 cytokines

With atopic asthma being the most common endotype, Th2 cells have long been considered the principal actor of asthma-associated lung inflammation[150]. It is believed that the nature of the allergen determines DC responses, which drive Th2 polarization during antigen priming and T cell activation[150,151]. Upon exposure to allergens and irritants, airway epithelial cells are activated via various TLRs and protease activated receptors, and release cytokines such as IL-1, GM-CSF,

IL-25 and TSLP[151,152]. These factors drive DC into the cDC2 phenotype, identified by the expression of IRF4, OX-40L, CD86, CCL17 and FcεRIa in human, which processes the antigens and migrates to draining lymph nodes to instruct Th2 priming via the MHC-II complex[151-153]. In addition, the relatively recent discoveries of ILCs has revealed that the activated ILC2 in the lung, which will be described in the following section, are a potent innate source of Th2 cytokines, including IL-5 and IL-13[154]. These cytokines act on several different cell types and further promote Th2 polarization[150,154].

However, the parameters regularly assessed during clinical diagnosis, such as eosinophilia, FeNO and IgE are often downstream consequence of Th2 inflammation[150]. In contrast, it rarely involves the direct characterization of Th2 populations, despite its central role in atopic asthma[150]. Th2 cells primarily feature the expression of transcription factor GATA3 and signature cytokines including IL-4, IL-5 and IL-13[150]. Their exact phenotype in terms of the activation and memory state differs among tissues and alters throughout the course of the disease[150]. The distinct characteristics of pathogenic Th2 cells are better understood thanks to recent advances of single cell transcriptomic approaches. Compared to Th2 cells in healthy individuals, the pathological Th2 cells in asthmatic patients demonstrate a distinct transcription profile, including the expression of hematopoietic PGD2 synthase (HPGDS), PPARG, CRTh2, IL17RA (IL-25 receptor) and develop a phenotype overlapping with Th9 cells via the expression of IL-9 and the transcription factor PU.1[155-157]. Th2 cells are enriched in asthmatic patients, and the number of allergen-specific Th2 cells and their cytokine production capacity rapidly increase during allergen exposure or viral exacerbation[158,159].

IL-4 is the first cytokine proven to be produced by the mast cell,[160,161] and is the key factor driving Th2 polarization[162]. IL-4Rα subunit is primarily associated with the intracellular STAT6 signaling pathway[163]. It dimerizes with either the common γ chain (γc) to form IL-4R, which is primarily expressed on hematopoietic cells, or with the IL-13Rα1 subunit to form IL-13R, found on a wider range of cell types[164]. T_{FH} is a major source of IL-4 in lymphoid tissues, which directs B cell IgE isotype switch and drives IgE and IgG1 synthesis[165]. IL-4 also induces the upregulation of FcεR expression on B cells, monocytes, mast cells and basophils[165], as well as stimulates the expression of adhesion molecules such as ICAM-1 and VCAM-1 in endothelial cells, which promotes inflammatory cell extravasation[166]. IL-4 has been demonstrated to act directly on airway epithelium and is responsible for goblet cell metaplasia and mucus hypersecretion[167]. Elevated IL-4 levels in sputum and serum of allergic patients, and the induction of IL-4 by allergen challenges have been reported[168-170]. The administration of nebulized IL-4 in asthmatic patients aggravated AHR and eosinophilia[171]. The humanized anti-IL-4Rα monoclonal antibody dupilumab is now a widely recognized therapeutic for severe asthma. Its efficacy in reducing the annualized asthma exacerbation rate (AAER), eosinophilia, IgE, FeNO and other Th2 inflammation markers, as well as improving asthma control and FEV_1 have been demonstrated in a phase 3 trial.[172]

With function and biology highly related to IL-4, IL-13 signaling through IL-13R activates STAT3 intracellular signaling pathway via the IL-13Rα1 subunit in addition to STAT6[164]. The soluble subunit IL-13Rα2 has been demonstrated to be expressed by epithelial cells, and act as a decoy receptor to dampen IL-13 signaling[173]. Clinically, IL-13 has been shown to be induced by allergen challenge, and its level in sputum correlates with eosinophilia[174,175]. An increase in IL-

13 expression in sputum or bronchial biopsy has also been reported among severe asthmatics and is correlated with decreased asthma control and loss of corticosteroid responsiveness[176]. Beside Th2 and ILCs in the airways, IL-13 is also produced by NK T cells, basophils, eosinophils, mast cells [173], and targets diverse structural cells. Like IL-4, IL-13 promotes isotype switching to IgE in B cells[177], acts on epithelial cells to drive goblet cell metaplasia and increase MUC5AC expression, as well as altering epithelial ion transport capacities to further enhance mucus secretion[178,179]. IL-13 promotes eosinophil recruitment via the upregulation of eotaxin-1[180]. It has been demonstrated that IL-13 plays the most prominent role in inducing AHR, while it appears to be independent of IL-4 or IL-5[181]. IL-13's effects on promoting ASM proliferation and contractility[111,182,183], mediating airway fibrosis via driving myofibroblast transformation and ECM deposition[184], and inducing mucus secretion and epithelial change as discussed above may collectively contribute to asthma-associated AHR[185]. However, despite the prominent roles of IL-13 in asthma immunopathology, clinical trials of the IL-13-targeting biologics to date overall have shown disappointing therapeutic efficacy[186]. Tralokinumab, an anti-IL-13 monoclonal antibody has been evaluated for its efficacy in improving asthma control, reducing exacerbation and sparing the use of corticosteroid among severe asthmatic in various clinical trials, and showed some effect on reducing exacerbations among patients with high serum periostin, a biomarker for the Th2-high endotype. However, it demonstrated very limited benefit for the rest of the abovementioned endpoints[187-190]. Similarly, lebrikizumab, an anti-IL-13 monoclonal antibody specifically blocking IL-13's interaction with the IL-4R subunit, showed certain efficiency in reducing the rate of exacerbation and increasing FEV_1 in phase 2 studies among patients with moderate to severe asthma, which failed to be reproduced in a phase 3 trial[191]. It has been proposed that anti-IL-13 therapy might only be effective for a subset of patients with

prominent AHR, ASM changes and mucus hypersecretion, as these features are the most predominately attributable to the action of IL-13[186]. It is also possible that the therapy might require dual blockade of another pathway to become effective, as many other cytokines have redundant functions[186].

IL-5 is an important cytokine for eosinophil functions as it has been shown direct eosinophil differentiation, maturation, migration and survival[192]. It is released by Th2 cells, ILC2s in response to alarmin stimulation, NK cells T cells, mast cells and eosinophils[193]. Upon IL-5 binding, the IL-5Rα chain associates with the nonspecific IL-5Rβ chain, which also shows affinity for the receptors of IL-3 and GM-CSF, to form a heterodimer receptor[193]. This activates intracellular signaling pathways including STAT1/3/5, which provides an eosinophil proliferative signal, as well as Ras-Raf-1 and NF-κB, which induce the expression of anti-apoptotic factors and pro-inflammatory cytokines[193]. In asthmatic patients, IL-5 has been shown to induce eosinophil differentiation in both the bone marrow and in the airway mucosa from CD34+ IL-5R+ progenitor cells following allergen challenges[194]. In mouse models of asthma, IL-5 has been demonstrated to play a role in airway remodelling, specifically subepithelial fibrosis[195]. Anti-IL-5 treatment in asthmatic patients also has shown an effect in reducing subepithelial ECM deposition assessed in airway biopsy[196]. High serum IL-5 has been reported in patients with severe disease[197]. As corticosteroid is efficient in inducing eosinophil apoptosis, eosinophilic asthma is generally responsive to corticosteroid treatment. However, there is still a subset of eosinophilic asthma patients demonstrating steroid resistance, which is caused by excessive IL-5 in the airway counteracting the effects of corticosteroids[198].

Currently, there are three monoclonal antibodies targeting the IL-5 signaling pathway approved for clinical use for treating asthma, however, the use of which is limited primarily to patients demonstrating the eosinophilic endotype[193]. Mepolizumab and reslizumab target the cytokine molecule itself to prevent its binding to IL-5R. Both have demonstrated efficacy in reducing exacerbations and improving FEV_1 among moderate to severe eosinophilic asthma patients[199-202]. Benralizumab, the anti IL-5Rα monoclonal antibody developed more recently, acts to prevent ligand binding as well as to initiate NK cell-mediated eosinophil killing via the engagement of FcγRIII on NK cells by the antibody's Fc region. In addition to inhibiting IL-5 signaling, it has been shown to be effective in depleting eosinophils and basophils in the circulation and tissue, and preventing their recruitment to the lung[192]. Clinical trials have shown its efficacy in reducing exacerbations, improving asthma control, FEV_1 and sparing oral corticosteroid intake among severe eosinophilic asthma patients[203-205].

ILC2s and alarmins

ILCs are a group of lymphoid cells lacking antigen specific T cell receptors, with subsets ILC1, ILC2 and ILC3 mirroring the phenotypes of Th1, Th2 and Th17, respectively. ILC2s are prominently found in the lung in human and mice, and the transcriptional factors RORα and GATA3 are necessary for its lineage commitment and polarization[206]. The ILC2 population undergoes activation and expansion upon stimulation by alarmins IL-25, IL-33 and TSLP, all of which have been detected at an increased level in asthma and in animal models[207-209]. The contribution of ILC2 to atopic asthma has been verified in murine models. Challenges with HDM or papain are sufficient to induce of IL-5 and IL-13 by ILC2s that facilitate the adaptive Th2 response[210,211]. Despite its rarity in number, the ILC2 is a prominent source of Th2

cytokines, and is sufficient to induce eosinophilia in Rag knockout mice, which lack T cell and B cell responses[206]. The ILC2 alone is also sufficient to induce goblet cell metaplasia and AHR in mice[212,213].

Similarly, among asthmatic patients, ILC2 numbers in BAL and blood, along with their Th2 cytokine-secreting capacities are increased[214,215]. Sputum and peripheral blood ILC2 frequencies show a positive correlation to eosinophilia and FeNO and negative correlation to FEV_1[216,217]. Additionally, ILC2 frequencies in blood and sputum have been shown to be increased in severe asthma compared to mild asthma[218]. However, given the overlap of ILC2 and Th2 functions, it is difficult to study the definitive isolated role of the former in allergic diseases in human, as there lacks an equivalent experimental model[150,151].

The ILC2-stimulating alarmins are considered to mostly originate from the airway epithelium, following injury or activation of a wide variety of pattern recognition receptors[219,220]. Their expression has also been shown in a large variety of other structural cells and leukocytes[219,220]. IL-25, (or IL-17E) was first described to induce Th2 responses by acting on its cognate receptor IL-25R (or IL-17RB)[221]. IL-25R consist of IL-17RA and IL-17RB subunits, and its activation results in intracellular signaling including the NF-κB, MAPK and STAT5 pathways[222]. Increased IL-25 expression has been linked to the Th2-high endotype, and positively correlates to AHR, eosinophilia and IgE levels[207]. Evidence also suggests that IL-25 may directly contribute to several features of airway remodelling, especially to thickening of the epithelial layer and to subepithelial fibrosis[207,223-225]. IL-25 may potentiate IL-33's action by upregulating the expression of its receptor[226]. Currently, there is no prominent anti-IL-25 biologic developed for clinical studies for treating asthma[227], although evidence suggests that anti-IL-25 therapy might

be particularly beneficial for treating virally-induced asthma exacerbations by reducing Th2 cytokine production while supporting anti-viral immunity[228].

IL-33, unlike most other cytokines regulated transcriptionally, is constitutively expressed and localized in the nuclei of epithelial cells[229]. It is released upon cellular injury, or alternatively, secreted by living cells via mechanisms yet to be fully elucidated[230]. The full-length IL-33 can be cleaved by allergen-derived proteases, or endogenous proteases including tryptase and chymase, which results in an increase of its signaling activity[231]. ST2 (or IL-1RL1), the receptor of IL-33, primarily activates NF-κB and MAPK intracellular signaling pathways. The elevated levels of IL-33 and its receptor ST2 in sputum, BAL, bronchial biopsy and serum have been linked to increased disease severity[208,232]. The serum IL-33 level is associated with eosinophilia and the Th2-high asthma endotype[233]. The SNPs in IL-33 and its receptor ST2 are among the ones most strongly linked to asthma susceptibility described in several genome-wide association studies[234-236]. Serum IL-33 has a Th2-inducing effect at mucosal sites more potently than IL-25[226]. IL-33 stimulates IL-13 release by mast cells, which promotes ASM contractility *in vitro*, a mechanism potentially explaining the positive correlation between IL-33 and AHR among asthmatic patients[237]. Various biologics targeting IL-33 are currently under investigation. Among which, itepekimab showed promising efficacy in reducing the incidence of loss of asthma control among adults with moderate to severe asthma[238]. However, its effect was not superior to dupilumab monotreatment, and the combination of the two treatments did not demonstrate additional benefit[238]. ST2-targeting astegolimab was also demonstrated to be effective in reducing AAER among severe asthmatic patients[239].

TSLP is closely related to IL-7[222]. Its receptor consists of a TSLPR and an IL-7Rα subunit, and activates intracellular signaling pathways including STAT3, STAT5, NF-κB,

MAPK and PI3K pathways upon ligand binding[222]. TSLP stimulates the expression of OX40L on DC, which promotes Th2 polarization during antigen presentation[240]. Increased TSLP levels can be detected in serum, BAL, sputum and exhaled breath condensate from asthmatic patients[208,241,242]. TSLP transcript level in airway epithelium is correlated to Th2-associated chemokines and disease severity[151,243]. Interestingly, increased TSLP level has also been found to associate with neutrophilia[151]. TSLP stimulation results in resistance to corticosteroid inhibition in ILC2 cells[209,198]. Tezepelumab, a humanized monoclonal antibody targeting TSLP has been approved in US and Canada for treatment of severe asthma. Its efficacy in reducing AAER, improving lung function and quality of life has been demonstrated in clinical trials[244]. Tezepelumab also effectively reduced serum IgE, FeNO and eosinophilia[244].

It is also important to recognize that the receptors for these alarmins are expressed in many cell types other than ILCs, namely T cells, macrophages and many structural cells, the functions of which largely awaits further exploration[222]. For example, direct actions of all three alarmins on CD4+ T cells to promote Th2 cytokine production have been demonstrated[222]. DCs transform into a phenotype that directs Th2 polarization and Th2 memory cell activation in response to all three alarmins[222]. IL-33 and TSLP both have been shown to support eosinophil adhesion and survival and drive M2-polarization of macrophages[222].

B cells and IgE

B cells mature and gain diversity in the bone marrow by undergoing V(D)J recombination. In the germinal center of the lymphoid tissues, B cells further gain diversity through somatic hypermutation, and antigen-specific cells undergo clonal expansion upon interaction with cognate follicular helper T cells[245]. In response to IL-4, IL-13 and OX40L signaling, B cells

undergo isotype switching to IgE directly from IgM, or indirectly through IgG and IgA intermediates[245]. The former commonly occur in lymphoid tissue and the latter in the mucosa[245,246]. Allergen challenge induce rapid expansion of mucosal IgE+ B cell population, primarily via isotype switching[247]. Activated B cell differentiate into plasma cells, some of which migrate to bone marrow and become a sustained source of serum IgE[246].

In asthma, the main pathological role of IgE is the activation of mast cells and basophils through its cognate receptors. This occurs when IgE molecules cross-link following binding adjacently onto antigen. The cross-linked IgE subsequently binds to two FcεRI subunits and brings them into close proximity to initiate intracellular signaling[245]. The FcεRI exists in tetrameric ($\alpha\beta\gamma_2$) and trimeric form ($\alpha\gamma_2$), with the former expressed in basophils and mast cells, primarily mediating cellular activation and degranulation, and latter expressed on B cells and other antigen-presenting cells, mediating facilitated antigen presentation and IgE elimination[247]. FcεRII, or CD23, is expressed on B cells which mediates B cell differentiation, IgE production and interaction with epithelial cells[247].

Omalizumab is a recombinant antibody in which a complementarity determining region of murine anti-human IgE antibody is incorporated into a human IgG1 framework. It binds to the Fc-binding region of IgE, thereby preventing the activation of mast cells and basophils[248]. Omalizumab is one of the earliest biologics used to treat atopic diseases, and in various phase III clinical trials, it effectively reduced serum IgE by over 90%, which directly contributed to the reduction of exacerbations, use of corticosteroid, and to an increase in the quality of life among children and adults with moderate to severe asthmatics[249-253].

Eosinophils

As a major effector cell type in the associated asthma endotypes, the eosinophil contributes to disease pathology by inducing tissue damage, promoting airway remodelling, AHR, and additional inflammation. Besides IL-3, IL-5 and GM-CSF that are widely recognized to mediate eosinophil differentiation from CD34+ progenitor cells, maturation and recruitment as previously mentioned, the chemotactic factors involved in eosinophil recruitment include eotaxin-1, 2 and 3 (CCL11, 24 and 26), CCL5 and PGD_2[25]. Eosinophils are a notable source of IL-13 and CysLTs, which act on ASM and epithelium and induce their respective changes associated with airway remodelling as described[254]. Eosinophil-derived TGF-β, MMP-9 and TIMP-1 also contribute to the subepithelial fibrosis aspect of airway remodelling[254]. Upon degranulation, eosinophils release eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO)[25]. These molecules, evolved for defense against extracellular pathogens, contribute to the pathogenesis of asthma upon aberrant release[255]. ECP and EDN induce cytotoxicity by acting on the cell membrane to increase its permeability[255], MBP contributes to AHR via inducing histamine release from mast cells and basophils[256]. EPO catalyzes the reactions that generate reactive oxygen species which are also toxic to the cells at high abundance[255]. Collectively, these proteins cause tissue damage, further contributing to subepithelial fibrosis[25]. Lastly, eosinophils express MHC II and co-stimulating ligands. *In vivo* evidence has demonstrated eosinophil's capacity for lymph node homing and antigen presentation to T cells[257]. However, it is unknown whether this process confers any non-redundant function in relation to other antigen-presenting cells[257].

Mast cells and basophils

Mast cells are tissue resident cells that play a central role in immediate allergic reactions, and an IgE-mediated reaction further results in increase in their number[246]. Beside IgE, they can also be activated by IgG through FcγR, TLR ligands and complement[258]. Upon activation, they release preformed mediators stored in granules, predominantly histamine, proteoglycans, and neutral proteases such as tryptase and chymase. Histamine induces smooth muscle contraction, mucus secretion and activates nerve endings[258]. The proteases activate fibroblasts, digest ECM to promote immune cell infiltration and enhance bronchoconstriction[258]. Lipid mediator secretion by mast cells is initiated by phospholipase A2-mediated release of arachidonic acid, which is converted to prostaglandins and leukotrienes. Many other protein mediators, such as TNF-α and Th2 cytokines, could be induced conventionally and preformed and stored in granules of mast cells at the same time[258].

Basophils are granulocytes but share many features with mast cells, including activation by IgE-FcεR interaction, complement and TLRs, and secretion of Th2 cytokines[258]. Basophils produce leukotrienes but not prostaglandins[246]. The exact roles of basophil in asthma pathophysiology are poorly understood, beside that they are found in increased quantity in fatal asthma, and contribute to Th2 inflammation to a certain degree via IL-4 secretion and antigen presentation[258-260].

Lipid mediators

Lipid mediators are derived primarily from mast cells, eosinophils and basophils among many cell types[261]. Arachidonic acid is released from membrane phospholipids by phospholipase A2,

and is further transformed into leukotrienes and hydroxy eicosatetraenoic acids (HETES) by lipoxygenases (LOX), or prostanoids by COX-1 and 2, or epoxyeicosatrienoic acids (EETs) and HETEs by cytochrome P450 pathways[261].

CysLTs, including LTC_4, LTD_4 and LTE_4, play the most prominent role in airway narrowing in asthma. First termed slow reacting substance of anaphylaxis upon discovery, CysLTs are agonists of ASM contraction and bronchospasm with potencies 1000 times higher than that of histamine[262]. Their receptors, CysLTR1 and 2, are preferentially expressed on Th2 cells upon induction by IL-4 and 13[263,264]. Conversely, CysLT stimulation promotes Th2 polarization by inducing Th2 cytokine production and reducing IFN-γ expression[263,265,266]. CysLTs are also proven to be responsible for processes including promoting eosinophil recruitment and survival[267,268], upregulating ST2 expression on CD4+ T cells[269], mediating ILC2 activation and migration[270]. Most importantly, evidence to date demonstrates that CysLTs is responsible for almost all features of airway remodelling[271]. Montelukast and zafirlukast are the two leukotriene receptor antagonists, specifically CysLTR1 antagonists, currently approved for treating asthma[272]. In experimental models of atopic asthma, montelukast attenuated Th2 cytokine expression[265], AHR[266], goblet and smooth muscle hyperplasia and subepithelial fibrosis[265]. Montelukast as a monotherapy, or add-on therapy to ICS has generally been proven effective, among patients with mild or moderate asthma in clinical trials[273]. This is reflected by various endpoints including FEV_1, exacerbation and hospitalization rates, as well as quality of life[273]. Though inferior to corticosteroid, montelukast is especially beneficial among patients who have poor adherence to ICS[273]. It is also effective in improving asthma control among moderate to severe asthmatics who respond poorly to ICS[273].

The products of COX-1 and 2, referred to as prostanoids, include PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$ and thromboxane A_2[274]. Interestingly, though PGE_2 is a pro-inflammatory mediator in other parts of the body, it demonstrates mainly anti-inflammatory actions in the lung. Among its four cognate receptors, EP_2 and $_4$ are associated with Gαs, which activates adenylyl cyclase and initiate cAMP production. These receptors contribute to PGE_2-mediated bronchodilation, as cAMP leads to smooth muscle relaxation via inhibition of MLCK activity[275]. Furthermore, EP_2 and $_4$ activation has also been shown to promote epithelial and vascular repair, and inhibit fibrosis by suppressing fibroblast functions[274]. Among immune cells, PGE_2 exerts overall suppressive effects via diverse pathways on different cell types, notably the suppression of eosinophil migration and degranulation and the reduction of T cell and ILC2 proliferation[274]. However, EP_1 and $_3$ are canonically associated with Gq that induces intracellular calcium signal activation via inositol triphosphate signaling, resulting in airway constriction in mice via neuronal pathways[275]. This likely explains the finding that intrapulmonary administration of PGE_2 induces acute coughing via vagal nerve stimulation, which renders it unsuitable as a therapeutic strategy[276]. However, selective EP_2 and EP_4 agonists instead have promising therapeutic potential for their bronchodilator and immunomodulatory effects[274].

PGD_2 is another prostanoid prominently featured in asthma that plays an overall proinflammatory role by directly contributing to the development of Th2 immunity. It acts via its receptors DP1, CRTH2 and the thromboxane receptor (TP). Although DP1, expressed broadly on both structural and immune cells, is associated with Gs which generally suppresses target cell functions, evidence suggests it has mixed functions on immune cells. Selective DP1 agonism has been shown to inhibit chemotaxis of eosinophil, basophil, DCs and CD4+ T cells[277], to promote

Th2 polarization by inhibiting Th1 cytokine secretion by NK cells and DCs[278,279], as well as to suppress degranulation of mast cells and basophils[277]. However, signaling via DP1 promotes eosinophil survival[280]. DP1 knockout in a mouse allergic asthma model reduced Th2 inflammation and airway hyperresponsiveness.[281] DP1-specific antagonists also inhibited eosinophil recruitment in a guinea pig asthma model[282]. In addition, DP1 activation mediates bronchodilation and vasodilation, and the latter is potentially relevant to the pathology of allergic rhinitis[283]. CRTH2 is closely related to other chemokine receptors and its expression is limited to the immune cells. Its role in mediating chemotaxis of Th2 cells, eosinophils and basophils in response to PGD_2 has been prominently described[282]. It also stimulates Th2 cytokine production by T cells and ILC2s, as well as facilitates ILC2 activation by alarmins[284-286]. CRTH2 antagonists have been extensively studied for their therapeutic values in asthma and other allergic diseases[287].

Other lipid mediators have been found in increased quantity in asthma. For example, thromboxane A_2 mediates bronchoconstriction and contributes to airway remodelling and AHR[288]. 15-HETE in exhaled breath condensate has been proposed as a biomarker for childhood asthma[289]. LTB_4, a neutrophil chemoattractant, has been shown to also mediate IL-13 production by T cells[290]. However, their roles in asthma pathophysiology are relatively poorly understood and further characterization is required to determine whether they are viable therapeutic targets.

1.3.2 Immunity associated with Th2-low asthma

Th1 immunity: Th1 cells, CD8+ T cells, NK cells and interferons

Th1 immunity is primarily responsible for mediating cytotoxic responses to eliminate intracellular pathogens and tumors. Th1 and Th2 cells and associated mediators demonstrate

mutually inhibitory relationships functionally, hence the theory of Th1/Th2 imbalance that contributes to the immunopathology of atopic asthma[291]. IL-12 reverses GATA-3 expression[292], and IFN-γ inhibits Th2 cell proliferation[293]. On the other hand, GATA-3 represses IL-12 signaling essential for Th1 polarization[294], and Th2-derived IL-4 and IL-10 are also responsible for inhibiting Th1 differentiation and effector functions[293,295,296]. It is important to note the Th1 vs Th2 compartments are sometimes poorly defined, especially in human, with the description of CD4+ T cells expressing both IL-4 and IFN-γ, and the type 2 counterpart of CD8+ T cells (Tc2) and NK cells (NK2), which are conventionally regarded as effector cells of cytotoxic responses only[297]. However, with increasing understanding of the immunity and the complexity of asthma endotypes, it is gradually recognized that such a paradigm is an oversimplification of the immunopathology[297]. Additionally, the roles of Th1 immunity in asthma pathophysiology are controversial and yet to be fully elucidated. Experimental data supporting their pathogenic and protective roles in asthma have both been presented.

IL-12, IL-18 and CD80/CD86 are antigen presenting cell-derived factors that primarily determine Th1 polarization[298]. The IL-12-STAT4 signal and the IFN-γ-STAT1 pathways synergize to direct Th1 cell differentiation from native CD4+ T cells[298]. T-bet expression is also essential for the polarization, as it mediates the expression of IFN-γ and CXCR3 [299], among other Th1-associated genes, and can directly suppress GATA-3 function and Th2 differentiation[300,301]. A decrease in T-bet-expressing cells is observed in airway tissues of asthmatic patients compared to non-asthmatic controls[302]. T-bet knockout mice develop spontaneous "asthma"[302]. Adoptive transfer studies of Th1 cells in animal models of asthma have yielded mixed results. When *in vivo*-derived Th1 and Th2 are transferred together, Th2 immunopathology is suppressed in an IFN-γ-dependent manner compared to recipients of Th2 only[303]. An adoptively transferred Th1

clone in WT BALB/c reduced AHR, eosinophilia, goblet cells, airway fibrosis and Th2 cytokines induced by OVA sensitization and challenge, but increased other cell types among BAL cells[304]. On the other hand, another report demonstrated that transfers of OVA-specific Th1 into SCID mice did not inhibit Th2-mediated AHR and exacerbated airway inflammation[305]. Furthermore, OVA-specific CD4+ T cells driven into a Th1 phenotype *in vitro* were shown to be able to equally induce AHR independent of any Th2 cytokines[306], and exacerbate both endogenous Th1 and Th2 inflammation[307].

Similar to Th1 cells, CD8+ T cell activation requires antigen presentation and signals including IL-2 and IL-12[308]. The canonical function of CD8+ T cells is to perform cytotoxic killing by the release of granzyme, perforin and activation of apoptotic signals by Fas ligand. This is mediated by the ligation of antigen-specific TCR on the activated CD8+ T cells with the complex of MHC I-antigen complex on the target cells[308]. In addition, CD8+ T cells are also a potent source of cytokines such as IFN-γ and TNFα[308]. As a key factor of Th1 immunity, it has been hypothesized that CD8+ T cells should exert an inhibitory action on Th2 immunity. Early studies have prominently indicated that CD8+ T cells are capable of supressing IgE production, a process proven to be dependent on IFN-γ, IL-12 and IL-18[309-312]. Various studies in rats yielded a consistent finding that depletion of CD8+ T cells exacerbated inflammation, airway remodelling and AHR induced by OVA sensitization and challenges[313-315]. Further investigation into CD8+ T cell compartments in a mouse model showed that only central memory CD8+ T cells confer the protective effect as described, while effector CD8+ T cells exacerbated the features of asthma[316]. Clinical studies regarding CD8+ T cells in asthma, however, overwhelmingly indicated their detrimental roles in the disease. The number of CD8+ T cells in the lung correlates to increased disease severity, fatal asthma cases and decline of lung

function[317,318]. Furthermore, Tc2, or CD8+ T cells producing IL-4, 5 and 13, are prominently observed in human, which could contribute to asthma pathophysiology similar to Th2[319]. IL-13 and BLT1-expressing CD8+ T cells in the airways correlated to increase in IgE and decrease in FEV_1 among asthmatic patients[320].

NK cells are lymphocytes lacking a variable TCR with the primary role of mediating cytotoxic responses as an innate immune response. With functions largely similar to cytotoxic CD8+ T cells, NK cells are activated by IL-12 and 18, perform cytotoxic killing also via granzyme, perforin and FasL, and secrete Th1-associated cytokines including TNFα and IFN-γ[321]. In addition, FcγRIII expressed on NK cells confers their ability to induce antibody-dependent cytotoxicity[322]. The natural toxicity receptors (NKp30, NKp44 and NKp46), which are expressed almost exclusively by NK cells, also contribute to signals corresponding to cytotoxic killing and immune regulation[323]. Some clinical observations suggest an impairment of NK cell functions in asthma, including a reduced number in BAL and blood and decreased cytotoxic killing capacity seen in severe asthma[324,325]. In animal models of asthma, NK cell activity has also been shown to dampen airway inflammation[326,327]. However, the pro-pathogenic roles of NK cells in asthma have been equally demonstrated. Increased IFN-γ and granzyme B secretion in NK cells has been described specifically in neutrophilic asthma[328]. The IL-4-secreting NK 2 cells have been described with increased number in the blood in asthmatic patients[329]. Depletion of NK cells prior to OVA sensitization and challenge also impaired the development of Th2 immunopathology in mice[330].

Interferons are the key cytokines of a Th1 response, which play roles in stimulating the functions of many associated cell types as discussed, and initiation of anti-viral cellular responses[331]. Type 1 IFNs include 13 IFN-α subtypes in human and IFN-β, which all signal

through the IFNAR1/2 complex to activate STAT1/2 and 3 pathways. Plasmacytoid DCs are the main source of IFN-α, while IFN-β is secreted by diverse innate immune cells and structural cells in response to viral triggers[332]. IFN-γ is a type 2 IFN that signals through the IFNGR1/2-STAT1 pathway. Its source is mostly limited to T cells and NK cells[332]. Type 3 IFNs, or IFN-λs, act via the IFNL1/IL-10RB-STAT1/2 pathway and induce signaling and gene transcription largely similarly to type 1 IFNs. However, their action is more restricted to the mucosal sites[333]. In animal models of atopic asthma involving the administration of exogenous IFNs, these cytokines are usually shown to be protective, as demonstrated by reduced inflammation and lung dysfunction via their overall inhibitory effects on T_H2 inflammation, and *vice versa* when their signaling is disrupted. Systemic IFN-β treatment suppressed AHR and airway inflammation in a mouse OVA model[334]. Intranasal IFN-λ protein or overexpression of IFN-λ using an adenoviral vector suppressed IL-33 and TSLP in addition to Th2 cytokines and eosinophilia in similar mouse models[335,336]. Administration of aerosolized IFN-γ reduced allergen-induced eosinophil and CD4+ T cell infiltration [337], as well as inhibiting IgE production and AHR in a mouse OVA model [338]. The transfection of a DNA vector containing the IFN-γ gene at airway mucosa yielded similar effects on airway inflammation induced by conalbumin or adoptive T_H2 transfer [339]. Compared to WT mice, IFN-γR1 knock-out mice demonstrated a higher IgE level and greater T_H2 cytokine production by T cells upon OVA challenges, as well as impaired resolution of eosinophilia within two months following challenges [340]. These data are consistent with observations from several clinical studies in asthmatic patients. With many type 1 interferons as therapeutics approved for treatment of other conditions, studies have been performed investigating the effect of long term (more than 5 months) IFN-α or -β administration in asthma. The data, in general, showed that type 1 interferons improved lung function and reduced

glucocorticoid doses required for treatment[341,342]. In addition, in a study with a small group of mild atopic asthmatic patients BAL eosinophilia was also reduced by inhaled IFN-γ aerosol[343]. A deficiency of interferon induction has been observed in asthma among both adult and children, and is thought to contribute to poor control of infection and therefore exacerbations[344]. IFN therapies showed certain efficacy in reducing the worsening of the symptoms during exacerbation[344]. Meanwhile, data supporting the pathogenic role of IFNs in asthma also exist, including the observation that increased IFN-γ marks the severe asthma phenotype[29].

Overall, with largely conflicting evidence on the effect of Th1 immunity in asthma, we hypothesize that there is no simple, generalizable explanation of its role. Instead, the source and the targets of Th1 cytokines, the specific pathological context of the disease, the spatial and temporal properties of Th1 immunity induction, and the asthma endotype could all influence how it would interact with existing asthma immunopathology.

Th17 and neutrophilic inflammation

Th17 is a subset of CD4 T cells characterized by expression of the transcription factor RORγt and secretion of IL-17A, IL-17F, IL-21 and IL-22, and it differentiates from naïve CD4+ T cells in response to TGF-β, IL-6, IL-1β, and IL-23[345]. In addition, ILC3 also differentiate in response to similar stimuli and demonstrate a cytokine secretion profile largely overlapping with Th17[346]. IL-17A triggers IL-17RA-NF-κB signals in target cells, which result in the induction of neutrophil-associated chemokines, mainly IL-8 in human[347]. The IL-17A signal in airway epithelial cells is responsible for induction of mucus hypersecretion and goblet cell metaplasia[348]. IL-17A has also been shown to induce ASMC proliferation, while increasing its contractility in a RhoA pathway-dependent manner[345]. These mechanisms have been proposed to contribute to

airway remodelling and AHR[345]. IL-17A and IL-17F are responsible for neutrophil maturation and activation[345].

Neutrophils in asthmatic airways show elevated expression of FcεRI, and respond to cross-linked IgE to release cell secretory granules[349]. Other neutrophil-derived factors, such as elastase, MMPs and TGF-β are also implicated in asthma-associated airway remodelling[350]. Increased neutrophil extracellular traps have been detected in asthma, which has been proposed to result in loss of epithelial barrier function. However, their exact role in asthma awaits further characterization[351]. An increased Th17 inflammatory signature is described in a subset of Th2-low asthma and has been linked to a more severe asthma phenotype and corticosteroid insensitivity[346]. RORγt overexpression in mice resulted in the development of a neutrophilic, steroid-insensitive phenotype following OVA sensitization and challenge[352]. Similar observations were also described in mice adoptively-transferred with OVA-specific Th17 cells derived *in vitro.* IL-17A and IL-17F are detected at an elevated level in sputum and BAL of asthmatic patients and are positively correlated to AHR and disease severity[346]. While steroids effectively inhibit Th2 responses and induce eosinophil apoptosis, they do not have the same actions on Th17 and neutrophils[353]. Additionally, Th17 has been demonstrated to confer corticosteroid insensitivity by directly impacting glucocorticoid receptor signaling[353].

1.4 Macrophage immunomodulatory functions, polarization, and their involvement in asthma

The macrophages in the lungs are commonly categorized into alveolar, interstitial and recruited (monocyte-derived) macrophage populations. Compared to the latter two that are derived from bone marrow hematopoietic stem cells, the former, defined as $F4/80^{+}$, $CD64^{+}$, $CD11b^{-}$, $CD11c^{hi}$

and SiglecF^{+} in mice, has distinct origins from fetal monocytes in the yolk sac. They are tissue resident cells populated throughout the lung and mostly self-replicate to perform functions essential for lung homeostasis, including surfactant maintenance, tissue repair and pathogen clearance[354]. They are also among the first cells activated in response to pulmonary infections and a major source of cytokines that mediate the mounting of an immune response[354].

Interstitial macrophages, defined as F4/80^{+}, CD64^{hi}, CD11b^{+}, Ly6C^{lo}, are a tissue-resident population that also perform phagocytosis, and have been described to possess prominent immunomodulatory properties via the secretion of IL-10, particularly in response to bacterial products LPS and CpG, which has been demonstrated to suppress allergen-induced inflammation in an asthma model in mice[355]. The IL-10 producing interstitial macrophage is responsible for supressing HDM-induced IL-13 and IL-17 expression as well as neutrophilia[356]. They are at least partially derived from monocytes[355], but one recent study suggests their diverse sources, such as lung and splenic resident monocytes[357].

Monocyte-derived macrophages, defined as F4/80^{+}, CD11b^{+}, Ly6C^{hi}, are recruited to the lung in a highly CCR2-dependent manner in response to inflammatory signals[358]. They are generally considered as the proinflammatory population. Through the production of inflammatory mediators and facilitating T cell activation, they play an important role in defense against bacterial and viral infections, as the hindrance of their recruitment results in impaired pathogen clearance[358]. They have also been demonstrated to contribute to other lung pathology, including pulmonary fibrosis and cystic fibrosis[358]. In a mouse model of asthma, IL-13 promotes CCL2 expression and enhances monocyte recruitment[359]. The recruited macrophage population contributes to eosinophil infiltration[360]. Blockage of CCL2-CCR2 signaling in a mouse OVA model reduced monocyte-derived macrophage recruitment and decreased Th2 inflammation.

However, the pro-resolution and immunoinhibitory function of monocyte-derived macrophages has been gaining increasing recognition[358]. A monocyte-like population extensively studied in the context of cancer, recognized as myeloid derived suppressor cells, has prominent immunosuppressive functions, characterized by the expression of factors such as IL-10, TGF-β, arginase-1 and PD-L1, and participates in immune evasion and metastasis of cancer[361]. Specifically in asthma, contrary to the evidence supporting the pro-Th2 role of monocyte-derived macrophage mentioned previously, Th2 immunopathology associated with OVA sensitization and challenge is more severe in CCR2 knockout mice, or with disruption of CCR2 signaling[362-364], suggesting a protective role of monocyte-derived macrophages. Furthermore, monocyte-derived macrophages induced by various TLR ligands, such as poly I:C and CpG has been shown to traffic to lymphoid organs and suppress T cell priming in an IL-10-dependent manner, and inhibit Th2 immunopathology in the mouse OVA model[365,366].

Within each subset compartment described, the macrophage could undergo phenotypic polarization towards mainly M1 or M2 subsets in response to immunological stimuli, a process reminiscent of T cell polarization. The differentiation of M1, or classically activated macrophages, is driven by GM-CSF, LPS and IFN-γ, and is considered the pro-inflammatory subset, featured by the upregulation of iNOS, MHC II, T cell activation co-stimulatory molecules CD80 and CD86 and pro-inflammatory cytokines including TNFα, IL-6, IL-1β and IL-12[367]. Therefore, M1 mainly mediates immune activation and pathogen clearance processes, causing tissue damage, and has been implicated in severe asthma[367]. M2, or alternatively activated macrophages, have many sub-classifications depending on the specific combination of differentiation stimuli, which include Th2 cytokines and immunosuppressive signals including

glucocorticoid, IL-10 and TGF-β[367]. The M2 subset, marked by expression of arginase-1, CD163, CD206, produces Th2 cytokines and chemokines that could promote atopic diseases, but is also associated with repair and anti-inflammatory functions via enhanced phagocytotic capacity and production of IL-10 and TGF-β[367]. It is important to note that *in vivo*, macrophages do not exist as strictly polarized phenotypes as described. Rather, they are largely heterogeneous and display phenotypes and functions along a continuum depending in specific disease and inflammatory conditions[368].

1.5 Transcriptional regulatory pathways associated with smooth muscle contractile versus synthetic/proliferative phenotype dichotomy

Apart from the immunology of asthma and its impacts on the functions of ASMCs, this thesis also includes studies of basic smooth muscle biology that is important in ASMC phenotype determination. This last section in the literature review addresses a specific transcriptional regulation pathway governing smooth muscle phenotypic differentiation, which is of important relevance to our stud.

Smooth muscle cells are terminally differentiated yet are capable of undergoing drastic phenotypic alteration in response to external stimuli[369]. These changes are frequently observed in pathological contexts, and the associated changes in smooth muscle function also actively contribute to disease pathogenesis. Overall, smooth muscle takes on either contractile or proliferative/synthetic phenotypes. The former features prominent expression of smooth muscle specific genes, including α-SMA, MYH11, CNN1 and low proliferative capacity. The latter is marked by downregulation of smooth muscle specific genes and increased cell cycle progression and synthesis of inflammatory mediators and ECM[369]. As smooth muscle physiology has been a

major focus of research in cardiovascular diseases, smooth muscle cell phenotype switching was first described in VSM. In general, VSM with prominent expression of contractile protein and minimal proliferation is associated with the homeostatic state. In contrast, VSM in atherosclerotic lesions undergoes nearly complete loss of contractile gene expression, and instead acquires pro-inflammatory and proliferative signatures that contribute to further inflammation and lesion formation[369]. Similarly, pathological VSM alteration towards a proliferative/synthetic phenotype is also described in pulmonary hypertension, intimal hyperplasia and restenosis[369,370].

Following the discovery of MyoCD, the transcriptional co-factor playing a pivotal role in smooth muscle development via initiating the transcription of almost all contractile protein genes, it was then described that its transcription activity relied on SRF, the same DNA-binding factor utilized by the ternary complex factor, mainly Elk-1, for initiating mitogenic processes[371]. SRF binds to a DNA motif named CArG box, which exists in the promoter regions of both contractile protein genes and mitogenic genes[371]. A subsequent study demonstrated that activated (phosphorylated) Elk-1 could bind to SRF-DNA complexes at the promoters of contractile protein genes, thereby competitively inhibiting MyoCD-mediated gene transcription while initiating cellular proliferation[372]. Although there is no known mechanism on whether inversely, MyoCD could inhibit Elk-1 activity, *in vitro* evidence also indicated that MyoCD and Elk-1 could compete for SRF occupation, therefore an increase in the quantity of MyoCD could signify a depletion of SRF for Elk-1-related mitogenic function[372]. Together, these pathways provided a molecular biological underpinning to the dichotomy of smooth muscle proliferative versus contractile phenotypes. Later, the same group described the mutual inhibitory mechanism between MyoCD

and NF-κB via direct binding, providing an explanation of the contractile versus synthetic polarization phenomenon in smooth muscle[373].

It is important to note that to date, these fundamental discoveries on pathways related smooth muscle phenotype determination are exclusively made in VSMCs or related cell types. With the phenotype switching paradigm adopted into the studies of ASM pathological changes in airway diseases, supported by evidence of changes towards both hypercontractile and proliferative states as described in the "ASM changes" section above, investigating the MyoCD-Elk-1-SRF interactions would yield a more systematic understanding of ASM phenotype modulation in asthma and shed a light on potential therapeutic strategies.

In addition, ASM-derived inflammatory mediators, especially chemotactic molecules, have been gradually gaining attention as contributors to inflammatory processes. CCL11, IL-8, CCL5 are known to be produced by ASM, which play roles in macrophage, neutrophil and eosinophil recruitment[374-377]. CXCR3 ligands derived from ASM have been proven to drive the migration of mast cells into smooth muscle bundles, which confers further pathological changes in the smooth muscle[120]. With our observations of the T cell-ASM interactions *in vitro* and *in vivo* demonstrating that the two cell types promote mutual asthma-related pathological changes [113,125,127,378], the investigation into the recruitment of and homing signals for T cells into ASM bundles is of particular interest, as the interruption of which could ameliorate asthma-associated airway structural changes and associated pathophysiology.

1.6 Rationale, hypothesis and objectives

As described in the sections above, ASM change is one of the main features of airway remodelling and directly contributes to asthma-associated pathophysiology[128]. Alterations in

ASM phenotype has been proposed to occur in asthma, but based on the current paradigm of smooth muscle contractile versus proliferative/synthetic phenotype dichotomy as determined by the interactions between MyoCD and Elk-1[372], research to date has provided conflicting evidence on the direction of polarization seen in asthmatic ASMs. Furthermore, the observations on elevated CD4+ T cell colocalized with asthmatic ASM bundles[113,125] imply that ASMCs produce chemokines that are responsible for T cell recruitment and infiltration. Lastly, our observations on ASM implicate the potential roles of Th1 immunity in asthma, which inspired us to establish a mouse model of atopic asthma to study the general interactions between Th1 and Th2 immunity, with a viral-like PAMP, PIC, being the inducer of the former.

We hypothesized that like VSM, ASMC phenotype is determined by the relative activity of the transcription factors MyoCD and Elk-1, which demonstrate mutual inhibitory relationship via competitive binding to SRF, and promote the hyper-contractile and proliferative phenotypes, respectively. Furthermore, relating to the synthetic phenotype, ASMCs are capable of producing chemotactic factors responsible for the recruitment of CD4+ T cells. Lastly, we anticipate that in the mouse model of atopic asthma, PIC co-administered with allergen induces additional inflammatory mediators, mainly Th1-associated cytokines, which results in exacerbated airway inflammation and a distinct asthma endotype that differs from the Th2-high endotype.

Our objectives included: 1. To characterize the MyoCD-Elk-1interactions and their roles in the determination of smooth muscle phenotype in cultured ASMCs; 2. To explore ASM synthetic capacity, specifically pertaining to the production of CD4+ T cell-reactive chemokine that is responsible for mediating their infiltration into ASM bundles in asthma; 3. To investigate the effect of PIC as an inducer of Th1 immunity on allergic airway inflammation in mice.

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SRF expression in excess permits a dual contractile-proliferative phenotype of airway smooth muscle.

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Running title: SRF quantity decides myocardin-Elk-1 competition.

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Abstract

The transcription factors (TFs) myocardin (MyoCD) and ETS Like-1 protein (Elk-1) competitively bind to serum response factor (SRF) and control myogenic- and mitogenic-related gene expression in smooth muscle, respectively. Their functions are therefore mutually inhibitory, which result in a contractile versus proliferative phenotype dichotomy. Airway smooth muscle cell (ASMC) phenotype alterations occur in various inflammatory airway diseases, promoting pathological remodelling and contributing to airflow obstruction. We characterized MyoCD and Elk-1 interactions and their roles in phenotype determination in human ASMCs. MyoCD overexpression in ASMCs increased smooth muscle gene expression, force generation, and partially restored the loss of smooth muscle protein associated with prolonged culturing, while inhibiting Elk-1 transcriptional activities and proliferation induced by epidermal growth factor (EGF). However, MyoCD overexpression failed to suppress these responses induced by fetal bovine serum (FBS) as FBS also upregulated SRF expression to a degree that allowed unopposed function of both TFs. Inhibition of the RhoA pathway reversed said SRF changes, allowing inhibition of Elk-1 by MyoCD overexpression and suppressing FBS-mediated contractile protein gene upregulation. Our study confirmed that MyoCD in increased abundance can competitively inhibit Elk-1 function. However, SRF upregulation permits a dual contractile-proliferative ASMC phenotype, anticipated to exacerbate pathological alterations, whereas therapies targeting SRF may inhibit both pathological ASMC proliferation and contractile protein gene expression.

Key words: myocardin, Elk-1, smooth muscle contractility, cellular proliferation, smooth muscle phenotype.

Background

Despite its differentiated state, smooth muscle demonstrates phenotypic plasticity, as its characteristics and functions may undergo substantial changes in response to environmental factors such as matrix composition, inflammatory signals and mechanical stresses (1-3). According to current understanding, smooth muscle cells exist as either contractile or proliferative/synthetic phenotypes (1, 2). Phenotype changes in airway smooth muscle (ASM) and vascular smooth muscle (VSM) have been described in disease contexts (4-7). For example, an increase in ASM mass, through ASMC hyperplasia, has been observed in both asthma patients (8) and in animal models of asthma (9). The increased mass is correlated to disease severity (10-14), as it likely contributes to airway hyperresponsiveness (15). Some studies also suggest asthmatic ASM possesses hypercontractile characteristics, including increased contractile stress (force/cross-sectional area) (16) or altered calcium handling (17-19). VSM has been shown to exist in a hyper-secretory phenotype in atherosclerotic lesions, in response to the pro-inflammatory environment. Indeed, contractile and proliferative VSM cells have been proposed to be distinct populations (20). In hypertension, increased contractility, proliferation and extracellular matrix protein synthesis have been reported, potentially causing arterial wall stiffening and subsequent vascular disorders (4, 21).

The molecular mechanism underlying smooth muscle contractile versus proliferative phenotypic dichotomy involves two distinct sets of genes differentially regulated by the same ubiquitous DNA-binding protein, serum response factor (SRF) (22). SRF forms a homodimer and binds to a DNA *cis* element called serum response element (SRE), at a specific sequence known as the CArG box normally found within the promoters or first introns. It has low intrinsic transcriptional activity, with its function being mainly determined by its cofactors (23). Elk-1 is a

member of the ternary complex factor activated by mitogen-activated protein kinases (MAPK)/ extracellular signal-regulated kinase (ERK) pathway via phosphorylation. Upon activation, the B box domain of Elk-1 binds to SRF while its A box domain bind to the ETS-binding domain on DNA to initiate transcription of growth-related genes, most prominently the immediate early genes including Fos, c-jun and Egr-1 (23). MyoCD is another co-factor of SRF with strong myogenic properties, as it specifically initiates transcription of smooth muscle and cardiac muscle genes upon association (24). Unlike growth-related genes which have a single CArG box at the promoter region, virtually all smooth muscle-specific genes have two or more CArG boxes in their promoter region (25). MyoCD forms homodimers via its leucine zipper domain, which in turn bind to two SRF homodimers at two CArG boxes to initiate transcription of smooth muscle genes (24). As MyoCD and Elk-1 share the same interface for SRF binding, there has been evidence suggesting they could compete for SRF for their respective transcriptional activities (22).

The effect of mitogens on suppressing MyoCD transcriptional activity has been well established in VSM. As the ETS-binding domains also exist in the smooth muscle gene promoters proximal to the CArG boxes, this also allows pElk-1 binding, which competitively inhibits MyoCD-SRF association and subsequent smooth muscle gene transcription (22, 26). Such a mechanism likely explains the loss of smooth muscle contractile properties generally observed when smooth muscle is exposed to growth factors and proinflammatory cytokines (27). Some have proposed that inversely, MyoCD could competitively inhibit pElk-1 transcriptional activity via the binding of free SRF (22). The possibility of such a property of MyoCD has only been demonstrated *in vitro* with competitive binding of recombinant pElk-1 and MyoCD to SRF (22), and is yet to be confirmed in any cellular system. Beyond the mechanism described,

MyoCD and NF-κB demonstrate cross-inhibitory actions in VSM via unknown mechanisms, which explains the opposition between smooth muscle contractile and inflammatory/synthetic phenotypes (28).

In this study, to characterize the interaction between MyoCD and Elk-1 and the associated human ASM phenotype regulation, we generated cultured primary ASMCs with stable overexpression of MyoCD using a lentiviral vector and assessed its effect on contractile responses, proliferation in response to mitogenic signals and inflammatory factor expression in response to cytokines.

Materials and Methods

Tissue procurement and ethical approval

The transplant-grade human lung tissues were procured from the National Disease Research Interchange and International Institute for the Advancement of Medicine. All procedures pertaining to the procurement and experimentation with human tissues were approved by the McGill University Health Center Research Ethics Board.

ASMC cultures

The generation and maintenance of ASMC cultures from tissues were performed as previously described (29). For all experiments, ASMCs were seeded at 1×10^4 cells/cm^2 density. Following overnight culturing for attachment, the cells were serum-starved for 24 hours prior to treatment with inhibitors, cytokines or growth factors for durations as specified.

Myocardin overexpression via lentiviral vectors

The overexpression of MYOCD and red fluorescent protein (RFP) as a negative control in ASMCs derived from seven distinct donors was achieved with custom ready-to-use lentiviral vectors. Following which, the transduced cells underwent positive selection, expansion and were preserved in frozen stocks. Additional information regarding the lentiviral vector constructs and transduction protocol can be found in Supplementary Material under “Lentiviral Vectors”.

Collagen gel contraction assay

ASMC-embedded collagen lattice preparation, contractile agonist challenge and imaging were performed according to procedures described in Supplementary Materials under “collagen gel contraction assay”.

Flow cytometry

ASMCs were first stained with eFluor780 fixable viability dye (eBioscience), fixed and permeabilized with Transcription Factor Staining Buffer Set (eBioscience), then stained with AlexaFluor488-conjugated anti Ki-67 antibody (Biolegend) in permeabilizing condition. Samples were analyzed with LSRFortessa X-20 flow cytometer (BD).

Western blot

ASMCs were washed with PBS and lysed with NP-40 cell lysis buffer (Invitrogen), ASM tissues were homogenized in RIPA buffer, both supplemented with protease and phosphatase inhibitor cocktail (ThermoFisher Scientific). The WB analysis was performed as previously described (29). The antibodies used are listed in Supplementary Table S2.

Co-immunoprecipitation

Immunoprecipitation of SRF was performed using anti-SRF antibody (Proteintech) with a commercial co-immunoprecipitation kit (ThermoFisher Scientific) according to the manufacturer's instructions. The immunoprecipitated samples were analyzed by Western Blot.

qRT-PCR

The RNA extraction, reverse transcription and qPCR was performed as previously described (29). Detailed procedures can be found in Supplementary Materials under "qRT-PCR".

Fluorescence microscopy

Immunofluorescence staining of α-smooth muscle actin (α-SMA) and cytosolic staining with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) was performed in ASMCs according to procedures in Supplementary Materials under "CFDA-SE and immunofluorescence staining". The stained cells were imaged with Zeiss Axio Imager M2.

Nuclear and cytosolic SRF quantification

ASMC cytosolic and nuclear fractions were separated and analyzed by Western blotting for their SRF content. Detailed procedures on fractionation can be found in Supplementary Materials under "Nuclear and cytosolic fractionation".

Chromatin immunoprecipitation (ChIP) and quantitative PCR

DNA-protein complexes were cross-linked and immunoprecipitated with anti-SRF antibody. To assess the enrichment of the promoter regions, quantitative PCR was performed with previously

described primers (30) detecting sequences proximal to their respective CArG boxes. Detailed procedures can be found in Supplementary Materials under “Chromatin immunoprecipitation”.

Statistical analysis

Student’s t-test, one-way and two-way ANOVA were performed as appropriate using GraphPad Prism software. A p value less than 0.05 was considered statistically significant.

Results

Lentiviral transduction of MyoCD resulted in upregulation of smooth muscle-specific contractile proteins, changes in cell morphology and formation of organized α-SMA fibers

qPCR analysis confirmed the successful transduction of MyoCD in primary human ASMCs (Figure 1A). Consequently, the treatment initiated the transcription of the smooth muscle contractile protein genes known to be MyoCD-inducible, including actin α2 (*ACTA2*, encoding α-SMA protein), myosin heavy chain 11 (*MYH11*, encoding smooth muscle myosin heavy chain (SMMHC) protein), *TAGLN* encoding transgelin (SM22) protein, calponin 1 (*CNN1*), but not *MYLK* (encoding myosin light chain kinase (MLCK1) protein) (Figure 1B-F). Consistent with these observations, MyoCD transduction resulted in increased protein levels of α-SMA and SMMHC, but not MLCK1, as assessed by WB (Figure 1G-J). Unexpectedly, though being a mitogen and Elk-1 activator that is anticipated to inhibit MyoCD function, high (10%) FBS exposure resulted in upregulation of MyoCD itself (Figure 1A) in the untransduced and RFP-transduced groups, along with increased *TAGLN* transcript in the untransduced and RFP-transduced groups, and decreased *CNN1* transcript in RFP- and MyoCD-transduced groups (Figure 1D-E) compared to the low (0.1%) FBS condition.

Immunofluorescent imaging revealed changes in cellular morphology induced by MyoCD overexpression. Cytosolic staining with CFDA-SE demonstrated that adherent MyoCD-transduced cells covered larger areas compared to control cells (Figure 1K-L), potentially as an adaptation to accommodate for the increased abundance in smooth muscle contractile proteins. Immunostaining of α-SMA once again showed its increased immunoreactivity in MyoCD transduced cells compared to RFP transduced cells, as well as the appearance of organized α-SMA fibers (Figure 1M).

Elevated force generation in MyoCD-transduced cells due to increased smooth muscle contractile proteins.

To verify whether MyoCD transduction contributed to an increased contractile response, we assessed the force generation of the transduced cells at the basal level (vehicle) and following KCl challenge using a collagen gel contraction assay. The representative image (Figure 2A) of the assay and the quantification of gel area reduction (Figure 2B) demonstrated that MyoCD-transduced cells caused a larger reduction of gel area at both basal level and in response to KCl compared to RFP-transduced cells. The gel area reduction due to cellular contraction was inhibited by Y-27632, which elevates myosin light chain phosphatase (MLCP) activity. This experiment was performed once only and shown in Figure 2A to further support that the gel area reduction was indeed due to cellular contraction rather than any artifact associated with the cell-embedded collagen gel.

An increase in smooth muscle force generation could either be associated with a higher content of contractile proteins in the cells, which has been confirmed to be the case in MyoCD-over-expressed cells (Figure 1), or elevated signaling of the contractile pathways, be it Ca^{2+} -

dependent MLCK1 activation or RhoA-Rho-associated protein kinase (ROCK)-MLCP activation. In an attempt to further understand the origin of increased force generation, we assessed regulatory light chain (RLC, also known as myosin light chain 2, or MLC2) phosphorylation in the transduced cells by WB in response to a 10 min challenge with the contractile agonists KCl and histamine. Both KCl and histamine induced greater magnitude of RLC phosphorylation in MyoCD-transduced cells (Figure 2E and F). However, we also observed that the total RLC content was elevated in MyoCD-transduced cells (Figure 2D), signifying that increased force generation is at least partially due to an increased expression of RLC in addition to the other contractile proteins.

MyoCD overexpression displaced SRF at the promoter sites of smooth muscle and proliferative genes and suppressed mitogen-induced *FOS* transcription

To investigate if MyoCD overexpression affected the transcriptional activity of Elk-1 by competitive occupation of SRF, as illustrated in Figure 3A, we first evaluated its impact on the presence of SRF at the 5' CArG boxes of the promoter sites of the smooth muscle genes *ACTA2* and *MYH11*, and the proliferative gene *FOS*, an immediate-early gene of proliferation regulated by the Elk-1-SRF complex, by ChIP-qPCR. As expected, MyoCD overexpression increased the MyoCD-SRF complex and increased SRF binding at the CArG boxes of *ACTA2* and *MYH11* genes (Figure 3B and C). Furthermore, we also observed a reduction in the *FOS* CArG box co-immunoprecipitated with SRF in MyoCD-transduced cells (Figure 3D), suggesting MyoCD overexpression de-stabilized the pElk-1-SRF complex at the promoter of proliferative genes. To consolidate this finding, we then assessed mitogen-induced transcription of *FOS* in the transduced cells. We chose to study the effects of EGF and FBS as the receptor for the former

has been shown to play an important role in asthma-related change in ASM mass, including hyperplasia in murine models (31, 32), and the latter is used as a common mitogen in cell culture studies. After 30 minutes of treatment with 10 ng/mL EGF or 10% FBS, a time point which has been widely described to correspond to maximal induction of *FOS* mRNA expression (33), we observed increases of the *FOS* transcript compared to the control treatment group, and such increases were reduced in MyoCD-transduced cells (Figure 3E-F). We also confirmed that the *FOS* mRNA level started to return to baseline by 1 hr (Figure S1) in both EGF and FBS-treated conditions.

MyoCD transduction suppressed proliferation and Elk-1-SRF association induced by EGF but not FBS

The association of Elk-1 and SRF was subsequently evaluated by co-immunoprecipitation. The EGF-induced increases in Elk-1 that co-immunoprecipitated with SRF were suppressed by MyoCD-transduction (Figure 3G-H), observed one day following the treatments. However, differing from the observations in ChIP-qPCR and *FOS* expression at early time points, FBS-treated RFP- and MyoCD-transduced cells demonstrated similar degrees of Elk-1-SRF association (Figure 3I-J).

Finally, to investigate if the observed changes in Elk-1 association with SRF and its transcriptional activities indeed confer differences in cellular mitogenic responses, we assessed the proliferation of RFP and MyoCD-transduced cells by quantifying Ki-67+ cells by flow cytometry, as well as by cell counts following EGF and FBS treatments. A 24-hour exposure to EGF induced a dose-dependent increase in Ki-67+ cells in both RFP- and MyoCD-transduced cells, with a peak response at 10 ng/mL. However, the response was significantly lower in

MyoCD-transduced cells at 10 and 100 ng/mL concentrations (Figure 3K-L). Consistent with our observations on Elk-1-SRF association, 10% FBS induced overall much higher percentages of Ki-67+ cells, but the values were similar between RFP- and MyoCD-transduced groups (Figure 3K-L). The proliferation assessed by cell count following a 7-day treatment with 10 ng/mL EGF, or 4 day treatment with 10% FBS in the two groups yielded similar observations (Figure 3M-N).

Differential response of smooth muscle contractile protein gene expression to mitogen-mediated suppression.

It is well established in VSM that Elk-1 activated by mitogenic signals inhibits smooth muscle gene expression by binding to the SRF-CArG box complex at the promoter regions and competitively inhibits the transcriptional activities of MyoCD, as demonstrated in Figure 4A (22). We therefore sought to describe this pathway in ASMCs using MyoCD transduced cells as a model system. Mitogen-mediated suppression of contractile protein genes was more prominent in MyoCD-transduced cells. EGF reduced *ACTA2* transcript and α-SMA protein levels (Figure 4B and E-F), while FBS led to a trend for increase in its transcript (p=0.076) (Figure 4B). *MYH11* expression at the transcript level showed a trend towards decrease in response to EGF, and a statistically significantly decreased in response to FBS (p=0.09) (Figure 4C). However, these changes were not observed at the protein level (Figure 4E and G). Interestingly, *TAGLN* expression was not suppressed by the mitogens, and FBS paradoxically induced an increase in *TAGLN* transcript in both RFP and MyoCD-transduced cells (Figure 4D). It is important to mention that though overall suppressed by mitogens, *MYH11* transcript level in MyoCD-transduced cells also saw a trend towards increase by day 3 following FBS treatment (Figure S2). Lastly, in RFP-transduced cells, neither EGF nor FBS had a substantial effect on contractile

protein gene expression, except that EGF reduced the *MYH11* transcript level (Figure 4C). This is likely because the expression levels of these genes were already too low to reflect any prominent change. Overall, we only observed a mild inhibitory effect of EGF on contractile protein gene expression, and FBS, though being a strong Elk-1 activator, paradoxically mediated upregulation of certain SM genes.

FBS upregulated total SRF expression and promoted its nuclear localization in a Rho-dependent manner

With further investigation, we demonstrated that FBS but not EGF caused an increase of the *SRF* transcript as early as 1 hour following treatment (Figure 5A), and a substantial increase in total SRF protein observed 1-day post-treatment (Figure 5B-C). It is also important to note that the *SRF* transcript level was not yet upregulated at 0.5 hr following FBS treatment (Figure S3), the time point where mitogen-induced *FOS* transcription was the most prominent. In addition, consistent with existing studies on SRF cellular distribution in ASM (34), Western blot analysis of isolated cytosolic and nuclear fractions revealed that FBS also promoted nuclear translocation of SRF (Figure 5D). Altogether, these data suggest that FBS increased the total SRF available for related transcriptional activities in the nuclei. As existing studies have indicated that SRF expression and nuclear translocation in various types of SMCs is associated with the RhoA-ROCK pathway (35, 36), we confirmed in our cellular system that inhibition of ROCK activity with Y-27632 reduced total SRF protein level (Figure 5E-F) and SRF nuclear translocation (Figure 5G) in FBS-treated cells.

Y-27632 limited SRF availability, restored the inhibitory effect of MyoCD overexpression on cellular proliferation, and inhibited FBS-induced contractile protein gene upregulation.

To further demonstrate the concept that a limiting amount of SRF is a prerequisite for MyoCD-Elk-1 cross-inhibitory action, and that such action is absent with FBS-mediated SRF expression and nuclear translocation, we incubated the transduced cells with Y-27632 prior to FBS treatment to inhibit its effects on SRF. MyoCD transduction did not affect cellular proliferation induced by 10% FBS in the vehicle treatment group, which is consistent with the data presented in Figure 3K-L, but significantly reduced the proportion of Ki-67+ cells in combination with Y-27632 treatment (Figure 6A-B). Y-27632 itself did not impact proliferation in the RFP-transduced group, which once again confirmed that the reduction in SRF quantity alone was not sufficient to restrict proliferation and that competitive inhibition by MyoCD was required (Figure 6A-B).

Given that the quantity of nuclear SRF is a confounding factor which determines if the interaction between MyoCD and Elk-1 occurs, we reasoned that the unexpected effect of FBS on contractile protein genes observed was also related to its effect on the upregulation of SRF. For this reason, we studied the effect of limiting SRF by ROCK inhibition on SM gene expression in FBS-treated cells. In MyoCD transduced cells, Y-27632 treatment suppressed FBS-mediated upregulation of *ACTA2* and *TAGLN* expression, while having no effect on *MYH11* transcript levels (Figure 6C-E). It is noteworthy that these effects were not due to Y-27632's potential effect on cellular health and protein synthesis, as it did not impact SM gene expression by itself, as reflected by the groups without serum treatment (Figure 6C-E).

MyoCD transduction in ASMCs is not sufficient to restore the level of contractile protein gene expression to that of native ASM tissues.

We next compared the expression of MyoCD and smooth muscle contractile protein genes on both transcript and protein levels among RFP-, MyoCD-transduced ASMCs and native ASM tissues. Consistent with the general understanding that there is a major loss in contractile properties when native ASMCs are cultured, the expression levels of MyoCD and of all the genes coding for contractile proteins examined in RFP-transduced cells, which generally represent cultured ASMCs, were only a fraction of those of the native tissue (Figure 7). MyoCD transduction resulted in its overexpression, exceeding the level in native tissues (Figure 7A). It restored the *ACTA2* transcript level to that similar to native tissues (Figure 7B), but only 10% of the native tissue *MYH11* transcript (Figure 7C).

However, quantification of contractile proteins by WB revealed that regardless of these observations on the transcripts, the levels of α-SMA, SMMHC and MLCK1 in both RFP- and MyoCD-transduced cells were far lower than in native tissues (Figure 7D-G), indicating that factors other than MyoCD-mediated gene expression contribute to loss of contractile functions when ASMCs are cultured.

MyoCD transduction does not affect the expression of NF-κB-inducible inflammatory genes.

Given existing reports of reciprocal inhibition between MyoCD and NF-κB signaling pathways, we assessed NF-κB-inducible inflammatory chemokines and cytokines, including CC motif ligand 5 (CCL5), CCL11, interleukin (IL)-8 and IL-6, as well as the adhesion molecules intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) in MyoCD-transduced cells following tumor necrosis factor α (TNFα) treatment by qRT-PCR.

Overall, we did not observe any suppression of NF-κB-inducible gene expression in MyoCD-transduced cells (Figure S4 A-F). On the contrary, TNFα-induced IL-8 and ICAM1 expression was elevated in MyoCD-transduced cells compared to RFP-transduced cells (Figure S4 C and E).

Discussion

Smooth muscle phenotype alterations have been described in various pathological contexts, notably for ASM in airway diseases and for VSM in cardiovascular diseases, with changes towards hyper-contractile and synthetic/proliferative phenotypes both being reported (1, 12, 16). The understanding of related molecular biology mechanisms in smooth muscle could therefore yield novel therapeutic approaches to these diseases (1, 37). In this study, we performed in-depth characterization of functional cross-inhibition between MyoCD and Elk-1, which has been proposed to be the transcriptional regulatory mechanism underlying the widely accepted smooth muscle contractile and proliferative phenotype dichotomy. In stable MyoCD-transduced primary human ASMCs, we observed the upregulation of smooth muscle contractile protein genes largely as expected. However, the expression of *MYLK*, which has been reported to be MyoCD-inducible (26, 38), was unaffected. This finding could be due to cell type-specific epigenetic modifications at its promoter that suppressed its interaction with the MyoCD-SRF transcription complex, as a similar regulatory phenomenon has been described in VSM (30, 39).

Consistent with the global contractile protein gene upregulation in MyoCD-transduced cells, the cells also demonstrated increased contractility, which is reflected in both the collagen contraction assay and increased agonist-induced RLC phosphorylation. Furthermore, MyoCD-transduced cells also featured increased total RLC expression, which likely directly contributed to the elevated phospho-RLC (pRLC) signal. Combining such observations with the fact that

MYLK expression was unaffected, it is reasonable to postulate that MyoCD increases ASMC force generation by elevating the quantity of contractile proteins including RLC. We limited our observations on the gel contraction assay to KCl, which, although less physiologically relevant than G-protein coupled receptor activation, gives a robust contractile response compared to histamine (data not shown). KCl induces receptor-independent calcium signals and force generation solely dependent on contractile protein expression. However, a definitive conclusion on whether MyoCD affects contraction-related signaling pathways requires specific investigation of the kinetics of calcium signaling, MLCK1 and RLC phosphatase activation.

MyoCD overexpression increased SRF bound at the promoters of *ACTA2* and *MYH11* as expected, while it decreased SRF bound at the promoter of *FOS* as assessed by ChIP-qPCR. This corresponded to inhibited transcription of *FOS* 30 minutes following EGF or FBS treatments. For the first time, these experimental results proved that in smooth muscle, increased MyoCD is indeed capable of competitively inhibiting Elk-1 transcriptional activity by occupying free SRF and reducing its availability for proliferative gene transcription (illustrated in the top left panel of Figure 6F). However, MyoCD transduction suppressed cellular proliferation and Elk-1-SRF association observed 24-hour post-treatment induced by EGF only, but not FBS. We then discovered FBS had the confounding effect of upregulating the quantity of SRF, as well as promoting its nuclear localization in ASMCs, in a RhoA-ROCK dependent manner, both of which have been described in other cell types in previous studies (34, 35). As MyoCD and Elk-1 cross-inhibition is dependent on their competition for SRF binding, we propose that an increase in nuclear SRF may eliminate the evidence of such interaction since there would be no significant competition (as depicted in the right panel of Figure 6F). Indeed, inhibition of RhoA-ROCK pathway with Y-27632 reversed SRF upregulation and nuclear localization mediated by

FBS, and subsequently inhibited FBS-induced proliferation in MyoCD-transduced cells, while having no impact on the proliferation of RFP-transduced cells. Overall, while we confirmed the existence of cross inhibitory actions between MyoCD and Elk-1 in ASMCs, we further proved that SRF being a limiting factor is a prerequisite for such interaction. An excess in nuclear SRF, as is the case of FBS-treated cells, permit MyoCD-transduced cells to possess both hypercontractile and hyperproliferative properties concurrently.

As to the discrepancies between early inhibition of *FOS* transcription by MyoCD-over expression in FBS-treated cells and the absence of inhibition of proliferation subsequently, we propose that it is due to the difference in the time-points of these observations in relation to that of SRF upregulation. The earliest FBS-mediated increases in SRF transcript we observed occurred 1 hr after treatment, by which time the *FOS* transcript level had already reached its peak and started to return to baseline. SRF was yet to be upregulated by FBS as confirmed by qPCR at 0.5 hr. Therefore, while overexpressed MyoCD inhibited SRF binding at the *FOS* promoter and *FOS* transcription early on, these inhibitory effects diminished quickly after the subsequent increase in nuclear SRF availability in FBS-treated cells. This eventually resulted in an absence of a difference in cellular proliferation observed on and after 24 hr.

The MyoCD-transduced ASMCs overcame the issue of significantly diminished expression level of contractile proteins due to culturing and provided an excellent model system to study Elk-1-medated inhibition of smooth muscle gene expression, which has yet to be described in this specific cell type. We observed that overall, different contractile protein genes respond differently to mitogen-induced Elk-1 activation. EGF suppressed *ACTA2* and *MYH11* expression in MyoCD-transduced cells, although the latter of which was observed at the transcript but not protein level, likely because the protein is relatively stable and is latent in

reflecting changes of expression. However, EGF did not affect the expression of *TAGLN*, although such effect has been reported in other smooth muscle cell types with another mitogen, platelet-derived growth factor (22, 26). Existing studies have demonstrated that smooth muscle genes have differential sensitivity to Elk-1-mediated inhibitory action. The binding of Elk-1 A box to ETS-binding domain on DNA, as well as its B box's interaction with SRF both partially determine its inhibitory effect on MyoCD-mediated gene transcription. However, their relative action could vary at the promoters of different contractile protein genes (26), which might explain our current observations. The exact mechanism conferring the differential sensitivity to Elk-1's inhibition awaits further investigation.

Interestingly, FBS paradoxically upregulated the transcript levels of *ACTA2*, *TAGLN* and *MYH11* (at a later time point than the former two genes). We hypothesize that the effect of FBS on increasing nuclear SRF availability further promoted MyoCD's transcriptional activity while eliminating the competitive inhibition effect of Elk-1, thereby conferring unexpected SM contractile protein gene upregulation. This is supported by the finding that suppressing SRF availability with Y-27632 resulted in reversal of FBS-induced *ACTA2* and *TAGLN* upregulation. It is important to clarify that although FBS promoted the expression of MyoCD itself, the magnitude of expression was trivial compared to that of lentiviral-mediated overexpression and did not cause statistically significant change in the MyoCD-transduced group, as demonstrated in the comparison between ASMCs cultured in low and high FBS conditions. Therefore, the observed reduction in the levels of *ACTA2* and *TAGLN* transcripts following Y-27632 treatment were therefore not attributable to changes in MyoCD quantity itself, but instead attributable to reduction in SRF availability.

As MyoCD overexpression only restored a fraction of the contractile protein levels of that in native tissues in culture, it is not the sole factor responsible for the loss of the contractile property of ASMCs in culture. It is likely that the *in vitro* culture conditions with prolonged exposure to proliferative signals resulted in permanent changes in cell phenotype. Other factors in the ASMC microenvironment have also been proven to play roles in determining their contractile and proliferative capacity (3, 40, 41).

Lastly, as a previous study described the cross-inhibitory action between MyoCD and p65 in the NF-κB signaling pathway (28), we assessed inflammatory gene expression in MyoCD-transduced ASMCs that were treated with TNFα, which is a canonical cytokine that activates the NF-κB pathway. Overall, the TNFα-inducible genes were not inhibited by MyoCD overexpression. On the contrary, we observed paradoxical increases in TNFα-induced IL-8 and ICAM1 expression in MyoCD-transduced cells. This was not a confounding effect of lentiviral transduction, as the cells in the RFP and MyoCD groups were exposed to the same viral vector treatment. However, whether this is a specific effect of MyoCD overexpression requires further exploration.

In summary, our findings shed light on the transcriptional regulatory mechanisms that are potentially responsible for pathological ASM phenotypic changes in airway diseases. Specifically, our results provide proof-of-concept evidence that smooth muscle may possess contractile and proliferative properties concomitantly. An upregulation of SRF could be especially detrimental as it allows dual activation of transcriptional responses associated with both contractile and proliferative pathways. Therefore, SRF could be a promising therapeutic target for ameliorating pathological changes in smooth muscle.

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Figure legends

Figure 1: Lentiviral transduction of MyoCD resulted in upregulation of contractile proteins, changes in cell morphology and formation of organized α-SMA fibers. A-F: mRNA levels of *MyoCD*, *ACTA2*, *MYH11*, *TAGLN*, *CNN1* and *MYLK* in untransduced (-), RFP-transduced control and MyoCD-transduced ASMCs cultured in 0.1% FBS or 10% FBS. Data are mean ± SEM, 2-way repeated-measures ANOVA with Bonferroni post-test. *:$p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$. G-J: representative WB image and quantifications of α-SMA, SMMHC, MLCK1 protein signals in the experimental groups mentioned. Data are mean ± SEM, 2-way repeated-measures ANOVA with Bonferroni post-test. *:$p<0.05$, **: $p<0.01$. K-L: representative CFDA-SE staining of adherent ASMC and quantification of cell area of RFP and MyoCD transduced cells. Scale bar represents 50 μm. Data represent individual cells sampled, unpaired Student's t-test for individual donors and all samples pooled, *:$p<0.05$, ***: $p<0.001$, ****: $p<0.0001$. M: representative immunofluorescent staining of α-SMA in RFP-transduced control and MyoCD-transduced ASMCs. Scale bar represents 50 μm.

Figure 2: Increased force generation in MyoCD-transduced cells was due to elevated presence of smooth muscle contractile elements. A-B : representative image of collagen gel contraction assay and quantification of gel area reduction mediated by in untransduced (-), RFP-transduced control and MyoCD-transduced ASMCs in response to 30-min vehicle (PBS) or KCl challenge. Data are mean ± SEM, 2-way repeated-measures ANOVA with Bonferroni post-test. *:$p<0.05$, ***: $p<0.001$, ****: $p<0.0001$. C-F: representative WB images and quantification of RLC and pRLC signals in RFP-transduced controls and MyoCD-transduced ASMCs in response to 10-min

vehicle (PBS), KCl or histamine challenges. Data are mean ± SEM, 2-way repeated-measures ANOVA with Bonferroni post-test. *:p<0.05, **: p<0.01.

Figure 3: MyoCD transduction shifted SRF distribution at promoter sites, inhibited early *FOS* transcription, and suppressed proliferation and Elk-1-SRF association induced by EGF but not FBS. A: An illustration of the proposed inhibitory mechanism of MyoCD overexpression on Elk-1 via the depletion of SRF. The illustration is created with BioRender.com. B-D: ChIP-qPCR analysis showing the abundance of SRF at the promoter 5'-CArG boxes of contractile protein genes *ACTA2* and *MYH11*, as well as that of proliferation gene *FOS* in untreated cells. Data are mean ± SEM, Paired Student's t-test, *:p<0.05, **: p<0.01. E-F: *FOS* transcription induced by 10 ng/mL EGF or 10% FBS 30 min following the treatment. Data are mean ± SEM, Paired two-way ANOVA with Bonferroni post-test (panel E) and paired Student's t-test (panel F), *:p<0.05, **: p<0.01, ***: p<0.001, p<0.0001. G-J: representative WB images and quantification of Elk-1 co-immunoprecipitated with SRF in RFP-transduced control and MyoCD-transduced ASMCs following 24 hr 10 ng/mL EGF or 10% FBS treatment. Data are mean ± SEM, 2-way repeated-measures ANOVA with Bonferroni post-test (panel H), and paired Student's t-test between (panel J). **: p<0.01, ***: p<0.001. K-L: Representative flow cytometry plot and quantification of proportions of Ki-67+ cells among RFP-transduced control and MyoCD-transduced ASMCs following overnight treatment of EGF at indicated concentrations or 10% FBS. Data are mean ± SEM, 2-way repeated-measure ANOVA with Bonferroni post-test among EGF-treated conditions, and paired Student's t-test between 10% FBS-treated conditions. *:p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001. M-N: Cellular proliferation assessed by cell count in RFP-transduced control and MyoCD-transduced ASMCs following 10 ng/mL EGF or 10% FBS

at indicated durations. Data are mean ± SEM, 2-way repeated-measure ANOVA with Bonferroni post-test (panel M), and paired Student's t-test (panel N). *:$p<0.05$.

Figure 4: The expression of ASMC contractile protein genes respond differently to the suppressive effect of mitogen signals. A: An illustration of proposed inhibitory mechanism of Elk-1 activation on MyoCD-mediated contractile protein gene expression. The illustration is created with BioRender.com. B-D: transcript levels of *ACTA2*, *MYH11* and *TAGLN* in RFP- and MyoCD-transduced cells following one-day control, 10 ng/mL EGF or 10% FBS treatments. Data are mean ± SEM, 2-way repeated-measures ANOVA with Bonferroni post-test. *:$p<0.05$, ***: $p<0.001$, ****$p<0.0001$. E-F: representative WB images and quantification of α-SMA and SMMHC signals in RFP- and MyoCD-transduced cells under three-day control, 10 ng/mL EGF or 10% FBS treatments. Data are mean ± SEM, 2-way repeated-measure ANOVA with Bonferroni post-test. *: $p<0.05$, **: $p<0.01$, ***:$p<0.001$.

Figure 5: FBS promotes SRF expression and nuclear localization in Rho-ROCK-dependent manner. A: qPCR analysis of *SRF* expression in RFP- or MyoCD-transduced ASMCs comparing control and 1 hr 10 ng/mL EGF or 10% FBS treatments. Data are mean ± SEM, 2-way repeated-measures ANOVA with Bonferroni post-test. ***: $p<0.001$, ****: $p<0.0001$. B-C: representative WB images and quantification of total SRF expression in RFP-transduced control and MyoCD-transduced ASMCs under control (-) or 3-day 10 ng/mL EGF- or 10% FBS-treated conditions. Data are mean ± SEM, 2-way repeated-measure ANOVA with Bonferroni post-test. *: $p<0.05$, **: $p<0.01$. D: WB images of SRF in cytosolic and nuclear fractions of ASMCs comparing control and EGF or FBS treatments. E-F: representative WB images and quantification of total

SRF expression in 10% FBS-treated ASMCs comparing effects of pretreatment with vehicle or Y-27632. Data are mean ± SEM, paired Student's t-test, *: $p<0.05$. G: WB images of SRF in cytosolic and nuclear fractions of 10% FBS-treated ASMCs comparing effects of pretreatment with vehicle or Y-27632.

Figure 6: Suppressing FBS-induced SRF activity via RhoA-ROCK pathway inhibition restored MyoCD's proliferation-suppressing effect, and reduced FBS-mediated contractile protein gene upregulation. A-B: Representative flow cytometry plots and quantification of FBS-induction cellular proliferation among RFP- and MyoCD-transduced ASMCs with vehicle or Y-27632 pretreatment. Data are mean ± SEM, 2-way repeated-measures ANOVA with Bonferroni post-test. *:$p<0.05$, **: $p<0.01$. C-D: transcript levels of *ACTA2*, *MYH11* and *TAGLN* in MyoCD-transduced cells with 10% FBS and/or Y-27632 pretreatment. Data are mean ± SEM, *:$p<0.05$,**:$p<0.01$ ***: $p<0.001$, ****$p<0.0001$. F: proposed mechanism showing the influence of nuclear SRF availability on the MyoCD-Elk-1 cross-inhibitory mechanism. Such mechanism is present when the quantity of SRF is a limiting factor (i.e. in absence of FBS or inhibition of RhoA-ROCK pathway). However, dual activation of MyoCD- and pElk-1-mediated gene transcription is permitted when SRF is in excess (i.e. in presence of FBS). The illustration is created with BioRender.com.

Figure 7: MyoCD transduction in ASMCs was not sufficient to restore the level of contractile protein expression in native ASM tissues. A-C: Comparison of mRNA levels of *MyoCD*, *ACTA2* and *MYH11* in RFP-transduced, MyoCD-transduced ASMCs and native ASM tissue lysates. Data are mean ± SEM, one-way ANOVA with Bonferroni post-test. *:$p<0.05$, **: $p<0.01$, ****:

p<0.0001. D-G: representative WB image and quantifications of α-SMA, SMMHC, and MLCK1 protein signals in the abovementioned groups. Data are mean ± SEM, unpaired one-way ANOVA with Bonferroni post-test. **: p<0.01, ***: p<0.001, ****: p<0.0001.

Figure 1

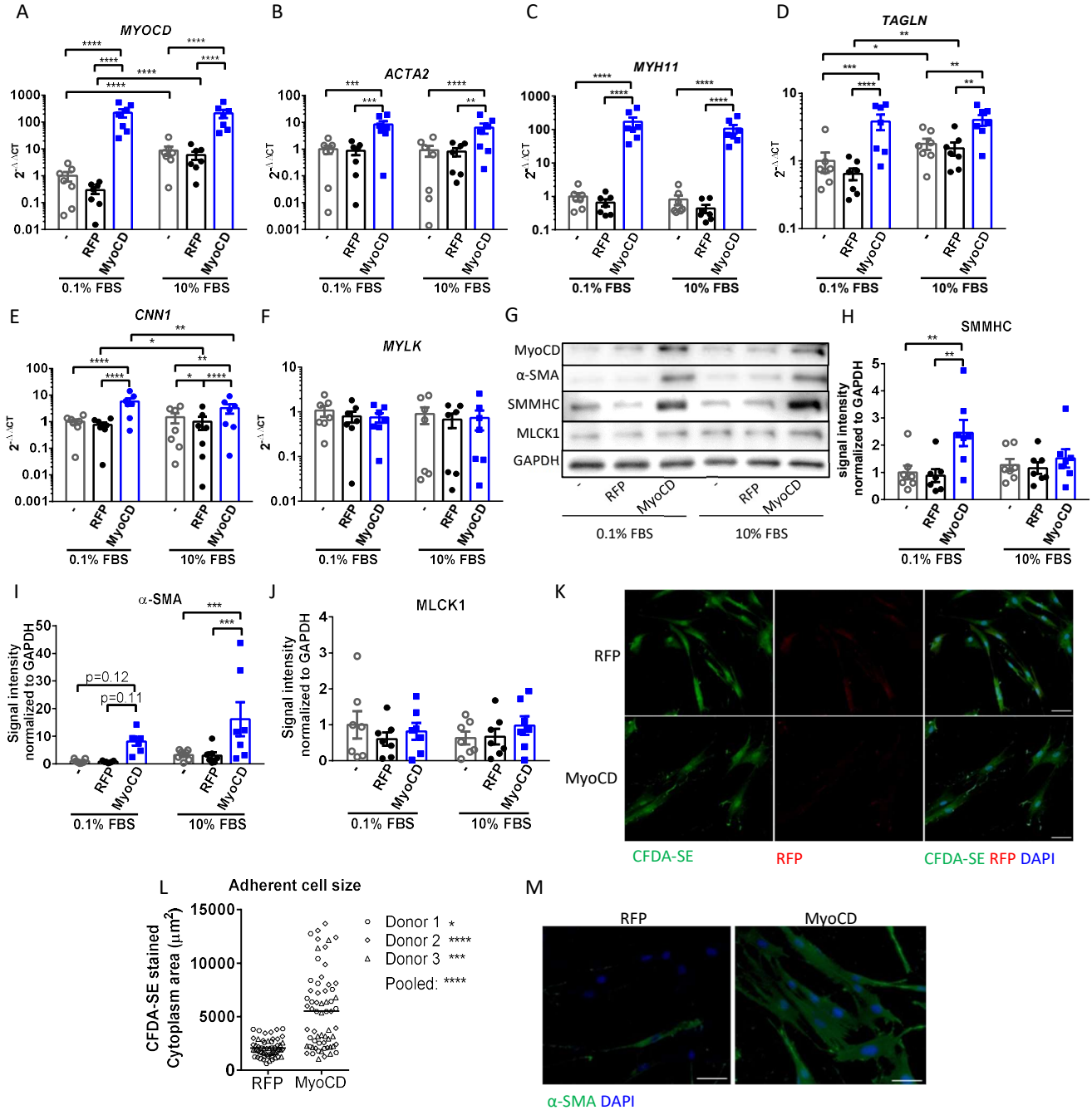


Figure 2

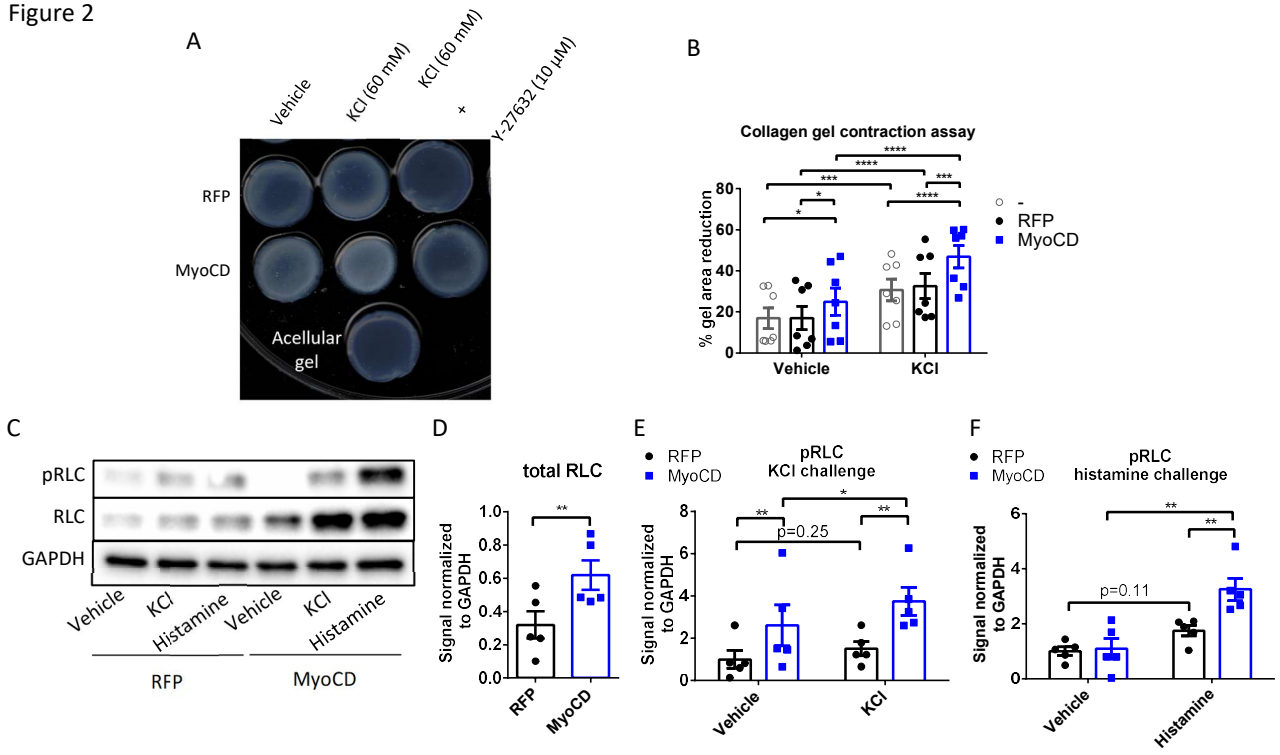
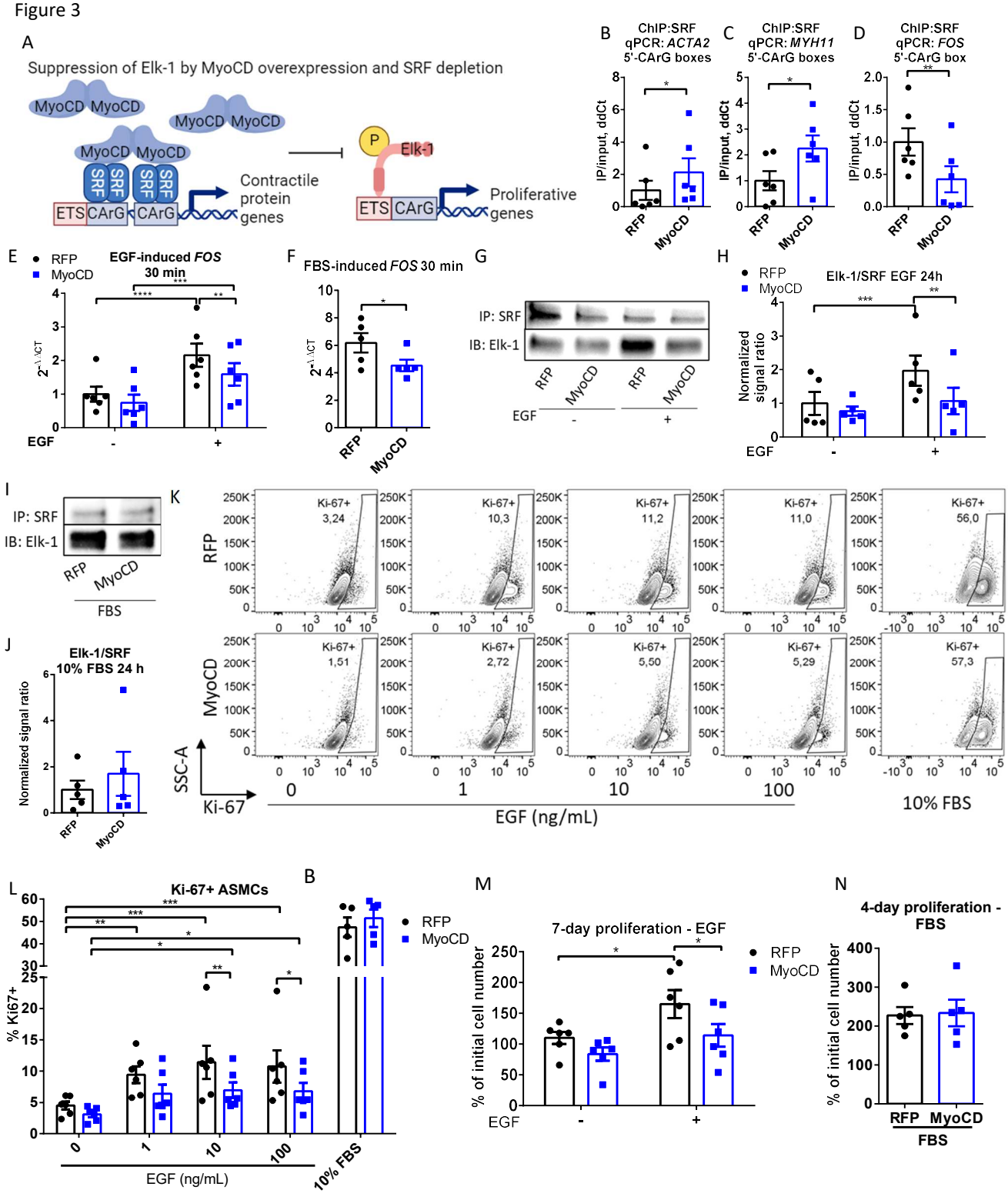


Figure 3



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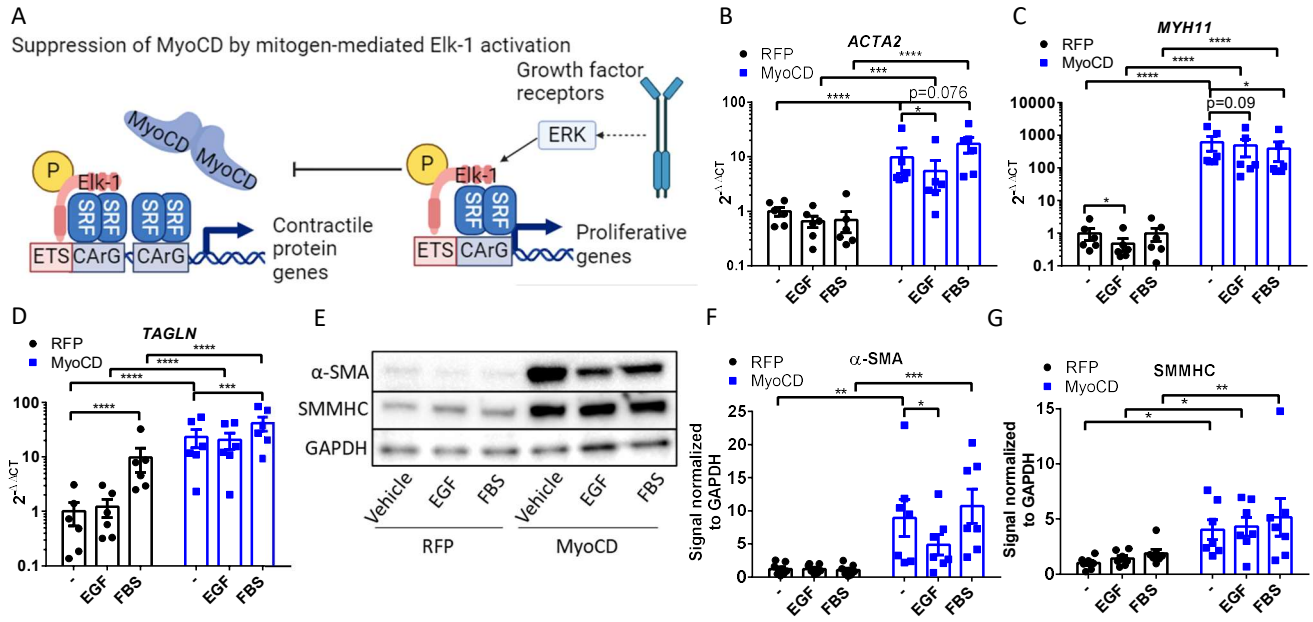


Figure 5

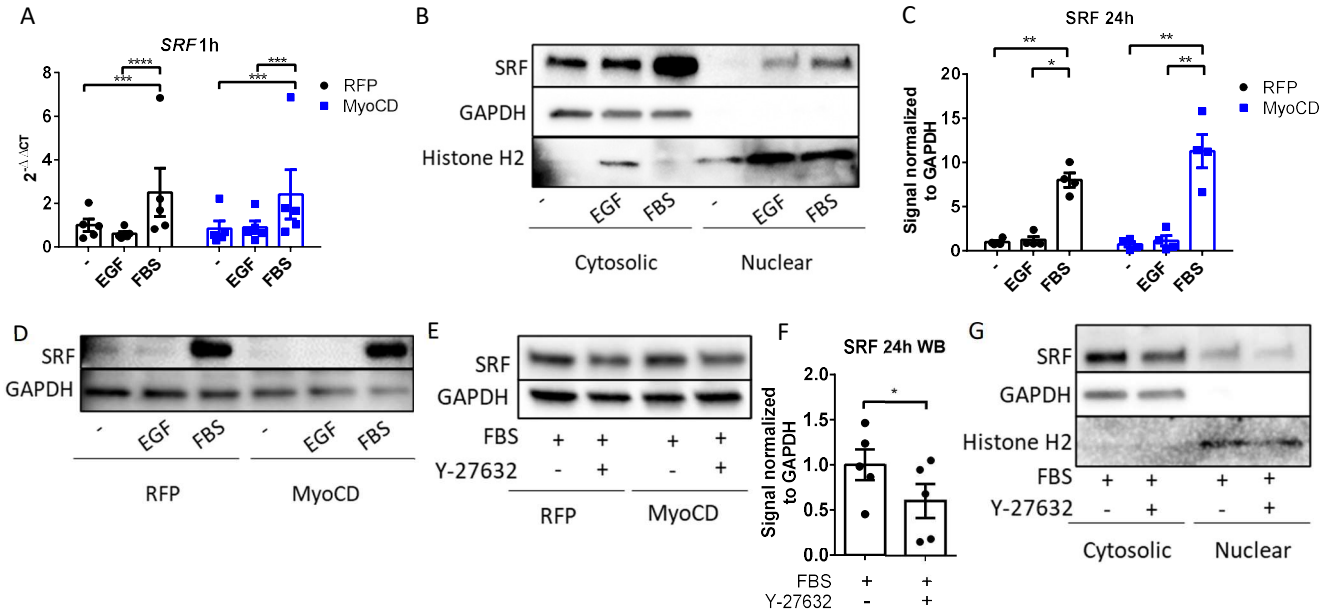


Figure 6

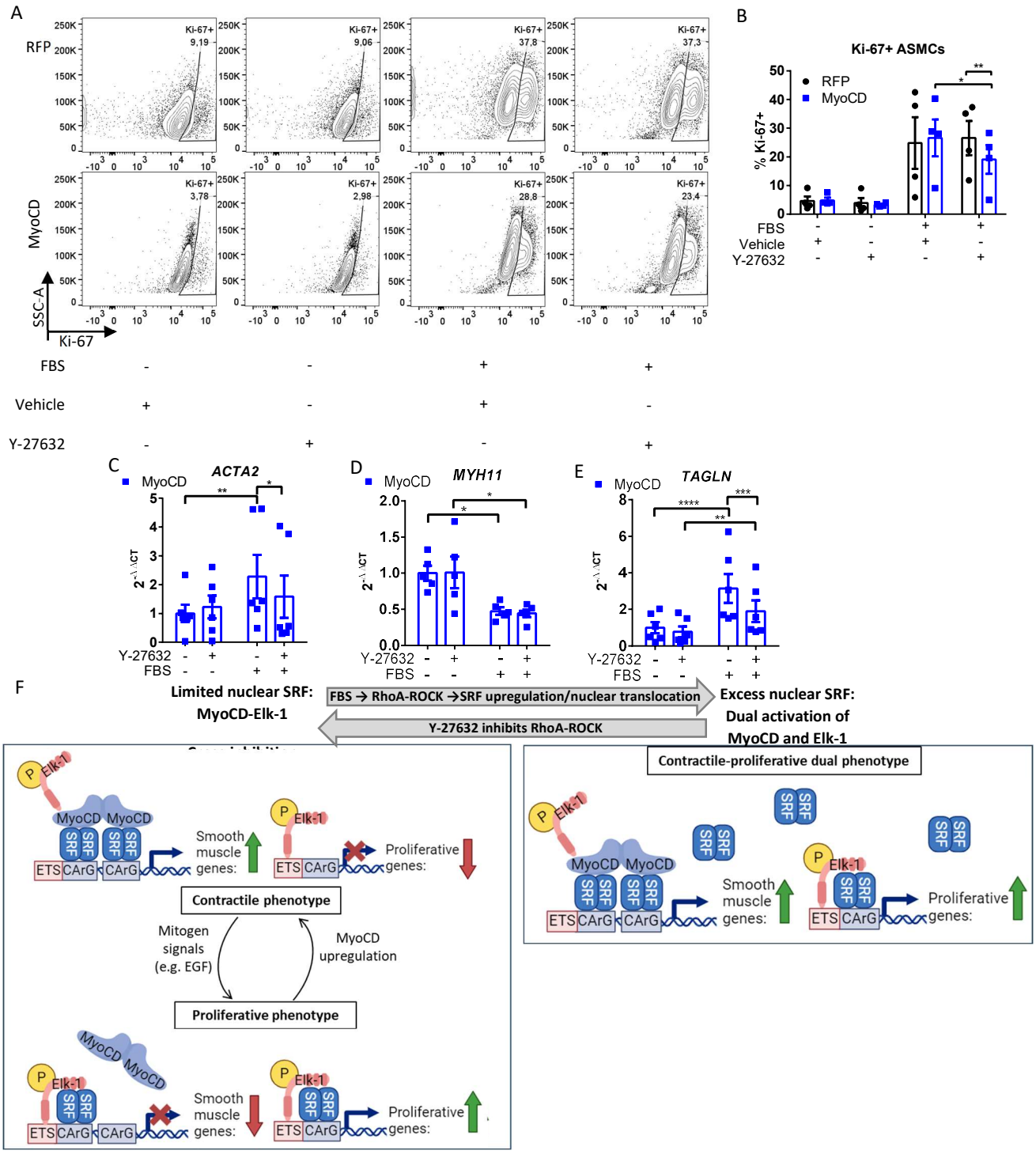
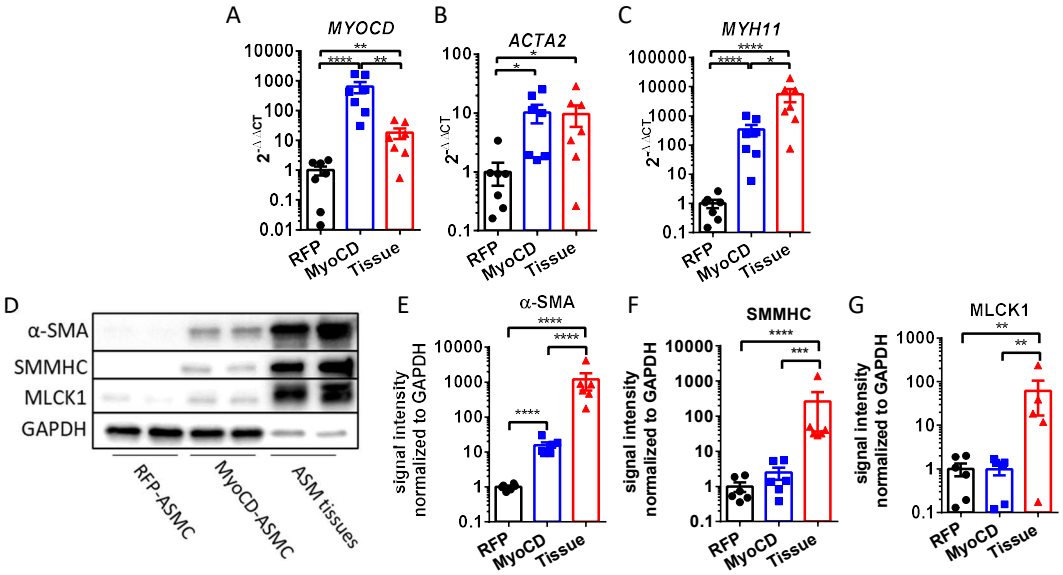


Figure 7



Supplementary Materials

Lentiviral vectors

The custom ready-to-use lentiviral particles carrying the gene expression vector containing open reading frame clone of human *MYOCD* (NM_001146312), and RFP as negative control, were purchased from OriGene. The expression vector backbone structure and sequence can be found on OriGene product website (Cat. #: PS100094). Briefly, the gene expression vectors contain a strong constitutive CMV promoter upstream of the gene of interest, and a puromycin resistance gene for the selection of successfully transduced cells.

For lentiviral transduction, ASMCs from seven donors were incubated with the described lentiviral particles, at a multiplicity of infection of 20 with the presence of 8 μg/mL polybrene (Sigma) for 20 hours. Following one-week positive selection of transduced cells with 2 μg/mL puromycin (Sigma) and further expansion, frozen stocks were generated for continued use.

Collagen gel contraction assay

ASMCs were mixed with neutralized rat tail collagen solution (Advanced Biomatrix) to achieve a final collagen concentration of 2 mg/mL and cell concentration of 2×106 /mL. 0.5 mL of the mixture was added to each well of 24-well plates and allowed for collagen cross-linking, followed by incubation in complete DMEM overnight to allow the cells to settle in the matrix. The collagen gels were detached from the wells and were challenged with vehicle or 60mM KCl (Sigma) as a contractile agonist. Thirty minutes following the challenge, the collagen gels were fixed with 4% buffered paraformaldehyde solution (Santa Cruz Biotechnology) and were imaged with a document scanner. The gel area was quantified with ImageJ software.

CFDA-SE and immunofluorescence staining

For immunostaining, ASMCs seeded on glass chamber slides were fixed with 4% PFA. Following permeabilization with 0.2% Triton X-100 and blocking with 1% BSA in PBS, the samples were stained with mouse anti-α-SMA antibody overnight (Invitrogen) and AlexaFluor 488-conjugated secondary antibody (Biolegend) for 1 hour. For CFDA-SE staining, the live adherent cells were stained with CFDA-SE (Invitrogen) prior to fixation. The slides were mounted with DAPI-containing mounting medium (Vector)

Nuclear and cytosolic fractionation

ASMCs were scraped off the culture plates and suspended in hypotonic cytoplasmic fractioning buffer containing 10 mM HEPES, 1.5 mM $MgCl_2$, 1 mM EDTA, 5mM TCEP, 0.05% NP-40, and protease/phosphatase inhibitors (all chemicals were from Sigma). The cells were passed through a 27G needle 10 times to facilitate lysis and were incubated on ice for 20 min. The lysate was centrifuged, and the supernatants were preserved as the cytoplasmic fraction. The pellet was washed and lysed with nuclear lysis buffer containing 5mM HEPES, 1.5 mM, 300 mM NaCl, 0.2 mM EDTA, 5 mM TCEP, 25% glycerol and protease/phosphatase inhibitors. The lysate was centrifuged, and the supernatants were preserved as the nuclear fraction. For quantification via Western blot, anti-GAPDH and anti-histone H2B antibodies were used as internal controls for cytosolic and nuclear fractions, respectively.

Chromatin Immunoprecipitation

To cross-link DNA-protein complexes, ASMCs were treated with 2mM disuccinimidyl glutarate (Cat. #: 13301, CovaChem) for 30 min and 1% formaldehyde for 15 min. For DNA

fragmentation, nuclei were isolated and sonicated for 25 minutes with 30 seconds on, 30 seconds off cycles using Bioruptor (Diagenode). The resulting lysate was incubated overnight with ChIP-compatible anti-SRF antibody (Cat. #: 5147S, Cell Signaling Technology) bound to protein A Dynabeads (Cat. #: 10001D, Invitrogen). Following reversal of crosslinking by overnight heating at 65°C, co-precipitated DNA was purified using the QIAquick PCR purification kit (Cat. #: 28104, Qiagen).

Primers detecting sequences proximal to the CArG boxes of actin α2 (*ACTA2*), myosin heavy chain 11 (*MYH11*) and *FOS* genes were used for qPCR. All primers for ChIP-qPCR were purchased from ThermoFisher, and the sequences for which are listed in Supplemental Table S1. $2^{-\Delta\Delta Ct}$ values were calculated with inputs of each sample as internal controls.

qRT-PCR

Total RNA from ASMCs was extracted with RNeasy kit (Qiagen). ASM tissues were homogenized in Trizol (Invitrogen) followed by RNA extraction according to the manufacturer's instructions. The reverse transcription reaction was performed using LunaScript RT SuperMix kit (New England Biolabs). Quantitative PCR was performed with SYBR Select Master Mix (Applied Biosystems) with 5 ng cDNA sample per assay. The expression levels of ribosomal protein S9 were used as an internal control. 2-ΔΔCt analysis was performed on the data obtained. All primers for qRT-PCR were purchased from ThermoFisher, and the sequences for which are listed in Supplemental Table S1.

Supplementary Tables

Target	Forward Primer	Reverse Primer
Primers for qRT-PCR		
MYOCD	TCAGCAATTTCAGAGGTAACACA	TGACTCCGGGTCATTTGC
ACTA2	AGCGTGGCTATTCCTTCGTT	GTGGTTTCATGGATGCCAGC
MYH11	AGACACAAGTATCACGGGAGAG	TTGGCTCCCACGATGTAACC
TAGLN	CACCCTCCATGGTCTTCAAGC	TGCTCCTGCGCTTTCTTCATAA
CNN1	TGGAGCACTGCGACACG	GCCCTAGGCGGAATTGTAGTA
MYLK	ACAGAAACGGGCAACCCATC	TGCTTCGCAAAACTTCCTTCTACT
FOS	GGAGGACCTTATCTGTGCGTG	ATACACACTCCATGCGTTTTGC
SRF	TCCTCACACTGCTGTCCTCT	GTTCCCTCCAACCCAGGAG
CCL11	ATACCCCTTCAGCGACTAGAG	GCTTTGGAGTTGGAGATTTTTGG
CCL55	CGCTGTCATCCTCATTGCTACTG	GCAGGGTGTGGTGTCCGAG
IL-8	CCACCGGAAGGAACCATCTC	TTCCTTGGGGTCCAGACAGA
IL-6	CATGTCTCCTTTCTCAGGGCTG	GTAGCCGCCCCACACAGA
ICAM1	ATGGCAACGACTCCTTCTCG	CGCCGGAAAGCTGTAGATGG
VCAM1	TTTGGATAATGTTTGCAGCTTCTCA	ACAGGATTCATTGTCAGCGT
Primers for ChIP-qPCR		
ACTA2 5' CArG boxes	AGCAGAACAGAGGAATGCAGTGGAAGAGAC	CCTCCCACTCGCCTCCCAAACAAGGAGC
MYH11 5' CArG boxes	CTGCGCGGGACCATATTTAGTCAGGGGGAG	CTGGGCGGGAGACAACCCAAAAAGGCCAGG
FOS 5' CArG box	CCGCCTCCCCCCGCACTGCACCCTCGGTG	CAGGGCTACAGGGAAAGGCCGTGGAAACCTG

Supplementary Table E1: Primer sequences

Supplementary Table E2: Antibodies for WB

Target	Supplier	Cat. #
GAPDH	Millipore	MAB374
MyoCD	Sigma-Aldrich	SAB4200539
α-SMA	Invitrogen	14-9760-82
SMMHC	Abcam	ab53219
MLCK1	Abcam	ab8978
pRLC	Cell Signaling Technology	3671
Total RLC	Cell Signaling Technology	3672
SRF	Cell Signaling Technology	5147
histone H2B	Biolegend	606301
HRP-conjugated anti mouse IgG	Biolegend	405306
HRP-conjugated anti-rat IgG	Biolegend	405405
HRP-conjugated anti-rabbit IgG	Biolegend	406401

Supplementary Figures

Figure E1

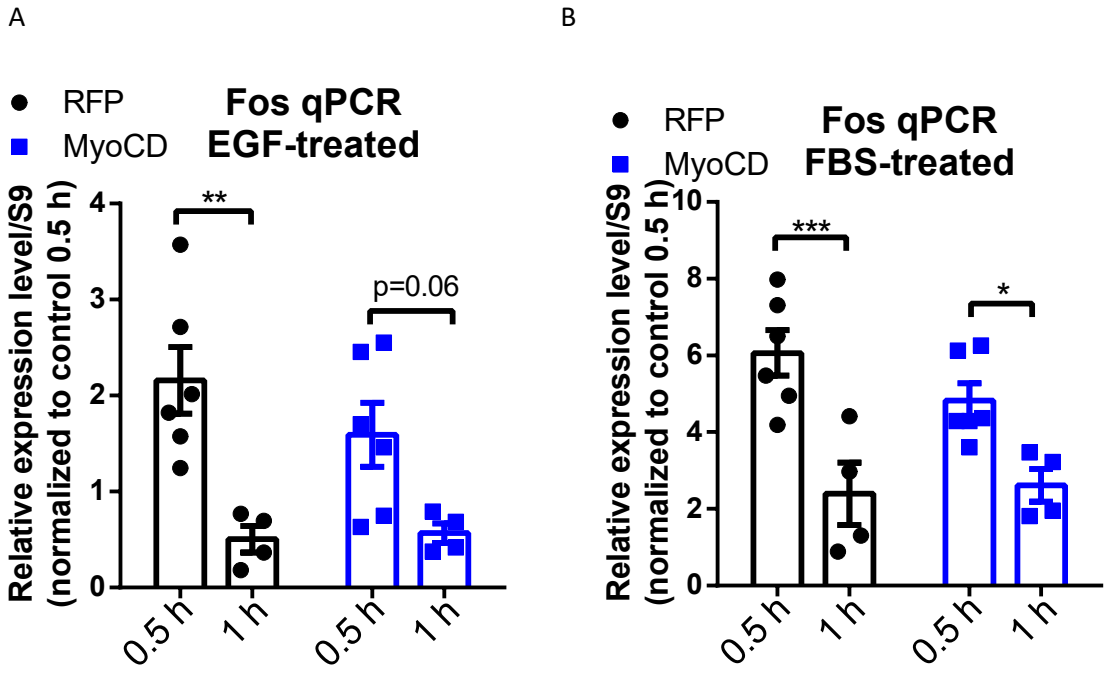


Figure E1: Mitogen-induced Fos expression diminished by 1 hour following treatment. A-B: mRNA level of Fos in RFP-transduced control and MyoCD-transduced ASMCs following 10 ng/mL EGF or 10% FBS treatments assessed at 0.5 hr and 1 hr. Data are mean ± SEM, 2-way repeated-measures ANOVA with Bonferroni post-test. *:p<0.05, **: p<0.01, ***: p<0.001.

Figure E2

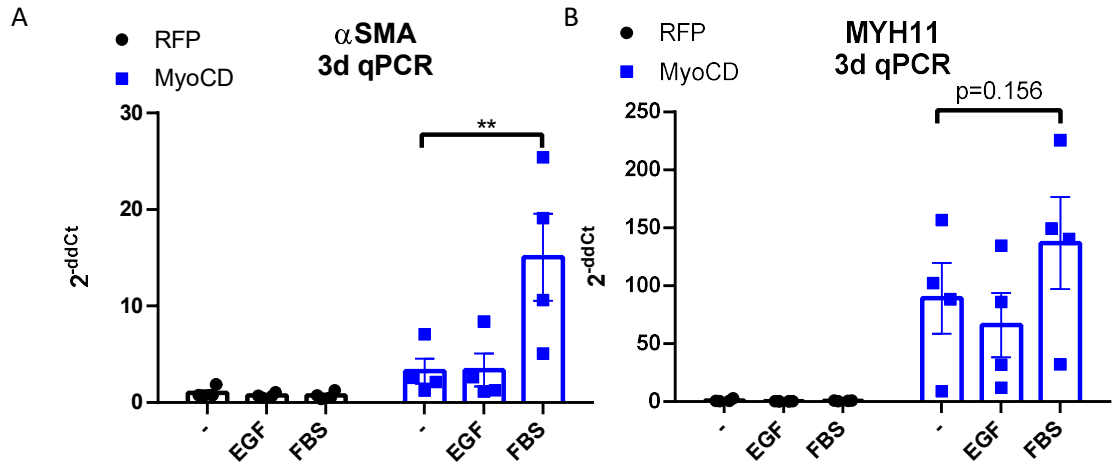


Figure E2: 3-day FBS treatment yielded a trending increase of MYH11 transcript level. A-B: mRNA level of α-SMA and MYH11 in RFP-transduced control and MyoCD-transduced ASMCs following 3-day 10 ng/mL EGF or 10% FBS treatments. Data are mean ± SEM, 2-way repeated-measures ANOVA with Bonferroni post-test, **: $p<0.01$.

Figure E3

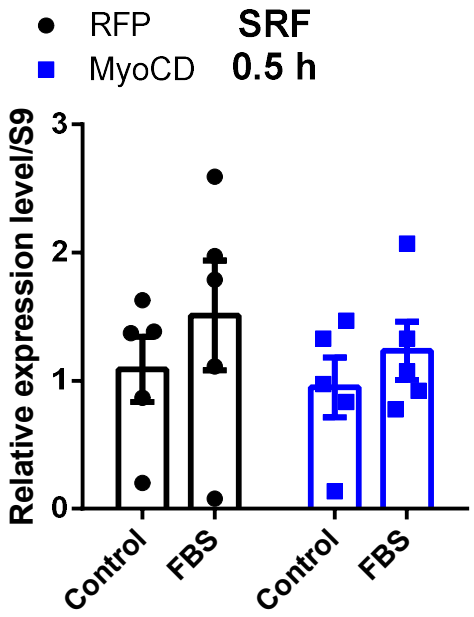


Figure E3: FBS-induced SRF upregulation was not observed at 0.5 hr. mRNA level of SRF in RFP-transduced control and MyoCD-transduced ASMCs following 10% FBS treatments assessed at 0.5 hr. Data are mean ± SEM, 2-way repeated-measures ANOVA with Bonferroni post-test.

Figure E4

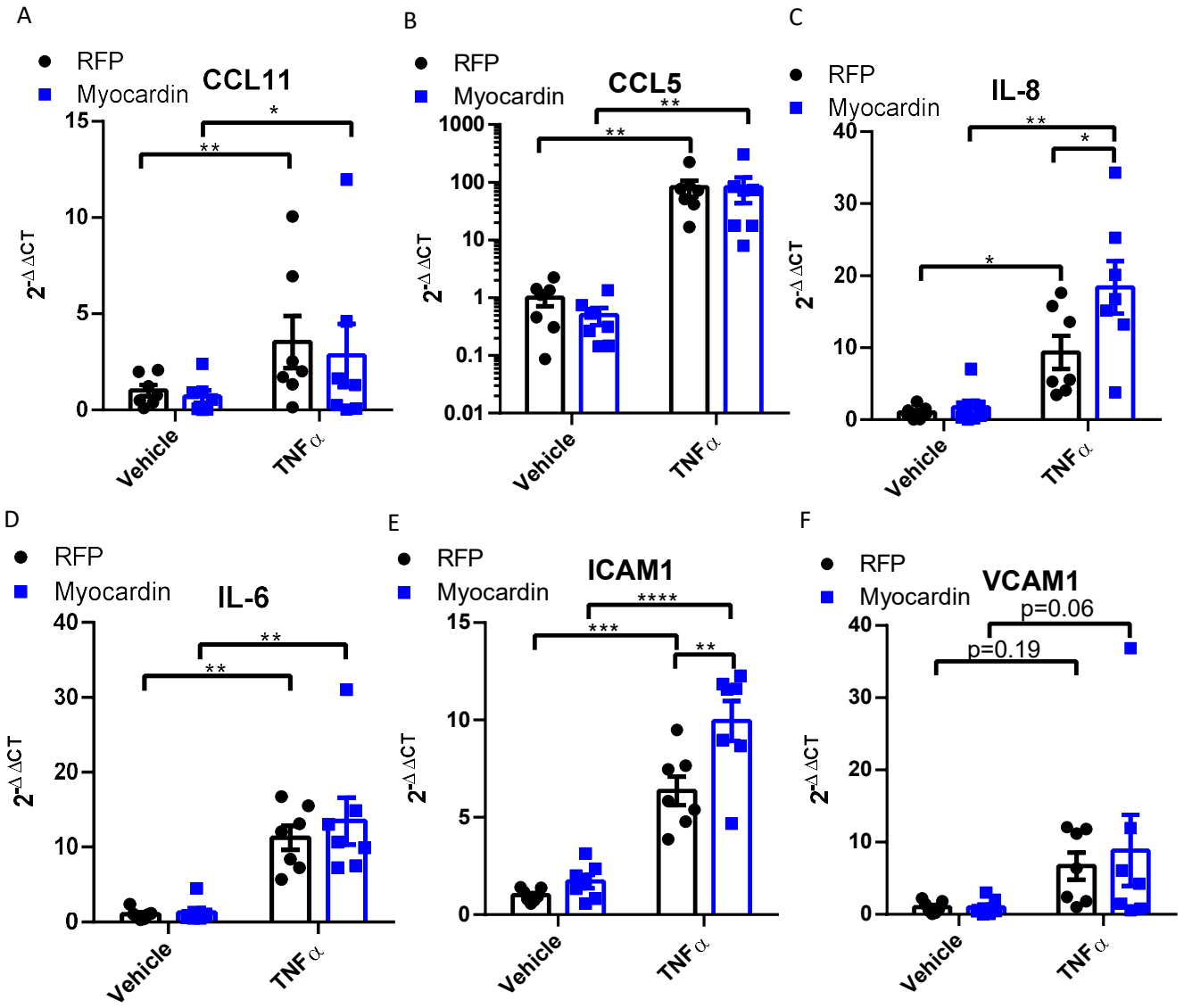


Figure E4: MyoCD transduction did not inhibit NF-κB-inducible inflammatory gene expression. A-F: mRNA levels of CCL11, CCL5, IL-8, IL-6, ICAM1 and VCAM1 in RFP-transduced control and MyoCD-transduced ASMCs following vehicle (PBS) or 10 ng/mL TNFα treatments. Data are mean ± SEM, 2-way repeated-measures ANOVA with Bonferroni post-test. *:p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001.

Preface to Chapter 3

In addition to the contractile versus proliferative smooth muscle phenotype dichotomy, the synthetic phenotype is frequently described in the literature, which is often also regarded as the counterpart of the contractile phenotype[1]. Indeed, ASM is capable of responding to inflammatory mediators and produces cytokines, chemokines[2], adhesion molecules[3] and ECM proteins[4]. Specifically in the context of inflammation, MyoCD-NF-κB mutual antagonism via direct interaction has been described in VSMC[5]. In the experiments described in Chapter 2, however, we failed to observe such a phenomenon in ASMC. As the molecular mechanism determining contractile versus synthetic phenotype polarization is yet to be fully established, it is difficult to conclude whether the two phenotypes indeed demonstrate such relationship.

In the following study, we investigated ASMC synthetic capacity with a particular focus on exploring ASMC-derived chemokines. This is a pertinent research question as mast cells and CD4+ T cells have both been found to infiltrate asthmatic ASM bundles, presumably in response to ASM-derived recruitment signals, and mediate pathological changes via contact-dependent interactions[6-8]. Motivated by our group's previous observations on T cell-ASM juxtapositioning[8], we used an *in vitro* cell migration assay to identify the ASMC-derived, T cell-reactive chemotactic factors.

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Interferon-γ amplifies airway smooth muscle-mediated CD4+ T cell recruitment by promoting the secretion of C-X-C-motif chemokine receptor 3 ligands

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Nonstandard abbreviations:

αSMA: α-smooth muscle actin

ASM: airway smooth muscle

ASMC: airway smooth muscle cell

CCL: C-C motif chemokine ligands

CCR: C-C motif chemokine receptors

CXCL: C-X-C motif chemokine ligands

CXCR: C-X-C motif chemokine receptors

FMO: Fluorescence minus one

IFN-γ: Interferon-γ

IFN-γR1: Interferon-γ receptor 1

MFI: Mean fluorescence intensity

qRT-PCR: quantitative RT-PCR

siRNA: small interference RNA

STAT1: Signal Transducer and Activator of Transcription 1

T_H: T helper cell

TNFα: tumor necrosis factor α

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Abstract

Asthmatic airways feature increased ASM mass that is largely attributable to hyperplasia, and which potentially contributes to excessive airway narrowing. T cells induce ASMC proliferation via contact-dependent mechanisms *in vitro* that may have importance for asthmatic ASM growth, as CD4+ T cells infiltrate ASM bundles in asthmatic human airways. In this study, we used an *in vitro* migration assay to investigate the pathways responsible for the trafficking of human CD4+ T cells to ASM. ASMCs induced chemotaxis of activated CD4+ T cells, which was inhibited by the CXCR3 antagonist AMG487 and neutralizing antibodies against its ligands CXCL10 and 11, but not CCR3 or CCR5 antagonists. CXCR3 expression was upregulated among all T cells following anti-CD3/CD28-activation. CD4+ T cells upregulated CXCL9, 10 and 11 expression in ASMCs in an IFN-γ/STAT1-dependent manner. Disruption of IFN-γ-signaling resulted in reduced T cell migration, along with inhibition of CD4+ T cell-mediated STAT1 activation and CXCR3 ligand secretion by ASMCs. ASMCs derived from healthy and asthmatic donors demonstrated similar T cell-recruiting capacities. *In vivo* CXCL10 and 11 expression by asthmatic ASM was confirmed by immunostaining. We conclude that the CXCL10/11-CXCR3 axis causes CD4+ T cell recruitment to ASM that is amplified by T cell-derived IFN-γ.

Key words: T cell chemotaxis, airway smooth muscle, CXCR3, CXCL10, CXCL11, IFN-γ

Introduction

Asthma is an inflammatory airway disease primarily characterized by reversible airflow obstruction, but frequently accompanied by structural alterations of the airway walls contributing to susceptibility of airflow limitation, referred to as airway remodeling (1). All tissues of the airways may be affected but the increase in airway smooth muscle (ASM) mass is one of the most prominent characteristics of airway remodelling, with its magnitude being a strong predictor of disease severity and persistent airflow limitation (2, 3). Both ASMC hypertrophy and hyperplasia are observed in asthma and are hypothesized to contribute to the enlarged bundles of ASM (4-6), although the exact biological mechanisms responsible are yet to be fully elucidated (7).

Evidence to date suggests that CD4+ T cells may be involved in mediating pathological changes of ASMs through contact-dependent induction of hyperplasia. *In vitro* studies have demonstrated that CD4+ T cells were capable of adhering to airway smooth muscle cells (ASMCs) through the interactions between integrin-adhesion molecules and/or CD44-hyaluronic acid, subsequently inducing DNA synthesis within ASMCs (8). The pro-proliferative effect of the CD4+ T cells on rat ASM is contact-dependent and is mediated by T cell-derived heparin-binding epidermal growth factor (9). In a rat ovalbumin-induced asthma model, adoptively transferred CD4+ T cells derived from ovalbumin-sensitized donors were localized in ASM bundles in naïve recipients upon ovalbumin exposures, and resulted in increased ASM mass (10). T cell infiltrates were also found in asthmatic human ASM bundles, the quantity of which positively correlated with ASM mass and disease severity (2). In addition, the T cells frequently were in juxtaposition with proliferating ASMCs, suggesting that the T cells promote the replication of ASMCs in close contact (2).

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It is evident that the two cell types undergo complex intercellular communication, likely playing a role in mediating increases in ASM mass associated with asthma. To our knowledge there has not been any study describing the signaling pathways responsible for the homing of CD4+ T cells to ASMs. However, it has been established that ASMCs are able to take on secretory functions and directly participate in inflammatory processes through the production of cytokines and chemokines (1, 11, 12). Therefore, we hypothesize that CD4+ T cells traffic to ASMs in response to certain ASMC-derived chemotactic factors. In the current study, we confirmed ASMC-mediated CD4+ T cell recruitment using an *in vitro* T cell migration model. We demonstrated that the CXCL 10 and 11-CXCR3 axis was the chemotactic pathway responsible for said process. We described the amplifying effect of CD4+ T cell-derived interferon-γ (IFN-γ) on the chemotactic pathway, which potentially provides a positive feedback mechanism mediating further T cell recruitment by ASMCs. Lastly, we compared ASMCs derived from healthy and asthmatic donors and showed that they had similar CXCR3 ligand secretion profiles and CD4+ T cell-recruiting capacity.

Materials and Methods

Cell and tissue procurement

Seventeen healthy blood donors (ten males and seven females) who were without a history of asthma and were aged eighteen years and above were recruited. Lung tissues from deceased organ donors, comprising six healthy individuals without a known history of asthma and five donors carrying a diagnosis of asthma, were procured by the International Institute for the Advancement of Medicine and National Tissue Research Interchange. The diagnosis of asthma was based on data in Donor Demographic Forms with limited information on disease severity.

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Additional donor information, including the age, gender and ethnicity of the donors are included in Supplementary Table 1. The procedures for procuring and using peripheral blood from volunteers and transplant grade lung tissues from deceased organ donors were approved by the Research Ethics Board of the McGill University Health Centre.

Antibodies and chemicals

Fetal bovine serum, PBS, 100× antibiotic-antimycotic solution, DMEM and RPMI culture media were purchased from Wisent (Saint-Jean-Baptiste, QC, Canada). Bovine serum albumin and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). The neutralizing antibodies against IFN-γ, CXCL9, 10 and 11 were purchased from R&D system (Minneapolis, MN, USA) and were re-suspended in PBS. Recombinant human IFN-γ was purchased from Stemcell Technologies (Vancouver, BC, Canada) and was dissolved in PBS. AMG487, SB328437 and Maraviroc were purchased from Tocris Bioscience (Bristol, United Kingdom) and dissolved in dimethyl sulfoxide.

CD4+ T cells isolation and activation

From each volunteer, 60mL of peripheral blood were drawn into sodium heparin-coated plastic tubes (BD Bioscience, San Jose, CA, USA) and diluted with an equal volume of PBS. The peripheral blood mononuclear cell fraction was recovered from the blood using density gradient centrifugation with Lymphoprep density gradient medium (Stemcell). CD4+ T cells were then isolated from PBMCs by immunomagnetic positive selection with human CD4+ microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The CD4+ T cells were activated by incubation with anti-CD3/anti-CD28 antibody-coated beads (Dynabeads Human T-Activator

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CD3/CD28, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) in 1:1 ratio for 72 hours in complete RPMI (10% fetal bovine serum and 1% antibiotic-antimycotic solution supplemented). Anti-CD3/anti-CD28 beads were used to activate the T cell receptor and co-stimulatory signals, respectively, so as to closely mimic antigen presenting cell-mediated CD4+ T cell activation *in vivo*.

ASMC culture

Tracheal or bronchial airway smooth muscle tissues were dissected from previously healthy donor lungs, and were treated with 0.4 mg/mL collagenase from Clostridium histolyticum (Sigma-Aldrich) dissolved in raw RPMI overnight to dissociate individual ASMCs from the tissue. The cells were then cultured in complete DMEM (10% fetal bovine serum and 1% antibiotic-antimycotic solution supplemented) in tissue culture-treated plates. The medium was refreshed every 2-3 days. Upon reaching 80-90% confluency, the ASMCs were passaged with 0.25% trypsin/2.25mM EDTA (Wisent). The ASMCs were used between passages 1-6 for all experiments. Only cells derived from healthy donors were used except for experiments in Figure 5, where ASMCs derived from healthy and asthmatic donors were compared to study the potential effects of asthma.

T cell migration assay

ASMC-mediated CD4+ T cell migration was assessed with a modified Boyden chamber assay based on Transwell cell culture inserts fitted with polycarbonate filters containing three-micron pores (Corning, NY, USA). Briefly, fifty thousand ASMCs were seeded in the lower chamber and cultured for 48 hours. For negative controls, the lower chambers were empty of ASMCs.

Upon the initiation of the assay, all medium in the apparatus was replaced with a 1:1 mixture of complete DMEM and complete RPMI. Five hundred thousand activated CD4+ T cells were placed into the upper chamber and the T cells that migrated through the polycarbonate filter into the lower wells were collected and counted manually using a hemacytometer. For experiments involving drug or antibody interventions, the T cells were pre-incubated with chemokine receptor antagonists or vehicle for thirty minutes before being placed into the upper chambers along with the antagonist-containing medium, and neutralizing antibodies or isotype controls were added to the lower wells at the start of the assay.

qRT-PCR

Total RNA from ASMCs was extracted with a commercial kit (RNeasy Mini Kit, Qiagen, Hilden, Germany) and quantified using a microplate reader (BioTek Instrument Inc., Winooski VT, USA). Reverse transcription and quantitative PCR were performed with commercial PCR mixes (Applied Biological Material Inc., Richmond, British Columbia) according to manufacturer instructions. Data were analyzed using $2^{-\Delta\Delta Ct}$ method with target gene expression normalized to ribosomal protein S9 level. The primers were purchased from Thermo Fisher Scientific, with sequences as follows: CXCL9 5'-ACTATCCACCTACAATCCTTGAAAGAC-3' forward, 5'-TCACATCTGCTGAATCTGGGTTTAG-3' reverse; CXCL10 5'-CTTCCAAGGATGGACCACACA-3' forward, 5'-CCTTCCTACAGGAGTAGTAGCAG-3' reverse; CXCL11 5'-AGAGGACGCTGTCTTTGCAT-3' forward, 5'-TGGGATTTAGGCATCGTTGT-3' reverse; S9 5'-CGCAGAGAGAAGTCGATGTG-3' forward, 5'-TGCTGACGCTTGATGAGAAG-3' reverse.

Flow cytometry

For CD4+ T cell phenotyping, the cells were stained first with a viability dye (eFluor™ 780, Invitrogen, Carlsbad, CA, USA) then with a panel of antibodies targeting surface markers for discriminating subsets. The antibodies used were as follows; BV510-conjugated anti-CD4 (BD Bioscience), PE-conjugated anti-CXCR3 (Biolegend, San Diego, CA, USA), APC-conjugated anti-CCR4 (Biolegend), PerCP-Cy5.5-conjugated anti-CCR6 (Biolegend), PE-Cy7-conjugated anti-CD25 (Biolegend) BV421-conjugated anti-CD127 (Biolegend), diluted in FACS buffer (1% bovine serum albumin in PBS) for 30 minutes on ice. The stained cells were treated with fixation reagent from Foxp3 / Transcription Factor Staining Buffer Set (Invitrogen) and stored until analysis.

For intracellular staining of chemokines, 6-hour GolgiStop™ (BD Bioscience)-treated ASMCs were incubated with trypsin-EDTA (Wisent) to detach them from the culture plates and to yield a single cell suspension. Following the staining with viability dye and BV421 anti-CD45 (Biolegend) for T cell exclusion, the cells were fixed and permeabilized according to the Buffer Set instructions and stained with APC-conjugated anti-human CXCL9 (Biolegend), PE-conjugated anti-human CXCL10 (Biolegend), and FITC-conjugated anti-human CXCL11 (LifeSpan BioSciences, Seattle, WA, United States) and diluted in permeabilization buffer for 30 minutes at room temperature. Stained cells were analysed on FACSCanto™ II analyzer (BD Bioscience), and data were analyzed using FlowJo software (BD Bioscience).

Western blot

ASMCs were washed and lysed with NP-40 cell lysis buffer (Invitrogen) supplemented with a protease and phosphatase inhibitor cocktail (Halt™, Invitrogen). The protein concentration in the

lysate was quantified using a Bradford protein assay (Quick Start™, Bio-Rad, Hercules, CA, USA). Ten micrograms of total protein were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% bovine serum albumin in TBS-Tween 20 (Sigma-Aldrich) at room temperature for 1 hour and incubated with primary antibodies, anti-IFN-γR1 (Santa Cruz Biotechnology, Dallas, TX, USA), anti-STAT1 phospho-Tyr701 (Biolegend) anti-total STAT1 (Cell Signaling Technologies, Danvers, MA, USA) and anti-GAPDH (MilliporeSigma, Burlington, MA, USA) overnight at 4°C. The appropriate horseradish peroxidase-conjugated secondary antibodies were then applied to the membrane and incubated at room temperature for 1 hour. The bands were visualized with Clarity ECL Western blotting substrate (Bio-rad).

Silencing of IFN-γR1 expression in ASMCs using siRNA

IFN-γR1 siRNA and control siRNA pools (Dharmacon, Lafayette, CO, USA) were pre-incubated with transfection reagent (Lipofectamine 2000, Thermo Fisher Scientific) dissolved in Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific). The mixture was then added to ASMC cultures at a final concentration of 10 nM siRNA. After two-day incubation, the cells were immediately used in the migration assay.

Chemokine analysis by ELISA

Supernatants from the cell cultures and the migration assay were collected and stored at -80°C until analysis. ELISA kits for human CXCL9, CXCL10 (both from R&D System) and CXCL11 (Sino Biological, Beijing, China) were used to measure the chemokine concentration in the supernatants.

Immunocytochemistry and immunohistochemistry

Migrated CD4+ T cells from the migration assay were deposited on glass slides using a Cytocentrifuge (Cytospin™ 4, ThermoFisher Scientific). Fresh tissues containing small airways from an asthmatic human lung procured by National Tissue Research Interchange (no further information regarding disease severity was available) were frozen in optimal cutting temperature compound. Four micron-thick sections were then obtained and loaded on glass slides. The cells and tissue sections were fixed with 4% paraformaldehyde (Sigma-Aldrich), permeabilized with 0.25% Triton X-100 (Sigma-Aldrich), and non-specific binding was blocked with 1% bovine serum albumin. The samples were then incubated with primary antibodies overnight at 4°C and secondary antibodies for 1 hour at room temperature. The primary antibodies used were mouse anti-CXCR3 (R&D System), mouse anti-αSMA (Sigma-Aldrich), goat anti-CXCL10 (R&D System) and rabbit anti-CXCL11 (Abcam, Cambridge, MA, USA). The corresponding secondary antibodies were PE conjugated anti-mouse (Biolegend), Alexa Fluor 555- and Alexa Fluor 488-conjugated anti-mouse (Thermo Fisher Scientifics), Alexa Fluor 555-conjugated anti-goat (Invitrogen) and Alexa Fluor 488-conjugated anti-rabbit (Thermo Fisher Scientifics), respectively. Coverslips were mounted with DAPI-containing mounting medium (Vector Laboratories, Burlingame, CA, USA) and the sections were visualized with confocal microscope (Zeiss LSM700, Carl Zeiss AG, Oberkochen, Germany).

Statistical analysis

All data are expressed as mean±SEM. Paired or unpaired 2-tailed Student's t-tests, one-way or two-way ANOVA with Bonferroni correction for multiple comparison were used as appropriate. A $p \leqslant 0.05$ was considered as statistically significant.

Results

ASMCs induce CD4+ T cell chemotaxis

We first examined if cultured ASMCs were capable of inducing migration of activated CD4+ T cells. Compared to the blank control, the presence of ASMCs in the lower chambers resulted in a significant increase in the number of T cells migrating through the porous filter (Fig. 1A) but only statistically significant at the 24-hour time point, with a magnitude of over 250% of the basal T cell migration rate. The increased number of T cells found in the lower chamber of the ASMC condition was not due to ASMC-induced T cell proliferation, as there was no significant difference in the total cell numbers (sum of migrated and un-migrated T cells, Supplemental Fig. 1).

We then determined if ASMC-induced CD4+ T cell migration was the result of chemotaxis or chemokinesis. ASMC-conditioned medium added to only the lower chamber induced an increase in T cell migration, which was diminished when the conditioned medium was added to both upper and lower chambers (Fig. 1B). This result indicates that the enhanced T cell migration was by chemotaxis as it is gradient-dependent, and that ASMCs constitutively secrete the T cell chemoattractant(s).

Blockade of the CXCL10, 11-CXCR3 axis inhibited ASMC-mediated CD4+ T cell chemotaxis

To identify the predominant ASMC-derived chemoattractant(s) responsible for the observed T cell migration, we chose to test the effects of pharmacological inhibitors against various chemokine receptors. ASMCs are known to secrete several T cell-associated chemokines, the most prominently described include CCL11 (eotaxin), CCL5 (RANTES) and CXCL10. The small molecule antagonists targeting the corresponding receptors for these chemokines were therefore first tested for their efficacy in blocking ASMC-mediated T cell migration. Compared to the vehicle control, 1μM AMG487, a CXCR3 antagonist, significantly inhibited ASMC-induced T cell chemotaxis. 10μM SB328437 (CCR3 antagonist) or 10μM maraviroc (an allosteric CCR5 antagonist) did not exert any notable effect (Fig. 1C).

We further confirmed the involvement of CXCR3 in mediating ASMC-induced T cell migration by blocking the action of its ligands, CXCL9, 10 and 11 using neutralizing antibodies. The neutralization of CXCL10 or 11 alone led to a statistically significant reduction in T cell migration (Fig. 1E and F). Combined neutralization of all three ligands yielded an even larger inhibitory effect (Fig. 1G).

CXCR3 was upregulated over the course of CD4+ T cell activation, leading to a homogeneous CXCR3+ CD4+ population

Following the observation regarding the involvement of CXCR3 in ASMC-mediated T cell recruitment, we characterized the expression pattern of the chemokine receptor among the T cells activated *in vitro*. In addition, we also sought to investigate if ASMCs preferentially attract CXCR3+ CD4+ T cells. FACS analysis revealed that activated CD4+ T cells are homogeneously CXCR3+ among both migrated and un-migrated populations (Fig. 2A).

As existing literature has described that freshly-isolated T cells demonstrate distinct CXCR3 positive and negative populations (13, 14), we postulated that anti-CD3/CD28 activation leads to global CXCR3 up-regulation. Indeed, approximately 50% of the CD4+ T cells were CXCR3+ prior to activation, and 24 hours of activation was sufficient to induce CXCR3+ upregulation in all T cells (Fig. 2B and C).

No phenotype selectivity observed in ASMC-mediated CD4+ T cell recruitment

CD4+ T cells have been described to express chemokine receptors in a largely heterogeneous pattern, which determines their differential homing properties and responses to specific inflammatory environments. In combination with CXCR3, which is widely recognized to be preferentially expressed by T_H1 cells, we utilized a plethora of other CD4+ T subset surface markers previously described to associate with T_H2, T_H9, T_H17 and T_{reg} (15) and the gating strategy shown in Supplemental Fig. 2A to determine if there was any subset selectivity associated with ASMC-mediated recruitment. We hypothesized that preferentially migrated CD4+ T subset should be enriched in the lower chambers. FACS analysis showed no difference in the abundance of CXCR3+CCR4-CCR6- (T_H1-like), CCR4+CCR6- (T_H2-like), CCR4+CCR6+(T_H17-like), CCR4-CCR6+ (T_H9-like) or CD25hiCD127- (T_{reg}) populations among migrated cells between those from the blank or ASMC conditions (Supplemental Fig. 2B).

Exposure to ASMCs induces CXCR3 internalization in CD4+ T cells

Flow cytometric analysis of T cell chemokine receptor expression also revealed that the migrated T cells when ASMC were present in the lower chamber displayed a markedly lower surface

CXCR3 expression level compared to the migrated T cells when the ASMCs were not in the lower chamber (Fig. 2D and E). The reduction was also observed among un-migrated cells in the ASMC-present condition, as well as the direct co-cultures of the two cell types (Fig. 2E), which further confirmed that the response was likely induced by soluble factors and not dependent on direct T cell-ASMC contact, or caused by the innate physical properties of migration assay apparatus. Such a pattern was not observed for the other chemokine receptors investigated (Supplemental Fig. 2C and D), indicating that the phenomenon was CXCR3-specific. Immunofluorescence staining of CXCR3 in migrated CD4+ T cells (Fig. 2F) indicated that exposure to ASMC led to receptor accumulation in intracellular compartments, likely in the endosomes (shown as aggregates highlighted by arrows).

CD4+ T cells upregulate secretion of CXCR3 ligands by ASMCs

We next characterized the expression and secretion of CXCR3 ligands by ASMCs. The mRNA of CXCL10 and 11 but not CXCL9 were detected in normal cultured ASMCs, and exposure to CD4+ T cells in the migration assay resulted in upregulation of the transcripts of all three chemokines in ASMCs (Fig. 3A). Analysis of culture medium by ELISA showed that only CXCL10 could be detected under basal conditions, following a period of 24 hours of culture of ASMCs from eight out of ten samples (Fig. 3B). Consistent with the observation by qPCR, all three ligands were upregulated, statistically significantly only at the 24 hour time point, when ASMCs were exposed to T cells in the migration assay (Fig. 3B). These CXCR3 ligands were unlikely to be T cell-derived, as they were not detected in supernatants from the blank condition, where only T cells were present (data not shown). To further confirm that the chemokines were ASMC-derived, we performed intracellular staining of CXCL9, 10 and 11 in ASMCs treated

with the protein transport inhibitor Golgistop™. The upregulation of only CXCL10 and 11 was observed within ASMCs upon exposure to CD4+ T cells in the migration assay (Fig. 3C). None of the CXCR3 ligands was observed by flow cytometry within CD4+ T cells (data not shown).

IFN-γ-STAT1 signaling is responsible for the induction of CXCL9, 10 and 11 in ASMCs.

As CXCL9, 10 and 11 are canonical interferon-inducible proteins, we then hypothesized that T cell-mediated upregulation of these chemokines in ASMCs is dependent on T cell-derived IFN-γ. We first addressed this possibility by investigating the activation state of STAT1, a transcription factor of the abovementioned chemokines in ASMCs, known to be associated with interferons. Exposure to CD4+ T cells in the migration assay led to a persistent elevation in the level of STAT1 phosphorylation, as well as the level of total STAT1, over the duration of the assay (24 hours) within ASMCs, but was only statistically significantly elevated at 24 hours (Fig. 4A). This finding supports the involvement of the interferons.

Treatment with a neutralizing antibody against IFN-γ in the migration assay resulted in reduced STAT1 activation within ASMCs as well as reduced production of CXCL9, 10 and 11 and CD4+ T cell recruitment by ASMCs (Fig. 4B-D). Further, a 24-hour incubation of ASMCs with T cell-conditioned medium also induced an increase in STAT1 activation and production of the abovementioned chemokines, which was inhibited by IFN-γ neutralization (Supplemental Fig. 3A and B). To confirm that these observations were not due to the effects of IFN-γ neutralization on T cells, we disrupted the signaling by treating ASMCs with IFN-γR1-targeting siRNA prior to the migration assay. Consistent with prior results, compared to control siRNA, IFN-γR1 siRNA reduced T cell-induced STAT1 activation, chemokine secretion and T cell

recruitment by ASMCs (Fig. 4E-G). Together, these data demonstrated that CD4+ T cell-derived IFN-γ drives CXCR3 ligand synthesis by ASMCs and amplifies their ability to recruit T cells.

ASMCs derived from healthy and asthmatic donors demonstrated similar IFN-γ-induced CXCR3 ligand secretion and CD4+ T cell recruitment

We investigated whether CXCR3 ligand expression and T cell recruitment by ASMCs derived from asthmatic individuals show inherently distinct properties. The healthy and asthmatic ASMCs demonstrated similar levels of CD4+ T cell-induced CXCL9, 10 and 11 secretion and T cell recruitment capacities (Figure 5A and B). Furthermore, no difference in basal level CXCL10 expression was observed, but compared to healthy ASMCs, asthmatic ASMCs secreted less CXCL10, yet similar quantities of CXCL9 and 11 upon the stimulation with 100 U/mL recombinant human IFN-γ (Figure 5C). Conditioned medium from recombinant IFN-γ-stimulated ASMCs increased T cell migration relative to medium from unstimulated control cells. However, the abovementioned distinction in CXCL10 expression between healthy and asthmatic cells did not translate into any differences in conditioned medium-induced T cell migration (Figure 5D).

Expression of CXCR3 ligands by asthmatic ASM in vivo

To relate our findings to the pathological context *in vivo*, we examined the expression of CXCL10 and 11 by ASM via immunostaining of airway tissues derived from an asthmatic lung. weak CXCL10 immunoreactivity was detected that was co-localized with ASM (Fig. 6, upper panels, chemokine positive areas highlighted by arrows). Strong CXCL11 immunoreactivity was

detected, and was localized to ASM as well as in the epithelium, but not in the vascular smooth muscle (Fig. 6, lower panels).

Discussion

T cells have been described to infiltrate ASM bundles in asthmatic subjects and to be in contact with ASMCs following their adoptive transfer in an animal asthma model, findings that correlate with increased ASM mass (2, 10). We hypothesized that certain ASMC-derived chemotactic factors are responsible for the T cell homing to ASM. In the current study, we established that ASMCs are capable of inducing CD4+ T cells chemotaxis *in vitro*, which is inhibited by the CXCR3 antagonist AMG487 and neutralizing antibodies against its cognate ligands CXCL10 and 11. We confirmed the *in vivo* expression of the two ligands by ASM in asthmatic airways by immunohistochemistry. We observed that anti-CD3/CD28-activation directly upregulated CXCR3 expression among CD4+ T cells. Lastly, exposure to CD4+ T cells led to increased CXCR3 ligand secretion by ASMCs, which was mediated by T cell-derived IFN-γ. Disruption of IFN-γ signaling inhibited STAT1 activation in ASMCs, which corresponds to reduced CXCR3 ligand secretion and T cell recruitment. Taken together, our findings revealed a potential pathway responsible for T cell trafficking to ASMs, and the relevance of T_H1 inflammation in this pathway.

Existing evidence supports the involvement of CXCR3-mediated immune cell recruitment in asthma pathogenesis. Concentrations of CXCL9 and 10 in bronchoalveolar lavage fluid were significantly higher among asthmatic subjects compared to healthy controls, which correlated positively with sputum eosinophil, neutrophil percentages and airflow limitation reflected by reduction in forced expiratory volume in 1 second (16). Segmental allergen challenges within asthmatic airways led to marked increase of CXCL10 in bronchoalveolar

lavage fluid (17). Similarly, in a mouse ovalbumin-induced asthma model, an increase in CXCR3 mRNA level was detected in the lungs following sensitization and challenges, and the associated inflammation and airway hyperresponsiveness were ameliorated by a dual inhibitor of CXCR3 and CCR5 (18). A single nucleotide polymorphism on the human CXCR3 gene was also found to be associated with a higher risk of developing asthma (19). Our study provides a potential mechanism through which CXCR3-mediated T cell recruitment could also plays a role in airway remodelling, specifically changes of ASMCs.

Besides mediating bronchomotor tone, ASMCs are immunoreactive cells known to secrete multiple cytokines, chemokines and growth factors and directly contribute to inflammatory processes (11). Expression of CXCR3 ligands by ASM has been widely described (20-22). Our study once again confirmed the secretion of all three ligands by ASMCs *in vitro* and the expression of CXCL10 and 11 in asthmatic ASM *in vivo*. CXCL9, 10 and 11 are known as interferon-inducible genes that primarily induce chemotaxis of lymphocytes and NK cells, among which, CXCL10 is usually present in the highest abundance, whereas CXCL11 shows the highest binding affinity towards CXCR3 (23, 24). Interleukin-1β, TNFα and IFN-γ are all capable of upregulating CXCL10 production by ASMCs individually and synergistically when combined, with IFN-γ eliciting the most potent response (22). c-Jun N-terminal kinase and nuclear factor-κB pathways are also involved in the induction of CXCL10 expression, potentially in a STAT1-independent manner (22). Notably, one study indicates that CXCL10 expression by ASMs is detected in bronchial biopsies from asthmatic patients only and not healthy controls, and that the chemokine directs mast cell migration to ASMCs (21), another cell type found to prominently infiltrate asthmatic ASM bundles (21, 25). Apart from the CXCR3 ligands, other described ASMC-derived chemokines that are potentially T cell-reactive include eotaxins, CCL5

and CXCL8 (11). Our results suggest that the former two chemokines might be less relevant in ASMC-mediated T cell recruitment in comparison to CXCR3 ligands, at least *in vitro*. However, the expression of chemokine receptors by local T cells in asthmatic airways, the nature of the inflammation and its impact on ASMs would dictate the actual chemotactic pathway responsible. Therefore, whether CXCR3 is involved in T cell homing to ASMs *in vivo* remains to be confirmed.

Our experiments comparing ASMCs derived from healthy and asthmatic donors showed no notable difference in their CD4+ T cell recruitment capabilities *in vitro*, nor in the magnitude of CXCL9 and 11 secretion. There was a marginally lesser CXCL10 secretion by IFN-γ-treated asthmatic ASMCs, of uncertain significance. These findings are somewhat in contrast to the findings of a previous study, in which treatment of IFN-γ combined with interleukin-1β and TNFα induced a significantly higher production of CXCL10 in ASMCs derived from asthmatic subjects compared to healthy controls (22). Beside the difference in the tissue source (fiberoptic bronchoscopy was performed in the referenced study) and the stimuli used, this discrepancy could also be due to the difference in the subject characteristics. The study applied inclusion criteria in the asthmatic cohort that included documented airway hyperresponsiveness and reversible airflow obstruction (22), whereas limited by the availability of tissues from deceased organ donors, our experiments may have included a more heterogeneous sample of asthma phenotypes. Thus it is possible that certain asthmatic subtypes may behave differently. Overall, our evidence suggests that asthma per se does not lead to programmed changes in the capacity of ASMCs to secrete CXCR3 ligands. Rather, it is the inflammatory environment in asthmatic airways harbouring increased T_H1 cytokines that leads to increased chemokine secretion by ASMCs and subsequent T cell recruitment.

Corresponding to the heterogeneity of CD4+ T cell phenotypes and their differential trafficking patterns in response to inflammations of various sorts, the chemokine receptor expression profiles of different CD4+ T cell subsets is diverse (26). Each subset has its set of primarily associated chemokine receptors, the expression of which is often under the direct regulation of the corresponding signature transcription factor (26). CXCR3, expressed mainly by effector and memory CD4+ and CD8+ T cells, is widely recognized to be associated with T_H1-immunity (26). Indeed, CXCR3+ T cells accumulate in pathological conditions harbouring T_H1 inflammation such as autoimmune diseases and viral infection, and this receptor is proven to be essential for their transmigration into these tissues (14, 27). However, CXCR3 was found to be expressed homogeneously on all anti-CD3/CD28 activated CD4+ T cells in our experimental conditions. Consistent with our data, other reports also described CXCR3 upregulation on total CD4+ T cells as a response to *in vitro* or *in vivo* antigen stimulation (13, 14). The homogeneity of expression of CXCR3 in turn explains the lack of subset selectivity of ASMC-mediated CD4+ T cell recruitment as defined by surface markers. However, assuming the CXCR3 axis is responsible for T cell homing to ASMs *in vivo,* a selective recruitment of T_H1 to ASMs would be expected to occur, given that the CXCR3+ CD4+ T population indeed preferentially demonstrates T_H1 polarity *in vivo* (28). This would favor a T_H1-high inflammatory microenvironment within the ASM bundles. Such hypothesis and its implications remain to be investigated.

CD4+ T cells exposed to ASMCs demonstrated a marked reduction in surface CXCR3 levels. The phenomenon is CXCR3-specific, as such changes are not observed for other chemokine receptors. Our immunocytochemical staining supports the hypothesis that the receptor internalizes via endocytosis following exposure of high levels of the cognate ligands.

CXCR3 internalization on T cells has been previously described in physiological settings. CXCL11 is found to be especially potent for inducing receptor internalization (29) through biased engagement of the β-arrestin signaling pathway (30). In agreement with our observations, T cells in the segmental allergen challenge of asthmatic airways demonstrated reduced levels of surface CXCR3, and the incubation of bronchoalveolar lavage- or peripheral blood-derived T cells with exogenous CXCL10 and CXCL11 led to a similar reduction in surface receptor levels while the total protein level was unchanged, proving the receptor undergoes activation-induced internalization (31). We propose the described phenomenon reflects the desensitization process of cell signaling, but whether it has any functional implications for T cell biology remains to be explored.

Lastly, our study indicated that T cell-derived IFN-γ, a major T_H1 cytokine, drives CXCR3 ligand production by ASMCs through the activation of the STAT1 pathway, suggesting that T_H1 inflammation is potentially involved in ASM remodelling processes by promoting CD4+ T cell recruitment and infiltration. Furthermore, the described ASMC-T cell communication invokes a positive feedback loop (illustrated in Fig. 7), leading to further T cell recruitment and associated pathological changes, which we hypothesize could be initiated or accelerated by conditions harbouring T_H1 inflammation, such as viral infection. IFN-γ, along with IFN-γ-secreting CD4+ and CD8+ T cells have been described to be present in higher numbers in asthmatic airways (32-36). IFN-γ is also suggested to play an especially prominent role in severe asthma (37, 38). However, the exact functional roles of the cytokine, and more generally, T_H1 inflammation in asthma have not been sufficiently explored. Although several animal studies involving exogenous IFN-γ administrations and gene manipulations support a protective role of T_H1 in asthma due to its T_H2-antagonizing effects (39-41), emerging evidence

indicates T_H1 inflammation positively contributes to the pathogenesis by promoting the development of airway hyperresponsiveness (42, 43) and exacerbating airway inflammation (43-46). In addition, the increasing understanding of the disease heterogeneity reveals that some phenotypes or endotypes harbour T_H1-high characteristics (47). Recent transcriptomic analysis on sputum cells revealed a distinct non-T_H2 asthmatic phenotype characterized by upregulation of genes associated with IFN and TNFα superfamily responses, which correlated with neutrophilia, lack of eosinophilia and increased inflammasome activation (48). Overall, our study suggests a potential pathway linking T_H1 inflammation to alterations of ASMC characteristics and functions via CD4+ T cell recruitment.

Previous *in vitro* studies revealed contact-dependent complex bi-directional communications between CD4+ T cells and ASMCs. Signaling through the ligations of integrin-intercellular adhesion molecule 1 and CD44-hyaluronan expressed on CD4+ T cells and ASMCs, respectively, induce ASMC proliferation (8, 9). CD4+ T cell-derived membrane conduits connecting the cytosols of the two cell types have also been described, which promotes T cell survival by mediating the transfer of anti-apoptotic protein myeloid cell leukemia-1 from ASMCs to T cells (49). This suggests ASMCs might play a role in modifying T cell-mediated immune reactions (49). Overall, it is likely that T cell-ASMC interactions contribute to the pathophysiology of asthma, specifically the structural changes of the ASM. The findings in the current study described the trafficking mechanism that is likely to be the prerequisite for these intercellular communications.

In summary, our study demonstrated the CXCL9, 10 and 11-CXCR3 axis as the primary chemotactic pathway involved in ASMC-mediated CD4+ T cell recruitment *in vitro*, and that elevated ASMC chemokine production induced by T cell-derived IFN-γ promotes further T cell

recruitment, invoking a positive feedback loop that likely exacerbates ASM remodelling and inflammation in asthma. The described pathways provide promising therapeutic targets for alleviating T cell-mediated inflammation and structural remodelling in asthma, although *in vivo* evidence is needed to conclude as to its relevance in disease. Moreover, our evidence also provides a novel mechanistic basis of the involvement of IFN-γ and T_H1 inflammation in asthma pathophysiology, many aspects of which are valuable for future explorations.

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Author Contributions

R. Sun and J. G. Martin designed the study; R. Sun and J. H. Jang performed the experiments; R. Sun analyzed the data; R. Sun and J. G. Martin wrote and edited the manuscript; A. M. Lauzon provided the biological samples, contributed to the processes of related ethics approval and edited the manuscript.

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Figure Captions

Fig. 1: ASMC-induced chemotaxis of activated CD4+ T cells is dependent on CXCR3 signaling. A) Number of T cell migrated overtime in the Boyden chambers with or without ASMC seeded in lower chamber. Data are means±SEM (n=4 for blank group, n=5 for ASMC group), ****P<0.0001, 2-way ANOVA with Bonferroni posttest. B) Quantification of 24-hour T cell migration in control compared to ASMC-conditioned medium added to either lower chamber only or both upper and lower chambers. Data are mean±SEM (n=6), *P<0.05, **P<0.01, 1-way repeated measures ANOVA with Bonferroni posttest. C) Quantification of 24-hour T cell migration in blank and ASMC conditions under the treatment of vehicle, 1μM AMG487 (CXCR3 antagonist), 10μM SB328437 (CCR3 antagonist), or 10μM Maraviroc (CCR5 antagonist). Data are mean±SEM (n=8), ****P<0.0001, 2-way repeated measures ANOVA with Bonferroni posttest. D-G) Quantification of 24-hour T cell migration in blank and ASMC conditions under single or combined neutralizing antibody treatments against CXCL9, 10 and 11. Data are mean±SEM (n=6), *P<0.05, **P<0.01, ****P<0.0001, 2-way repeated measures ANOVA with Bonferroni post-test.

Fig. 2: CXCR3+ upregulation upon CD4+ T cell activation and receptor internalization following exposure to ASMC. A) Percentage of CXCR3+ CD4+ T cells in migration assay. Data are mean±SEM (n=15), 2-way repeated measures ANOVA with Bonferroni posttest. B) Representative flow cytometry plot demonstrating the effect of anti-CD3/CD28 activation on CXCR3+ expression on CD4+ T cells. C) Quantification of the proportion of CXCR3+ T cells comparing control cells and cells being activated over 24 or 72 hours. Data are mean±SEM (n=3-5), **P<0.01, ****P<0.0001 compared to 0 hr, ##P<0.01 compared to corresponding time points

in control, 1-way ANOVA with Bonferroni posttest. D) Representative flow cytometry plot demonstrating the exposure to ASMC led to reduction in CXCR3 signal among migrated T cells. E) Comparison of mean fluorescence intensity (MFI) of CXCR3 between T cells exposed or unexposed to ASMCs, in migrated, unmigrated populations and direct co-cultures. Data are mean±SEM (n=6 for control and n=15 for the remaining groups), ****P<0.0001, 2-way ANOVA with Bonferroni post-test. F) Immunofluorescence staining of CXCR3 demonstrating the subcellular location of the receptor in migrated cells from blank or ASMC condition. Scale bars represent 50μm. Arrows indicate intracellular foci of internalized receptors.

Fig. 3: CD4+ T cells upregulates the expression of CXCR3 ligands in ASMCs. A) Relative RNA levels of CXCL9, 10 and 11 in control ASMCs and T cell-exposed ASMCs from migration assay, measured by qPCR. Data are mean±SEM (n=8 for control ASMC and n=14 for T cell-exposed group), n.d.: not detected, *P<0.05, **P<0.01, 2-tailed, unpaired Student's t-test. B) ELISA measuring the levels of CXCL9, 10 and 11 in the supernatant of control ASMCs versus the supernatants from the lower chambers of ASMC condition in migration assay over 24 hours. Data are mean±SEM (n=8-10 for control ASMC and n=6-7 for T cell-exposed group), ****P<0.0001 compared to the same condition at 1 hr, ####P<0.0001 compared to corresponding time points in control ASMC groups, 2-way ANOVA with Bonferroni post-test. C) Representative flow cytometry plots and quantification on MFI of intracellular CXCL9, 10 and 11 comparing control ASMCs and T cell-exposed ASMCs from migration assay. Data are mean±SEM (n=8 for control group and n=16 for T cell-exposed group), *P<0.05, **P<0.01, 2-tailed, unpaired Student's t-test.

Fig. 4: CD4+ T cell-derived IFN-γ mediates upregulation of CXCR3 ligand secretion by ASMCs and further amplifies T cell recruitment. A) Representative Western blots, quantification of phospho-STAT1 and total STAT1 within ASMCs overtime in migration assay. Data are mean±SEM (n=5 for control ASMC group and n=7 for remaining groups) * P<0.05, **P<0.01 compared to corresponding time points in control ASMC group, 2-way ANOVA with Bonferroni posttest. B) Representative Western blots, quantification of STAT1 activation as well as total STAT1 comparing ASMCs in migration assay with isotype control or anti-IFN-γ antibody treatment. Data are mean±SEM (n=4), *P<0.05, 2-tailed, paired Student's t-test. C) ELISA quantification of CXCL9, 10 and 11 in lower chamber supernatants from migration assay with isotype control or anti-IFN-γ antibody treatments. Data are mean±SEM (n=9), *P<0.05, **P<0.01, 2-tailed, paired Student's t-test. D) Quantification of 24-hour T cell migration in blank and ASMC conditions under isotype control or anti-IFN-γ antibody treatments. Data are mean±SEM (n=9), *P<0.05, ****P<0.0001, 2-way repeated measure ANOVA with Bonferroni posttest. E) Representative Western blots, quantification of STAT1 activation as well as total STAT1 comparing ASMCs in migration assay with control or IFN-γR1 siRNA treatment. Data are mean±SEM (n=4), **P<0.01, 2-tailed, paired Student's t-test. F) ELISA quantification of CXCL9, 10 and 11 in lower chamber supernatants from migration assay with control and IFN-γR1 siRNA-treated ASMCs. Data are mean±SEM (n=6), *P<0.05, 2-tailed, paired Student's t-test. G) Quantification of 24-hour T cell migration induced by control siRNA-treated and IFN-γR1-treated ASMCs. Data are mean±SEM (n=4), *P<0.05, 2-tailed, paired Student's t-test.

Fig. 5: ASMCs derived from healthy and asthmatic donors demonstrate similar CXCR3 ligand secretion profile and CD4+ T cell recruiting capacity. A) ELISA quantification of

CD4+ T cell-induced secretion of CXCL9, 10 and 11 by ASMCs derived from healthy and asthmatic donors (H-ASMC and A-ASMC, respectively). Data are mean±SEM (n=5), 2-tailed, unpaired Student's t-test. B) Quantification of H-ASMC- and A-ASMC-mediated 24-hour CD4+ T cell migration. Data are mean±SEM (n=5), *P<0.05, 1-way ANOVA with Bonferroni posttest. C) ELISA quantification of of CXCL9, 10 and 11 secretion by H-ASMCs and A-ASMCs under untreated or 100 U/mL IFN-γ-treated conditions. Data are mean±SEM (n=5), n.d.: not detected, *P<0.05, ** P<0.01, **** P<0.0001, 2-way ANOVA with Bonferroni posttest. D) Quantification of 7-hour and 24-hour CD4+ T cell migration induced by 5×10^4/mL H-ASMC- or A-ASMC-conditioned medium under untreated or IFN-γ-treated conditions. Data are mean±SEM (n=10), *** P<0.001, **** P<0.0001, 2-way ANOVA with Bonferroni post-test.

Fig. 6: Expression of CXCR3 ligands by ASM *in vivo*. Immunofluorescence staining of CXCL10 (upper panels, green arrows highlight CXCL10+ ASM regions) or CXCL11 (lower panels) along with αSMA in frozen sections of human asthmatic airways. Scale bars represent 50 μm. ASM: airway smooth muscle, VSM: vascular smooth muscle, Epi.: epithelial cells.

Fig. 7: Proposed signaling pathways of ASMC-mediated CD4+ T cell recruitment and pathophysiology related to asthma. In asthmatic airways, ASM secrete CXCR3 ligands (CXCL10 is constitutive, and CXCL9, 10 and 11 are IFN-γ-inducible), recruiting CXCR3+ CD4+ T cells to ASMs. These T cells act as a source of IFN-γ, which stimulates ASMCs to further secrete mentioned chemokines. This results in subsequent amplification of CD4+ T cell recruitment, and potentially creates a positive feedback mechanism which contributes to further airway inflammation and ASM changes. Relating the signaling mechanism described to the

pathophysiology of asthma, many endotypes of asthma harbours T_H1-high environment and elevated level of IFN-γ in the airways. In addition, virus-induced exacerbations also produce IFN-γ-high inflammatory responses. All of these mechanisms could trigger chemokine secretion by ASMC and subsequent T cell recruitment. Furthermore, contact-dependent ASMC-T cell interactions lead to ASM hyperplasia, contributing to airway hyperresponsiveness. High T_H1 inflammation has also been correlated to corticosteroid insensitivity. Illustration created with BioRender.com.

Fig. 1

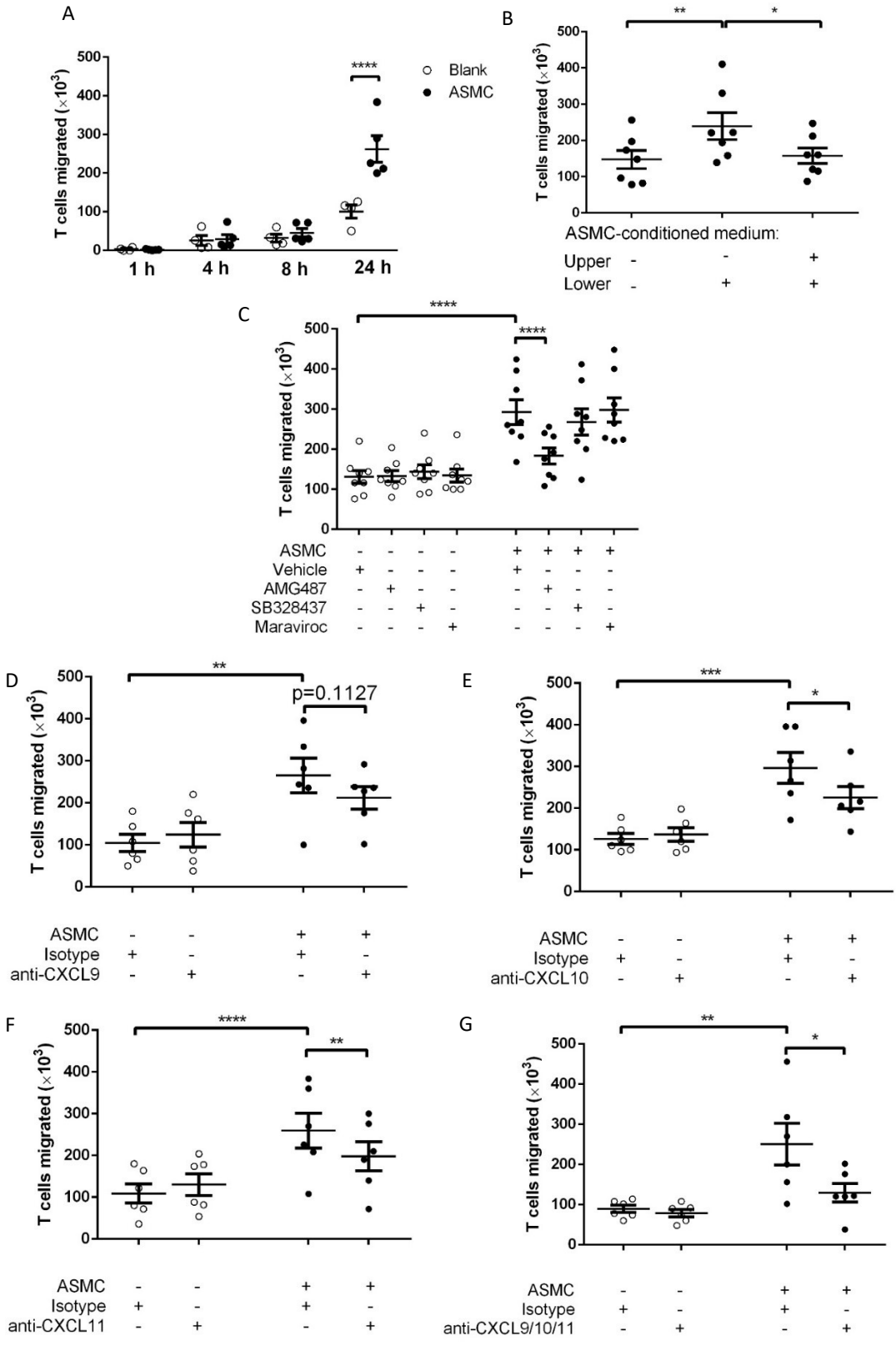

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Fig. 2

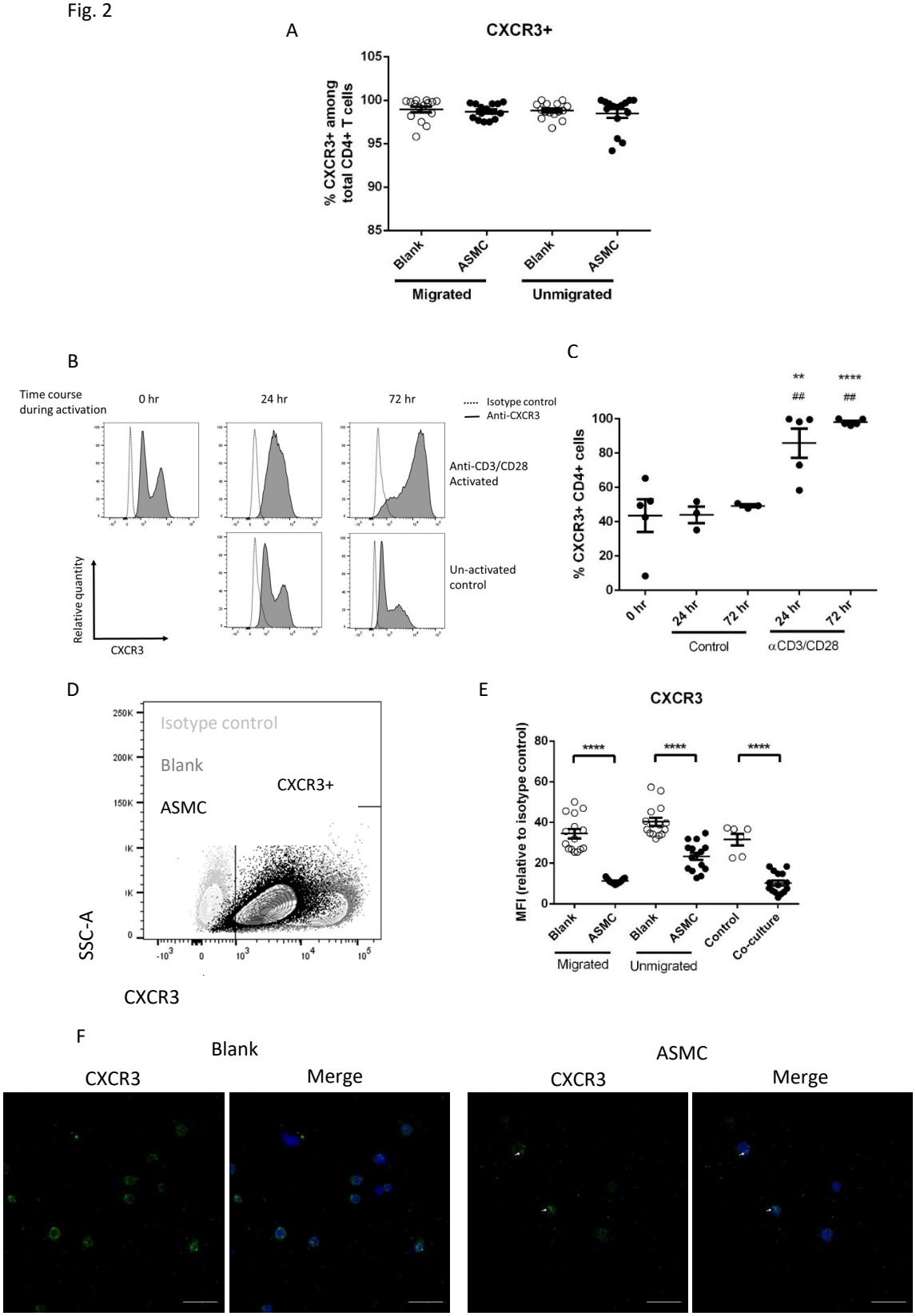

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Fig. 3

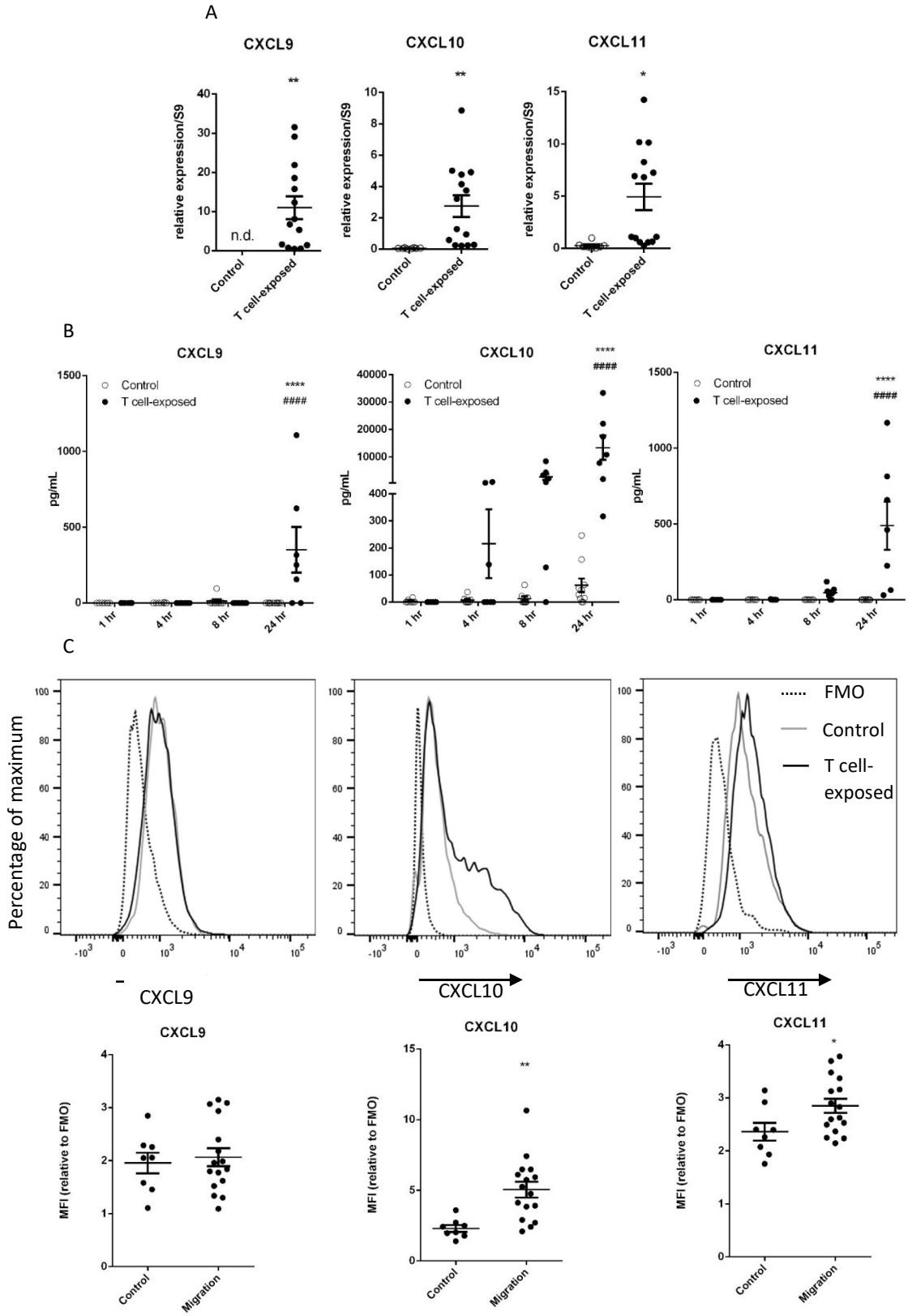

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Fig. 4

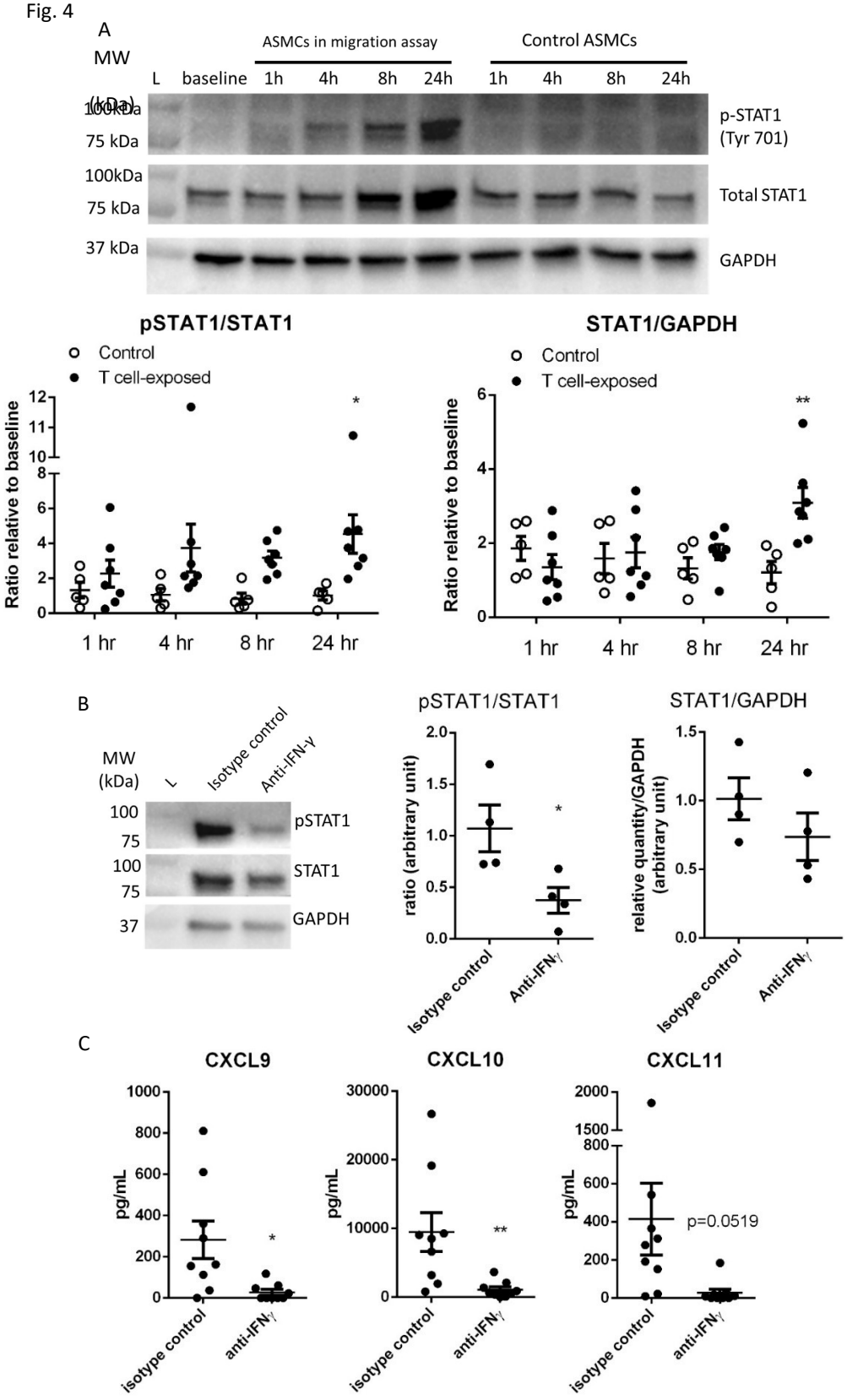

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Fig. 4 continued

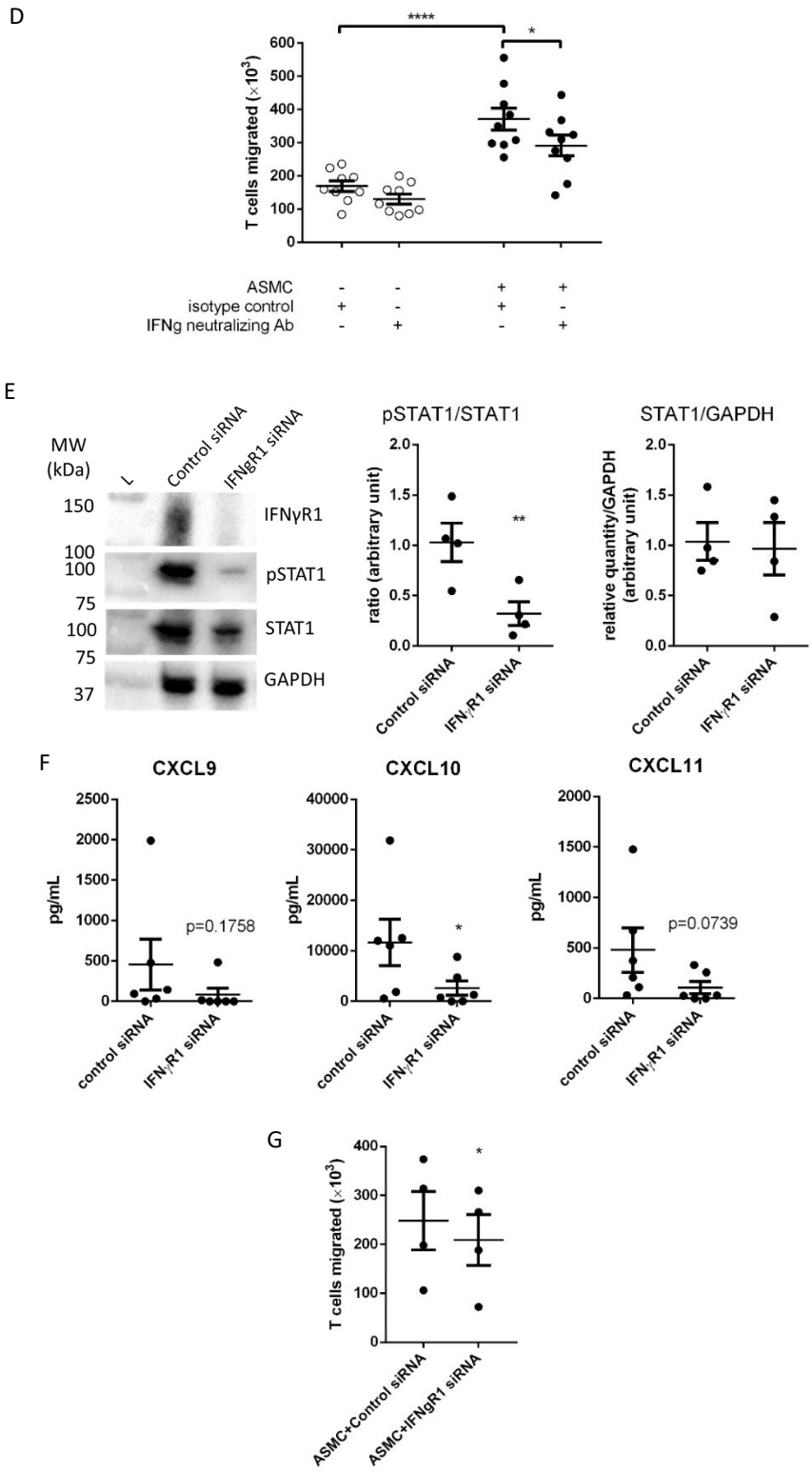

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Fig. 5

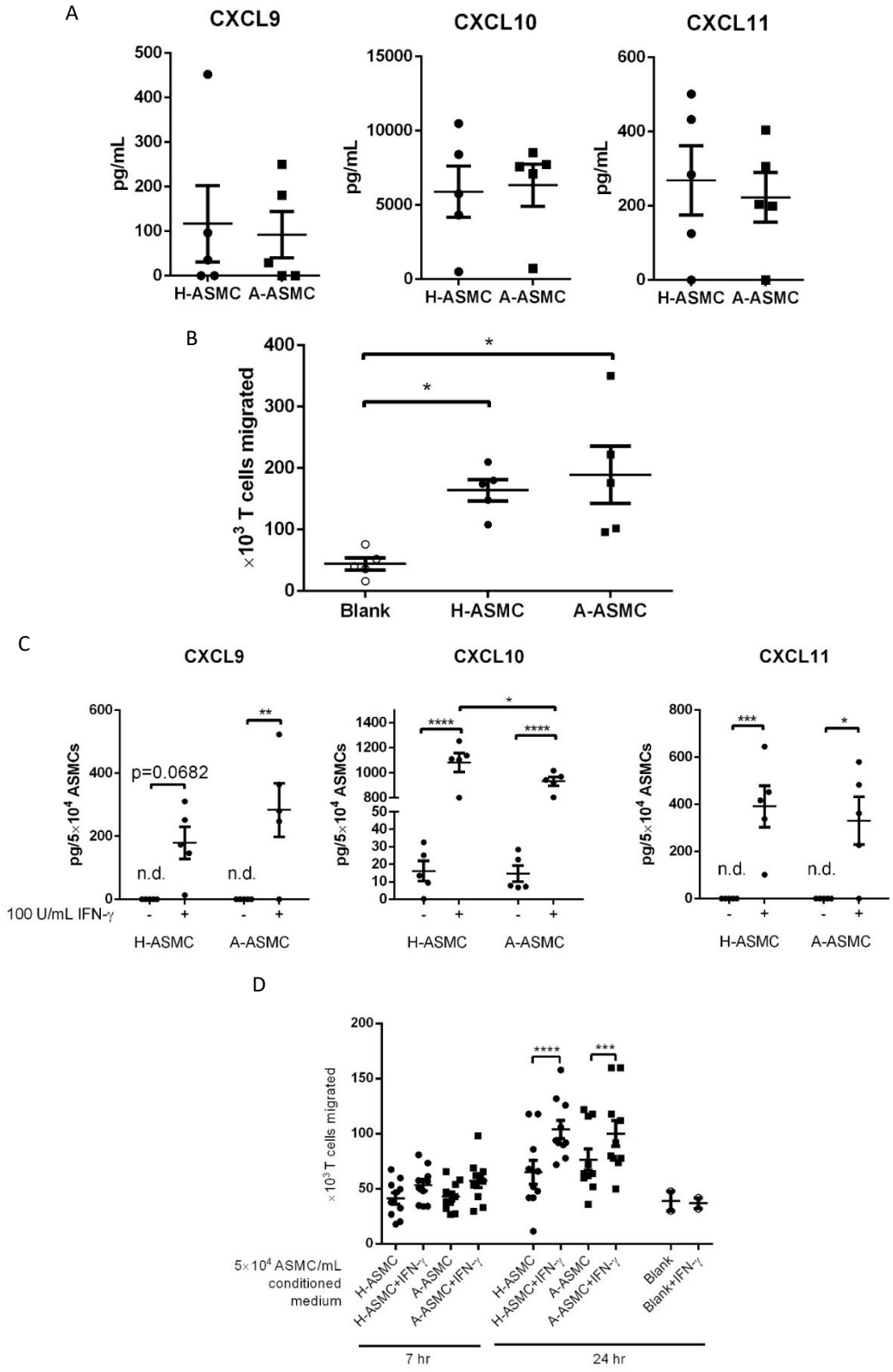
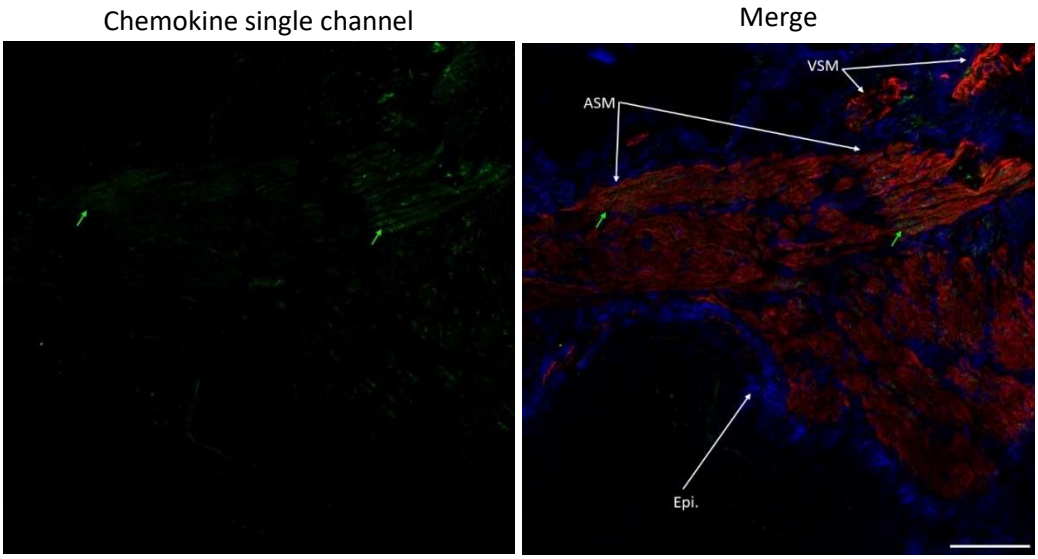
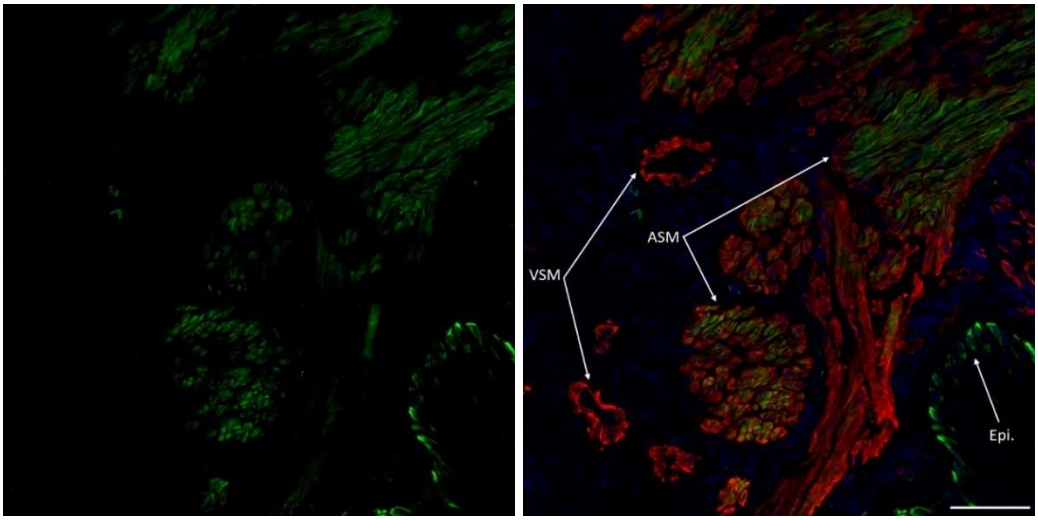


Fig. 6

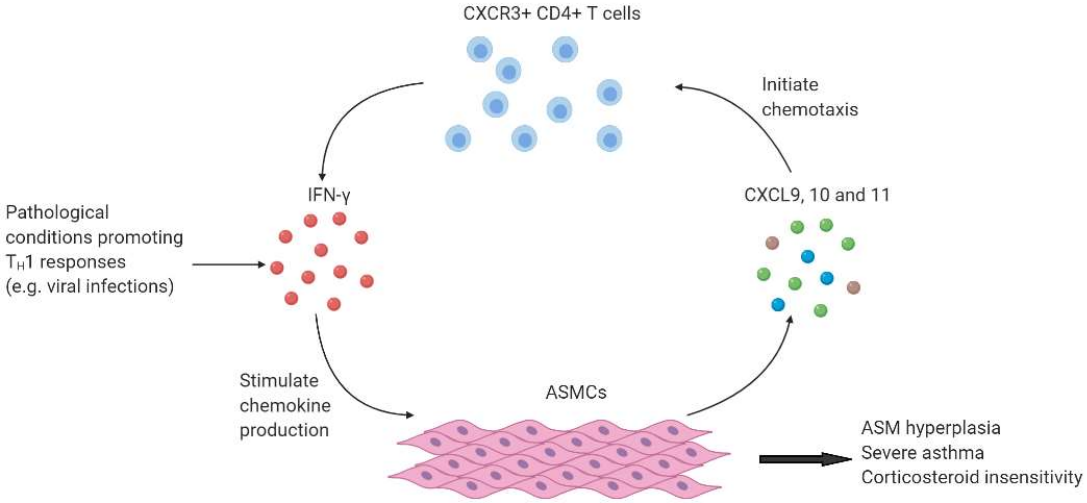


CXCL10/αSMA/DAPI



CXCL11/αSMA/DAPI

Fig. 7



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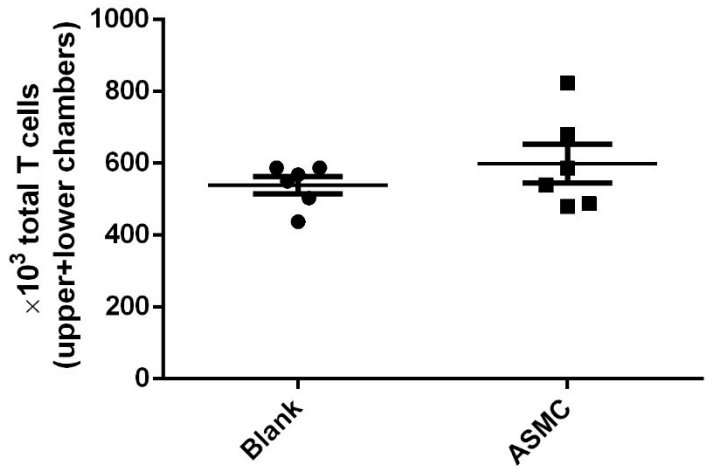
Supplementary Materials

Supplemental Table 1: Information of lung tissue donors for ASMC cultures:

Healthy			Asthmatic		
Age	Gender	Ethnicity	Age	Gender	Ethnicity
55	Male	Caucasian	60	Female	Caucasian
35	Male	Caucasian	40	Male	Asian
54	Female	Caucasian	23	Male	Hispanic
55	Male	Black/African American	52	Female	Black/African American
18	Male	Caucasian	56	Female	Black/African American
37	Male	Caucasian			

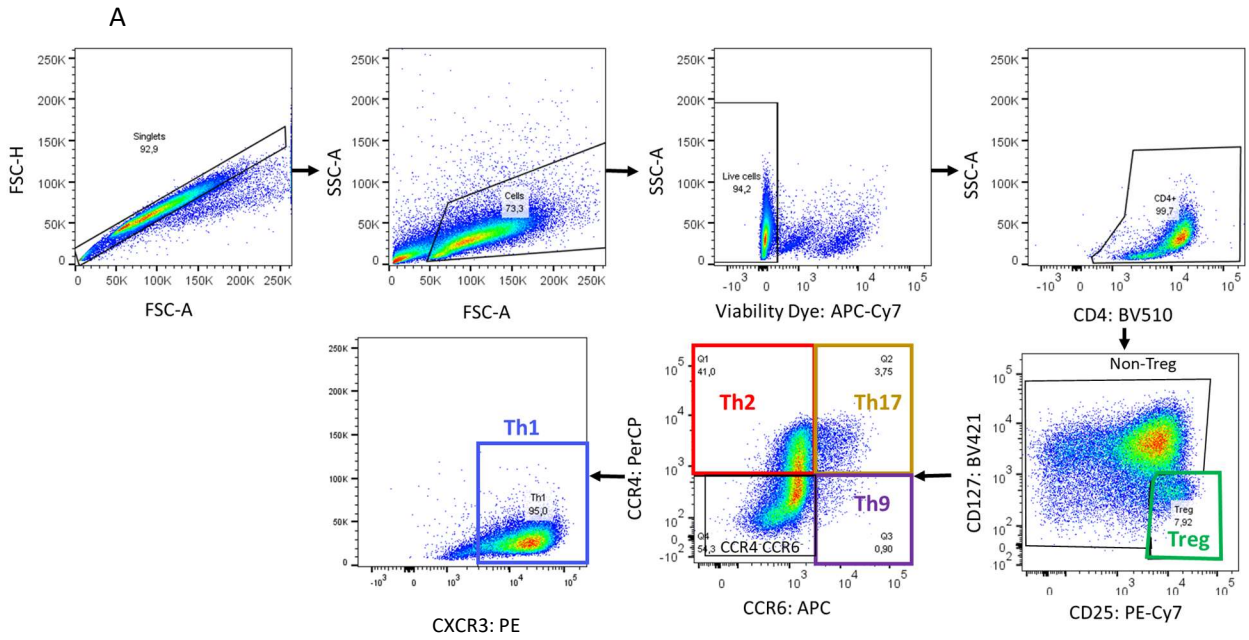
Supplemental Figures

Supplemental Fig. 1



Supplemental Fig. 1: No significant difference in CD4+ T cell proliferation between control and ASMC conditions in migration assay. Quantification of the total CD4+ T cells (upper chamber and lower chamber) in two conditions of the migration assay. Data are mean±SEM (n=6), 2-tailed, paired Student's t-test.

Supplemental Fig. 2



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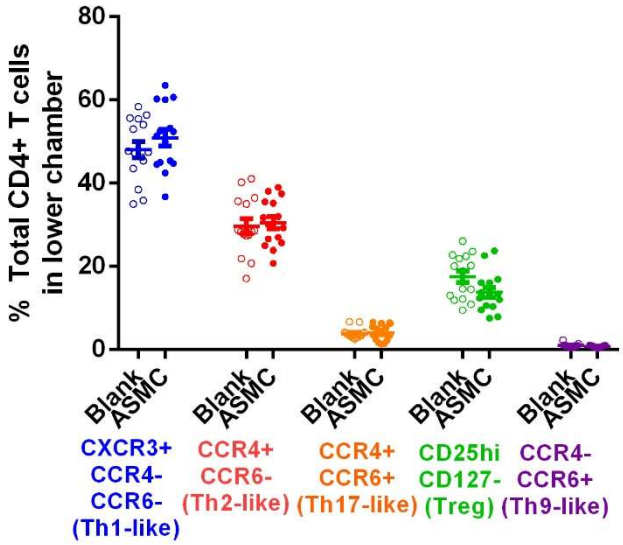

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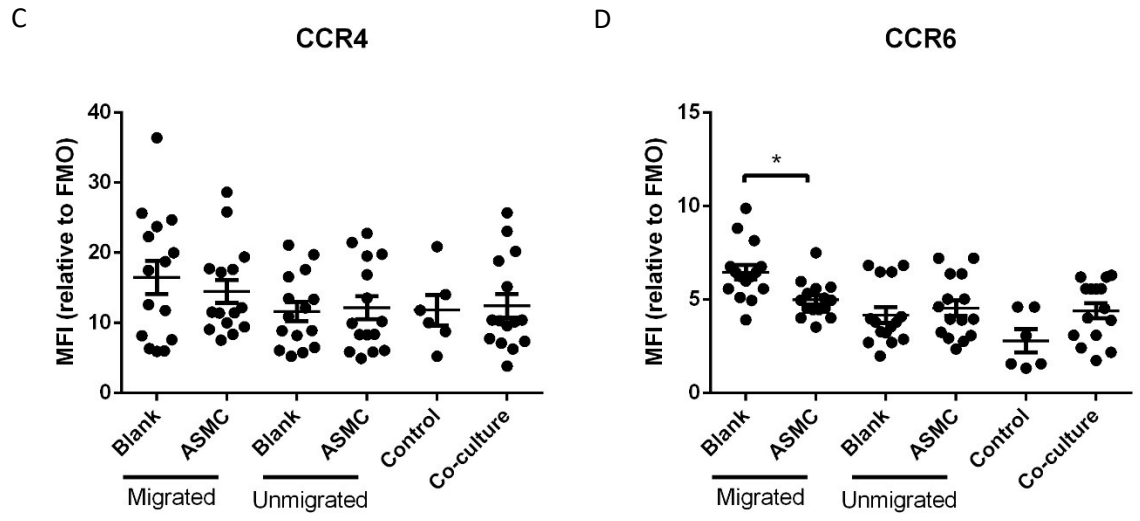
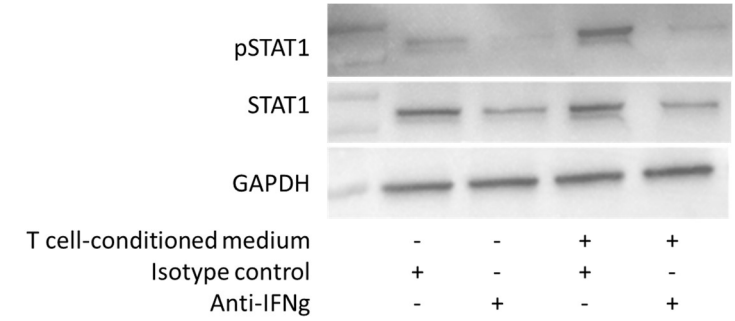


Supplemental Fig. 2: No phenotype selectivity was demonstrated in ASMC-mediated CD4+ T cell recruitment as characterized by the subset surface markers. A) The gating strategies for distinguishing T_H1-like, T_H2-like, T_H17-like, T_H9-like and T_{reg}-like populations among activated CD4+ T cells based on surface markers. B) Comparisons of proportions of each mentioned populations among migrated CD4+ T cells between blank and ASMC conditions. Data are mean±SEM (n=15), 1-way ANOVA with Bonferroni posttest. C) The MFI of CCR4 and CCR6 among total CD4+ T cells in migration assay and direct co-culture experiment. Data are mean±SEM (n=15), *P<0.05, 1-way ANOVA with Bonferroni posttest.

Supplemental Fig. 3

A



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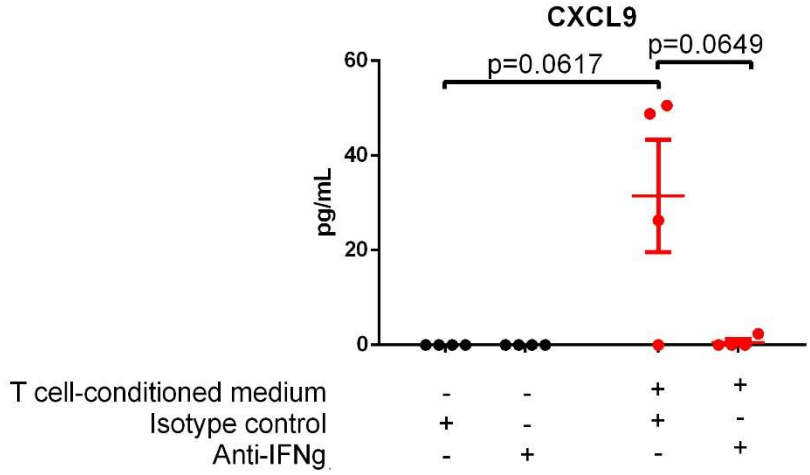
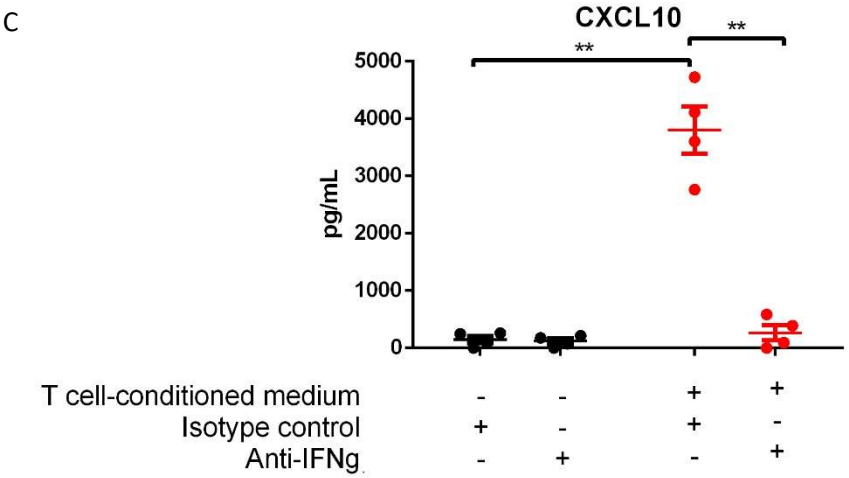
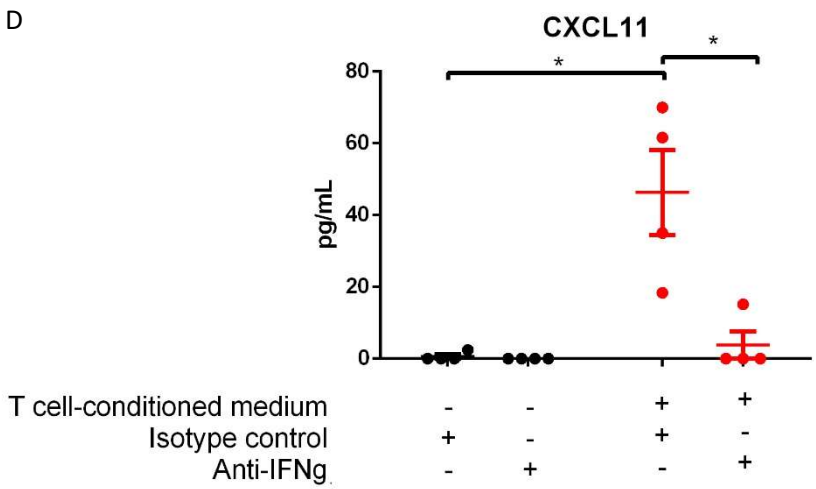


C





Supplemental Fig. 3: CD4+ T cell-conditioned medium induces STAT1 activation and CXCR3 ligand production by ASMCs in IFN-γ-dependent manner. A) Representative western blots showing STAT1 phosphorylation and total STAT1 within ASMCs when treated with or without T cell-conditioned medium or IFN-γ neutralizing antibodies. B-D) CXCL9, 10 and 11 secretion by ASMCs when treated with or without T cell-conditioned medium or IFN-γ neutralizing antibodies. Data are mean±SEM (n=4), *P<0.05, **P<0.01, 2-way repeated measures ANOVA with Bonferroni posttest.

Preface to Chapter 4

CXCR3 ligands, which were found to mediate mast cell migration to ASM bundles[1], are now demonstrated in our study to be responsible for also mediating CD4+ T cell chemotaxis *in vitro*, suggesting that ASM-derived CXCR3 ligands could in general play a prominent role in leukocyte recruitment *in vivo*. Being interferon-inducible genes, CXCR3 ligands have been associated with Th1 immunity in the context of autoimmunity and anti-viral responses[2]. As the roles of Th1 immunity are poorly understood in asthma, our evidence proposed a novel role that Th1 immunity could contribute to asthma-associated airway structural change and pathophysiology.

To expand the scope of our investigations and to further understand the involvement of Th1 immunity in asthma as the next step, we aimed to establish an animal model of Th2-high asthma, with concurrent Th1-inducing stimuli, in order to study the interaction between the two immunological compartments. Contradicting the observation that Th1-associated cytokines demonstrate an inhibitory effect on Th2 immunopathology and therefore alleviate asthma-associated inflammation[3], an elevation of these cytokines also marks more severe disease in human[4]. In the following study, we chose to use PIC, a synthetic double-stranded RNA that mimics a viral-like PAMP, as an inducer of Th1-associated response. We propose that PIC could confer a distinct severe asthma endotype in mice via the induction of Th1 immunity and additional inflammation when co-administered with allergen. We anticipated the described treatment would yield a Th2-low asthma model that would help us better understand the roles of Th1 immunity in asthma and its link to disease severity.

1 Brightling, C. E. *et al.* The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle. *Am J Respir Crit Care Med* **171**, 1103-1108 (2005).

2 Groom, J. R. & Luster, A. D. CXCR3 in T cell function. *Exp Cell Res* **317**, 620-631 (2011).
3 Maeda, Y., Musoh, K., Shichijo, M., Tanaka, H. & Nagai, H. Interferon-beta prevents antigen-induced bronchial inflammation and airway hyperreactivity in mice. *Pharmacology* **55**, 32-43 (1997).
4 Raundhal, M. *et al.* High IFN-gamma and low SLPI mark severe asthma in mice and humans. *J Clin Invest* **125**, 3037-3050 (2015).

Poly I:C alleviates T helper 2 immunopathology by recruiting anti-inflammatory monocyte-derived macrophages to the lungs in a murine antigen-specific asthma model

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Keywords: allergic asthma, T helper 2 immunopathology, alternatively activated macrophage, interleukin-10, arginase-1.

Abstract

The interaction between antiviral responses and the T helper 2 (Th2) immunopathology in asthma remains an important focus. While infections contribute to asthma exacerbation due to dysregulated immune responses, exposure to pathogen-associated molecular patterns (PAMPs) may lead to the development of regulatory pathways that inhibit Th2 immunopathology through the induction of regulatory macrophages. In this study, we aimed to investigate the crosstalk between the pulmonary Th2 immune and antiviral responses in an ovalbumin (OVA) sensitization and challenge-induced mouse. For a viral-associated immune stimulus, we used poly I:C, a mimic of double-stranded RNA found in various respiratory viruses, which acts through several pattern recognition receptors to initiate interferon and inflammatory cytokine production. When co-administered during OVA challenges, it dampened airway hyperresponsiveness, eosinophilia and Th2 cytokine expression. However, this was not due to the cross-inhibitory action of poly I:C-induced interferons, interferon-inducible genes and inflammatory cytokines, as they were also suppressed in poly I:C/OVA co-administered group. Instead, an enrichment of monocyte-derived macrophages was seen uniquely in the lungs of co-administrated mice. These cells featured a prominent alternatively activated (M2) phenotype and the expression of immunoregulatory factors. Their regulatory function was confirmed in an *in vitro* T cell inhibition assay. Lastly, poly I:C failed to confer a Th2-suppressing effect in mice depleted of monocytes with anti-Ly6C antibody, and in c-c motif chemokine receptor 2 (CCR2) knockout mice with impaired monocyte recruitment. Overall, our data contributed to the understanding of macrophage-mediated immunomodulation initiated by PAMP that suppress allergic airway reactions.

Background

Asthma remains one of the most common chronic diseases globally, with rising prevalence since the 1950s[1]. Atopic, T helper 2 (Th2)-high asthma is the most prevalent asthma endotype, featuring antigen-specific CD4+ T cells as the central actor that confers immunopathology via the secretion of Th2 cytokines, mainly IL-4, 5 and 13[2,3]. Specifically, IL-4 mediates additional Th2 polarization during T cell activation, and directs B cell isotype switching to IgE, which is primarily responsible for inducing mast cell-mediated allergic reactions[4]. IL-5 promotes eosinophil maturation and recruitment to the lungs[4]. IL-13 has a wide variety of structural and immune cell targets and plays a prominent role in airway hyperresponsiveness (AHR) and airway remodelling[4]. However, with improving understanding of asthma-associated immunopathology, it has been recognized that the disease covers complex endotypes, and that atopic responses and Th2 immunity is not the sole explanation for its pathogenesis[2]. Therefore, more recent research efforts have been increasingly focusing on exploring the non-type 2 aspects of the the disease immunopathology.

The interactions between viral stimuli and asthma pathophysiology have long been a topic of interest in research, mainly due to the role of viruses in the majority of exacerbations, which are the leading contributor to severity, hospitalization and asthma-related death[5]. Anti-viral response is mediated by Th1 immunity, involving components conferring cytotoxic responses, including CD8+ T cells, NK cells, interferons and other associated inflammatory cytokines[6]. As a Th1 response demonstrates a mutual inhibitory relationship with Th2 immunity[6], virally-induced asthma exacerbations therefore pose a paradox. It is likely that factors beyond a simple Th1-Th2 balance exist contributing to worsening of the disease,

especially in aspects pertaining to host tolerance or resistance to the pathogens that are impacted upon by asthma[7].

However, several recent reports have indicated that intrapulmonary exposures to viral and bacterial associated toll-like receptor (TLR) ligands induced regulatory macrophages in mice, marked particularly by the secretion of immunosuppressive cytokine IL-10, a feature shared by all these studies[8-11]. These populations are classified as either monocyte-derived or interstitial macrophages, that could act both in the lung and lymph node to restrict T cell functions, including in models of asthma[8-11]. These data suggest that microbe-associated immunological signals could be important in the development of immunoregulatory mechanisms necessary for the prevention of atopic disorders, an idea supported by a number of epidemiological studies[8,12-14]. Collectively, these studies introduced a novel player in the immune regulation associated with responses to pathogens in asthma[8-11].

Polyinosinic:polycytidylic acid, or poly I:C, is a synthetic double-stranded RNA[15], which mimics viral pathogen associated molecular patterns (PAMPs) found in common respiratory viruses including coronavirus, rhinovirus and respiratory syncytial virus[16]. It binds to pattern recognition receptors including TLR3 located on the endosomal membrane[15], and cytoplasmic receptors retinoic-acid-inducible protein I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5)[17]. These receptors mediate downstream activation of interferon regulatory factor 3 (IRF3) to initiate the production of type 1 interferons and nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPK) to upregulate the expression of inflammatory cytokines including TNFα and IL-6[18]. Experimentally, poly I:C has been used as an adjuvant for the induction of Th1-related components of immune reactions, such as CD8+ T cells, NK cells and interferon-mediated anti-viral responses[19].

In this study, to investigate the interaction between Th2 immunity in asthma and Th1 reactions triggered by viral like stimuli, we characterized the immune responses to intrapulmonary poly I:C co-administered during allergen challenges in a mouse model of atopic asthma. Unexpectedly we demonstrated that poly I:C suppressed Th2 immunopathology independently from the cross-regulatory effect of Th1 immunity. Instead, it induced a protective monocyte-derived macrophage population that is responsible for dampening asthma-associated immunopathology. Our data indicate that a unique signal initiated by the combination of viral-like PAMP and Th2 immune milieu is responsible for the induction and recruitment of an immunomodulatory myeloid population novel to the context of asthma, which poses therapeutic potential and facilitates our understanding of potential tolerogenic pathways beneficial for alleviating the disease.

Materials and Methods

Animals and ethics

BALB/c mice were purchased from Charles River Laboratories. Breeding pairs of C57BL/6 and CCR2 KO mice on C57BL/6 background were purchased from Jackson Laboratory and sustained in-house. All colonies were kept in a standard animal housing unit. All experiments involving mice were performed following the guidelines of the Canadian Council for Animal Care, with protocols approved by the Animal Care Committee of McGill University.

Regimens for OVA sensitization and challenge, poly I:C co-administration and antibody-injection for cell depletion

In all experiments, female BALB/c mice aged 8-12 weeks were used unless otherwise stated. As illustrated in Figure 1A, mice were sensitized with intraperitoneal (i.p.) injections of 20 μg OVA

(Sigma) absorbed onto 1 mg aluminum hydroxide (ThermoFisher Scientific) in 0.2 mL PBS on day 0 and day 7. On days 14, 15 and 16, the mice were intranasally challenged daily with one of the following: PBS as vehicle control, 50 μg OVA, 40 μg poly I:C, or a combination of OVA and poly I:C diluted in 40 μL PBS. The mice were sacrificed on day 17 or 18 for 1- or 2-day post challenge endpoints (1dpc and 2dpc), respectively. For antibody-mediated cell depletion, anti-Ly6C antibody and isotype control purchased from BioXCell were injected intraperitoneally at a dose of 80mg/kg on day 13, 15 and 16 in addition to the appropriate sensitization and challenge regimens.

Measurement of respiratory system mechanics and methacholine challenges.

The mice were anesthetized with i.p. injections of 10 mg/kg xylazine and 50mg/kg pentobarbital, followed by tracheal cannulation with an 18-gauge cannula. The mice were then connected to small animal ventilator and lung mechanical measurement unit (flexiVent, Scireq, Montreal) and were injected i.p. with 1 mg/kg pancuronium bromide as a paralytic. To characterize airway hyperresponsiveness, aerosols containing increasing concentrations of methacholine dissolved in PBS ranging from 6.25 to 50 mg/mL were delivered via the cannula, followed by the assessment of respiratory resistance and elastance after each delivered dose.

Flow cytometry

The mice were euthanized by CO_2 asphyxiation or pentobarbital overdose. The lung tissues were dissected and diced into pieces smaller than 1 mm with a surgical scalpel and digested with RPMI containing 150 U/mL collagenase from *Clostridium histolyticum* (Sigma) and 20 μg/mL DNaseI (Roche) for 45 minutes at 37°C. The lung draining lymph nodes were teased apart with a 26-gauge needle prior to digestion with the abovementioned collagenase/DNase cocktail. The tissues were then passed through an 18-gauge needle then filtered through 40 μm cell strainer,

resulting in a single cell suspension. Blood samples were obtained via cardiac puncture and collected in EDTA-containing tubes. Red blood cells in all samples were lysed with RBC lysis buffer (Biolegend). For intracellular cytokine staining, the cells were activated with a PMA/ionomycin/Brefeldin A cocktail (Biolegend) dissolved in RPMI supplemented with 10% FBS and antibiotic/antimycotic solutions for 5 hours in a standard incubator.

The cells were incubated with eFluor780 viability dye (Invitrogen) and anti-CD16/32 Fc blocking antibody (Biolegend) prior to staining with surface antibodies diluted in FACS buffer (1% bovine serum albumin in PBS). The cells were then fixed and permeabilized with a commercially available buffer set (Invitrogen) and stained with intracellular antibodies diluted in the permeabilization buffer. The samples were acquired on LSRFortessa X-20 flow cytometer (BD). All antibodies used for flow cytometry analysis can be found in Supplementary Table 1.

BAL collection and differential counting of BAL cells

BAL was obtained from euthanized mice by injecting and withdrawing 1 mL PBS through the tracheal cannula. The BAL were then centrifuged at 500×g for 5 min, and the supernatants were stored at -80°C, the pelleted cells where resuspended in 0.2 mL PBS, and the total cell number was quantified with a hemacytometer. Fifty thousand cells were spun onto microscope glass slides using a Cytospin 4 centrifuge (Epredia). Following air-drying, the slides were stained with a Diff-Quik staining kit (Fisher) and mounted with cover slips. The cells were differentially counted based on morphology as macrophage/monocytes, lymphocytes, neutrophils and eosinophils. A total of 300 cells was counted. Based on the percentage of each cell type and total BAL cells, the absolute cell numbers were calculated.

qRT-PCR

Lung tissues were snap-frozen in liquid nitrogen immediately following dissection. Following homogenization in Trizol (Cat. #: 15596026, Invitrogen), total RNA was extracted according to the manufacturer's instructions. The RNA concentration was measured by absorbance at 260nm by spectrophotometer (BioTek Instruments), and the reverse transcription was performed using LunaScript RT SuperMix kit (Cat. #: E3010L, New England Biolabs) with 500 ng total RNA according to manufacturer's instructions. 5 ng cDNA from each sample was mixed with SYBR Select Master Mix (Cat. #: 4472908, Applied Biosystems) for quantitative real-time PCR. GAPDH were used as the housekeeping gene for normalization. $2^{-\Delta\Delta Ct}$ values were calculated on the data obtained. All primers for qRT-PCR were synthesized by ThermoFisher, and their sequences are listed in supplementary Table 2.

Cell sorting

The single cell suspension from digested lung tissues were labelled with viability dye and surface antibody as described in the previous section, and the live cells were sorted with a FACSAria Fusion Flow Cytometer (BD) for SiglecF-, Ly6G-, CD11b+, F4/80+, Ly6C+ and SiglecF-, Ly6G-, CD11b+, F4/80+, Ly6C- macrophage populations. The sorted cells were immediately placed in Trizol for qRT-PCR analysis or cultured in complete RPMI for 24 hours for the measurement of secreted factors.

T cell inhibition assay

CD4+ T cells were isolated from lung-draining lymph nodes of OVA-challenged mice with a negative selection cell isolation kit (Stemcell Technology) and labelled with CFDA-SE (ThermoFisher). F4/80-targeting positive selection kit (Stemcell Technology) was used to enrich lung macrophages from OVA- or OVA-poly I:C-challenged mice. The resultant cells were seeded in tissue culture plates to allow adhesion of the macrophages. After 2 hours, non-adherent

cells, which consisted of mostly eosinophils, were rinsed off, yielding isolated pan-macrophage population with purity over 90%. One hundred thousand CD4+ T cell we mixed with the same number of anti-CD3/CD28 T cell activation beads and were cultured alone, or co-cultured with one million lung macrophages for three days. The cells were collected and analyzed via flow cytometry for activation status and proliferation, which is reflected by reduction in CFDA-SE labelling intensity.

ELISA

The level of CCL2 in the BAL fluid was assessed with a sandwich ELISA kit (R&D Systems) according to manufacturer's instructions.

Statistical analysis

Each data point in the figures represents an individual animal. Student's t-test, one-way and two-way ANOVA were performed as appropriate using GraphPad Prism software. A p value less than 0.05 was considered statistically significant.

Results

Poly I:C co-administration alleviated airway hyperresponsiveness and eosinophilia following antigen challenges

Consistent with existing descriptions of allergen models in mice, OVA sensitization and challenge elicited AHR on 2dpc, reflected by an exacerbated elevation of total respiratory resistance and elastance upon MCh challenges (Figure 1B-C). poly I:C-co-administration significantly attenuated these responses, while poly I:C alone did not evoke any change in respiratory mechanics (Figure 1B-C).

The differential counting of BAL cells collected 2dpc revealed that OVA sensitization and challenge induced atopic airway inflammation as expected, reflected by a significant increase of total cells and eosinophils, as well as a trending increase of macrophages (Figure 1D). poly I:C alone did not result in any marked changes in BAL cell population compared to PBS control (Figure 1D). However, its co-administration during antigen challenge resulted in a significant reduction in eosinophils and significant increase of lymphocytes compared to OVA challenge alone (Figure 1D). Supporting these findings, total cells and eosinophil number assessed by flow cytometry in lung tissue digests were increased by OVA sensitization and challenge on 1dpc and 2dpc, with an increased magnitude of eosinophilia seen on 2dpc (Figure 1E-F). poly I:C-co-administration did not impact the number of total lung cells yielded by digestion (Figure 1E) but significantly suppressed eosinophilia compared to the OVA-only challenged group on 2dpc (Figure 1F). poly I:C did not lead to neutrophil infiltration on either 1dpc or 2dpc (Figure 1G). OVA challenge also increased CD4+ T cell infiltration on 2dpc which was not altered by poly I:C co-administration (Figure 1H)

Poly I:C-co-administration inhibited IL-5 and IL-13 expression by CD4+ T cells

Consistent with the observations on AHR and lung eosinophilia, qRT-PCR analysis of lung tissue revealed that poly I:C co-administration suppressed IL-5 and IL-13 upregulation induced by OVA challenge on both 1dpc and 2dpc, without affecting the expression of IL-4 (Figure 2A-C). Furthermore, OVA challenge-induced upregulation of transcripts of Th2-associated chemokine CCL17 and eosinophil chemoattractant CCL11 was suppressed by poly I:C co-administration, with the former observed on both 1 and 2dpc and the latter on 2dpc only (Figure 2D-E). Similarly, induction of IL-5- and IL-13-secreting CD4+ T cells by OVA challenge was

suppressed in OVA-poly I:C co-administration group on 2dpc only, without affecting IFN-γ+ or IL-4+ populations, as assessed by flow cytometry following *ex vivo* stimulation (Figure 2F-J).

Poly I:C-sensing pattern recognition receptors, and poly I:C-induced inflammatory and interferon-associated gene upregulation is diminished in allergen-challenged mice

As existing studies have demonstrated Th1-associated cytokines, especially type 1 and type 2 IFNs, could suppress Th2 immunopathology and alleviate inflammation in animal models of asthma, which is consistent with the mutual inhibitory relationship between Th1 and Th2 immunity, we hypothesized that poly I:C-induced production of IFNs and associated genes were responsible for the suppression of Th2 responses in our model. We first confirmed the expression of the poly I:C-sensing pattern recognition receptors and cytokines known to be induced by poly I:C by qRT-PCR. We observed that RIG-I and MDA-5 expression were upregulated in response to poly I:C administration, and that all three receptors saw a downregulation in OVA- and OVA/poly I:C-challenged mice as assessed at 1dpc. Consistent with these observations, IFN-β, IFN-γ, CXCL10, CCL5, TNFα, CXCL1 and IL-6 were prominently induced in the lung by poly I:C administration, however, the induction of all these factors beside IL-6 were impaired in OVA-poly I:C co-administered mice (Figure 3).

Poly I:C stimulated monocyte-derived macrophage recruitment and resulted in an enrichment of an M2-like, IL-10-secreting macrophage population.

Knowing the suppression of Th2 immunopathology by poly I:C was not due to the inhibitory effect of Th1-associated cytokines, we performed additional characterization of the lung immune landscape. We observed that poly I:C administration resulted in an enrichment of a monocyte-derived macrophage population, marked by F4/80+, CD11b+ and Ly6C+ in the lung (Figure 4A). This effect was amplified in the group co-administered OVA and poly I:C, which was most

pronounced on 1dpc but persisted until 2dpc (Figure 4A). In addition, the F4/80+, CD11b+ and Ly6C- population, which consists of interstitial macrophages and non-classical monocytes[20,21], was present in the lung in increased numbers in OVA-challenged mice on both 1dpc and 2dpc (Figure 4B). poly I:C co-administration did not further impact its number (Figure 4B). Poly I:C induced the expression of CCL2, the chemokine primarily responsible for monocyte egress from the bone marrow and recruitment to the tissues by binding to CCR2[22,23], as confirmed by qPCR and ELISA in BALF on 1dpc, though the level of which was slightly suppressed in the OVA/poly I:C co-administration group (Figure 4C and D), similar to the observation of other poly I:C-inducible genes. This signifies that CCL2-CCR2 axis was still the major chemotactic axis governing the recruitment of monocyte-derived macrophage in this experimental model. Additional phenotyping revealed the pronounced M2 characteristics of the monocyte-derived macrophage populations induced by OVA-poly I:C-co-administration, marked by arginase-1, CD206 and CD163 expression (Figure 4E-H). M2 macrophages exercise their immunomodulatory functions via the secretion of anti-inflammatory factors. We found that the Ly6C+ macrophages induced by OVA-poly I:C co-administration markedly increased the expression of IL-10, verified by flow cytometry (Figure 4E and I) and by qPCR following cell sorting (Figure 4L). These observations corresponded to an increased level of lung arginase-1 and IL-10 transcripts in the co-administered group compared to all other experimental groups, the two factors with functions that could directly contribute to the inhibition of T cell functions (Figure J and K). Furthermore, the Ly6C+ population demonstrated increased expression of the additional suppressor marker PD-L1 (Figure M).

OVA-poly I:C co-administration-induced lung macrophages inhibited T cell activation and proliferation *in vitro*

After demonstrating the prominent suppressor phenotype of lung macrophages induced in OVA-poly I:C co-administered group, we then investigated whether they were functionally inhibitory, specifically in regard to CD4+ T cells. Lung-draining lymph node (LN) CD4+ T cells from OVA sensitized and challenged mice were cultured alone or co-cultured with lung macrophages from OVA or OVA-poly I:C administered mice in the presence of anti-CD3/CD28 activation beads, as illustrated in Figure 5A. Compared to T cells cultured alone, those co-cultured with lung macrophages from OVA-challenged mice demonstrated enhanced activation, with an increase in proliferation reflected by the cells with reduced CFDA-SE labelling, and an increase in the CD44+ CD62L- effector-like phenotype (Figure 5B-D). This suggested that macrophages from the lungs of allergen-challenged mice promote T cells activation. However, T cells co-cultured with lung macrophages from the OVA/poly I:C-co-administered mice demonstrated reduced proliferation and activation in comparison to the other two group, again confirming the suppressive functions of OVA/poly I:C co-administration-induced macrophages (Figure 5B-D).

poly I:C co-administration failed to suppress Th2 responses in the absence of Ly6C+ macrophages

Finally, we investigated whether poly I:C would suppress Th2 immunopathology in the absence of Ly6C+ macrophages to provide further proof of the immunomodulatory function of this cell population *in vivo*. To achieve this objective, we first injected anti-Ly6C antibody prior to administration of OVA and poly I:C to deplete the Ly6C+ macrophages. We observed that compared to mice injected with isotype control, anti-Ly6C antibody only selectively depleted macrophages with the highest Ly6C expression (Figure 6A and B). In response to this treatment, an increase in lung eosinophils was observed following poly I:C-OVA co-administration compared to isotype control group (Figure 6C). To adopt a model with more prominent Ly6C+

macrophage depletion, we tested our hypothesis with CCR2 KO mice on the C57BL/6 background. We first confirmed that poly I:C co-administration induced Ly6C+ macrophage infiltration and suppressed eosinophilia in this WT mouse strain, similar to BALB/c (Figure 6D-F). However, in CCR2- KO mice, the number of Ly6C+ macrophages in lung tissue was overall much lower compared to WT mice and was not induced by poly I:C (Figure 6D and E). Most importantly, OVA/poly I:C co-administered CCR2 KO mice still demonstrated prominent eosinophilia similar to that of the OVA-only challenged CCR2 KO group, with the eosinophil number significantly higher than in the OVA/poly I:C co-administered WT group (Figure 6D and F). Consistent with these findings, poly I:C co-administration suppressed allergen-induced IL-5 and IL-13 expression as assessed by qPCR, in WT C57BL/6 mice but not CCR2 KO mice (Figure 6G and H).

Discussion

Th2 cells play a central role in atopic asthma via the production of Th2 cytokines, which mediate IgE production, eosinophil recruitment, AHR and airway remodelling characteristic of the disease[3]. In this study, we described the cellular and molecular mechanisms associated with poly I:C-mediated suppression of Th2 immunopathology in a mouse model of allergic asthma. poly I:C is a potent inducer of interferons and Th1-associated factors as it mimics viral PAMPs to initiate cytotoxic responses. It has been investigated as an adjuvant to boost the efficacy of vaccines against respiratory viruses and anti-cancer immunotherapies[19,24]. Emerging evidence demonstrated that intrapulmonary exposure to PAMP, like poly I:C and the bacteria-associated TLR9 agonist CpG, induces immunomodulatory macrophages that are capable of dampening inflammation, including in asthma models, via anti-inflammatory mediators, notably IL-

10[8,9,11,25-27]. These macrophages not only act at the mucosal site in the lung, but also have been shown to migrate to the lymph nodes to suppress T cell priming[9,11]. It has been hypothesized that this population is a part of the mechanisms that acts as a brake on inflammatory processes to prevent dysregulated immune activation[11,28]. Our study provided evidence supporting the immunomodulatory functions of such macrophage populations and demonstrated that the induction of these cells by poly I:C during allergen challenges provided a protective effect that suppressed asthma-associated Th2 immunopathology in a mouse model.

First, we established that poly I:C co-administration during antigen challenges alleviated asthma-associated pathobiology, including Th2 cytokine expression, eosinophilia and AHR. Moreover, this was not due to the cross-inhibitory effect of Th1 responses, as the interferons, interferon-inducible genes and other inflammatory factors known to be stimulated by poly I:C were also downregulated in the poly I:C-OVA co-administered group compared to poly I:C-only controls. This is worth noting as the reduced interferon induction by poly I:C in OVA-challenged mice is consistent with the deficiency of interferon responses to respiratory viral infections observed among asthmatic patients, which results in poor pathogen clearance and consequently exacerbation[29-32]. The implication of these observations in the context of viral infection merits further exploration. However, as poly I:C-induced non-Th2 cytokines could also contribute to inflammation and AHR[33], the downregulation of these factors is an additional indication of the existence of a protective pathway, and that its immunosuppressive effect is not only limited to Th2-associated factors. Collectively, the global suppression of inflammatory mediators hinted at the existence of a novel immunosuppression pathway specifically induced by OVA-poly I:C co-administration.

With additional immunophenotyping, we identified a prominent enrichment of Ly6C+ monocyte-derived macrophages in the lungs of OVA-poly I:C co-administered mice, the magnitude of which was higher than the effects of either stimulus alone. Additionally, these cells were also longer lasting compared to those in all other groups and persisted in the lung tissue up to at least 2dpc. We also observed that contrary to the general type 1-inducing effect of poly I:C, the resultant monocyte-derived macrophages demonstrated a prominent M2 phenotype and expressed molecules associated with suppressor functions, notably IL-10 and PD-L1. Though the Ly6C- population, primarily containing non-classical monocytes and interstitial macrophages[20,21], was not elevated in numbers by poly I:C/OVA co-administration, they also demonstrated the M2 phenotype and upregulated expression of IL-10. The specific mechanism as to how poly I:C and allergen-induced responses cooperatively mediated the derivation of the described macrophage populations is yet to be explained, but we hypothesize that as illustrated in Figure 6, poly I:C acts mainly as an inducer of the monocyte recruitment signal. The resultant monocyte-derived macrophages were subsequently converted into an alternatively-activated suppressor-like phenotype in response to the Th2-high milieu in the lung, particularly with the induction by allergen of IL-4 and IL-13, which are factors canonically driving M2 polarization[34]. In addition, IL-10 derived from these macrophages might act in an autocrine/paracrine fashion to further promote the M2 phenotypic conversion[35].

The lack of neutrophil induction by poly I:C is expected and is consistent with the observations in existing studies, which have demonstrated that a dose of poly I:C less than 50 μg does not evoke an increase in neutrophils at 24 hours after challenge[36,37]. We conclude that low-dose poly I:C confers only a transient neutrophil recruitment and retention signal. As our earliest observation occurs also on 1dpc, the mild neutrophilia may have resolved by this time. However,

the efficacy of poly I:C administration in our model was confirmed by an induction of monocyte-derived macrophages, along with interferons, interferon-inducible genes and other inflammatory mediators in the poly I:C-only group.

Furthermore, we established the potential protective functions of the monocyte-derived macrophages induced by poly I:C/OVA co-administration by demonstrating the upregulation of immunosuppressive factors, particularly arginase-1 and IL-10 compared to OVA only. Arginase-1, widely considered as an M2 marker, suppresses T cell activation through affecting arginine metabolism[38,39]. IL-10 inhibits T cell function by directly acting on T cells to induce anergy, and by inhibition of antigen presenting cell functions[40,41]. However, we have yet to definitively prove whether the protective effect of these macrophage was directly attributable to these factors. As this population also expresses other markers characteristic of suppressor-like cells, including PD-L1, there seems to be a trend of overall conversion towards a suppressor cell phenotype. Therefore, more extensive phenotyping of this macrophage population would be of interest to ascertain the molecular source of the inhibitory action. The immunosuppressive functions of the macrophages have been extensively studied in the context of cancer, with defined populations such as tumor-associated macrophages and myeloid-derived suppressor cells that contribute to tumor growth and immune evasion[42,43]. Our study provides proof-of-concept evidence that macrophages with similar phenotype can be induced to alleviate immunopathology in the context of inflammatory disorders.

To further demonstrate the immunosuppressive function of these macrophages, we performed an *in vitro* T cell inhibition assay by co-culturing lung macrophages isolated from either the OVA- or OVA/poly I:C-challenged group with LN CD4+ T cells in the presence of the anti-CD3/CD28 T cell activator. We observed that while macrophages from OVA-challenged

lungs promoted T cell proliferation and activation in comparison to the control with only T cell activators, those from OVA/poly I:C-challenged lungs inhibited both responses, supporting our understanding of the function of these macrophages.

Finally, we provided *in vivo* proof that the monocyte-derived macrophages are necessary for the suppression of Th2 immunopathology by poly I:C. This is demonstrated with OVA/poly I:C-co-administration experiments in models deficient in such population in the lungs. In mice depleted of monocytes using anti-Ly6C antibody, and CCR2 KO mice with an impaired monocyte recruitment signal, poly I:C co-administration failed to restrict eosinophilia compared to their respective controls. Interestingly, previous studies involving OVA sensitization and challenge in CCR2 KO mice also showed exacerbated Th2 immunopathology in the KO mice compared to wild type mice, hinting at a protective role of monocyte-derived macrophages[44,45]. Our study, besides being overall consistent with these observations, further demonstrated the induction of monocyte-derived macrophage corresponds to the diminished allergen-induced Th2 response. These findings further support the immunomodulatory functions of monocyte-derived macrophages in the context of asthma.

The interaction of viral stimuli such as poly I:C and Th2 responses have been investigated previously, especially in the context of virally induced asthma exacerbations. One study reported an exacerbated AHR induced by poly I:C without changes in inflammation[36]. Differing from these studies where poly I:C challenges are performed following the completion of allergen challenges, it was co-administered throughout the entire course of allergen challenges in our model. We believe that the concurrent presence of the immunomodulatory macrophages described during allergen exposures is the key to result in the suppression Th2 immunopathology. This is further supported by the temporal relationships between the induction

of Th2 response and the infiltration of the macrophage population. OVA-induced eosinophilia and Th2 cytokine-producing CD4+ T cells were most prominent on 2dpc, and the Th2-suppressing effect of poly I:C was also seen only on 2dpc. However, the peak induction of M2-like populations was on 1dpc, suggesting the presence of these macrophages during the allergen exposure and the development of Th2 response is important for their immunosuppressive effect.

Overall, our experimental model implicates the protective roles of viral-like PAMP against allergic responses, reminiscent of theories related to the hygiene hypothesis[46]. Though these theories have been criticized for their limitations due to the proposed mechanism being largely restricted to Th1-Th2 imbalance[12], ample evidence suggests early life exposure to bacterial and viral pathogens is indeed associated with decreased risk of the development of atopic diseases, suggesting a broader sense of such a hypothesis might still be relevant and explanatory for the rise of allergic disorders in the past decades[46,47]. Our study, along with many others[47], provides evidence of potential mechanisms, particularly involving immunomodulatory macrophages, through which microbe exposures could influence immunity and dampen allergic responses.

In conclusion, we determined that poly I:C co-administration during allergen challenges suppressed Th2 immunopathology in a mouse model of atopic asthma, via the induction of an M2-like, IL-10-secreting monocyte-derived macrophages. Further research should be dedicated to the mechanisms responsible for the development of such macrophage phenotype, and their implications in the clinical context.

Statements and declarations

This study was supported by a project grant from Canadian Institutes of Health Research to J. G. Martin, as well as a doctoral training award from Fonds de Recherche du Québec – Santé received by R. Sun. The authors have no relevant financial or non-financial conflict of interests to disclose. R. Sun and J. G. Martin designed the study; R. Sun, E. Ward and D. Pyo contributed to the experimental work; R. Sun and E. Ward analyzed the data; R. Sun and J. G. Martin wrote and edited the manuscript. All authors approved the manuscript.

Figure legends

Figure 1: Poly I:C co-administration during allergen challenges dampened asthma-associated airway hyperresponsiveness and eosinophilic inflammation. A: The regimen for OVA sensitization and challenge for establishing atopic asthma model in mice, and co-administration of poly I:C. B and C: Total respiratory resistance and elastance following methacholine challenges in mice administered with PBS vehicle, OVA and/or poly I:C, assessed at 2dpc. Data are mean ± SEM, 2-way ANOVA with Bonferroni post-test. *:$p<0.05$, **: $p<0.01$, ****:$p<0.0001$. D: total and differential counting of the BAL cells in the four experimental groups indicated. Data are mean ± SEM, 2-way ANOVA with Bonferroni post-test. *:$p<0.05$, ***: $p<0.001$. E-H: numbers of total cells yielded from lung tissue digestion and single cell preparation, as well as numbers of eosinophils, neutrophils and CD4+ T cells assessed via flow cytometry from the four experimental groups indicated, on 1 and 2dpc. Data are mean ± SEM, 2-way ANOVA with Bonferroni post-test. *:$p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****:$p<0.0001$.

Figure 2: Poly I:C co-administration during allergen challenges alleviated Th2 immunopathology. A-E: the expression levels of IL-4, IL-5, IL-13 CCL11 and CCL17 in the lung tissues from the four experimental groups indicated as measured by qPCR on 1 and 2dpc. Data are mean ± SEM, 2-way ANOVA with Bonferroni post-test. *:p<0.05, **: p<0.01, ***: p<0.001, ****:p<0.0001. F-J: Representative flow cytometry plots and quantification of IFN-γ+, IL-4+, IL-5+ and IL-13+ in the lung tissues from the four experimental groups indicated on 1 and 2dpc. Data are mean ± SEM, 2-way ANOVA with Bonferroni post-test. *:p<0.05, **: p<0.01, ***: p<0.001, ****:p<0.0001.

Figure 3: The receptors of poly I:C and poly I:C-inducible cytokines are deficient in allergen-challenged mice. A-K: The expression levels of TLR3, RIG-I, MDA-5, IFN-α, IFN-β, IFN-γ, CXCL10, CCL5, TNFα, CXCL1 and IL-6 in the lung tissues from the four experimental groups indicated as measured by qPCR on 1dpc. Data are mean ± SEM, 2-way ANOVA with Bonferroni post-test. *:p<0.05, **: p<0.01, ***: p<0.001, ****:p<0.0001.

Figure 4: Poly I:C/OVA co-administration resulted in an enrichment of M2-like monocyte-derived macrophages with regulatory phenotypes in the lungs. A-B: Quantification of Ly6C+ (monocyte-derived) and Ly6C- (non-classical and interstitial) macrophages in the lung tissues from the four experimental groups indicated as measured by flow cytometry on 1 and 2dpc. Data are mean ± SEM, 2-way ANOVA with Bonferroni post-test. *:p<0.05, **: p<0.01, ***: p<0.001, ****:p<0.0001. C-D: Expression level of CCL2 in the lung tissue as measured by qPCR, and BAL as measured by ELISA, from the four experimental groups indicated on 1dpc. Data are mean ± SEM, 2-way ANOVA with Bonferroni post-test. **: p<0.01, ***: p<0.001,

****:p<0.0001. E-I: Representative flow plots and quantification of arginase-1, CD206, CD163 and IL-10 expression in Ly6C+ and Ly6C- macrophage populations on 1dpc. Data are mean ± SEM, 2-way ANOVA with Bonferroni post-test. *:p<0.05, **: p<0.01, ****:p<0.0001. J-K: The expression levels of arginase-1 and IL-10 in the lung tissues from the four experimental groups indicated as measured by qPCR on 1dpc. Data are mean ± SEM, 2-way ANOVA with Bonferroni post-test. *:p<0.05, **: p<0.01, ****:p<0.0001. L-M: The expression levels of IL-10 and PD-L1 in FACS-sorted Ly6C+ macrophages from OVA- and OVA/poly I:C-challenged groups as measured by qPCR on 1dpc. Data are mean ± SEM, unpaired Student's t-test. *:p<0.05, ***: p<0.001, ****:p<0.0001.

Figure 5: OVA-induced lung macrophages enhanced, while OVA/poly I:C-induced lung macrophages suppressed CD4+ T cell activation *in vitro*. A: An illustration of the setup of *in vitro* T cell suppression assay. bronchial lymph nodes (LN) CD4+ T cells were activated with anti-CD3/CD28 activation beads while cultured alone or co-cultured with lung macrophages from mice challenged with OVA only or OVA and poly I:C. B-D: Representative flow cytometry plots and quantification of proliferating cells, marked by reduction in CFSE labelling intensity, and activation status, marked CD44+, CD62L- effector-like phenotype. Data are mean ± SEM, One-way ANOVA with Bonferroni post-test. *:p<0.05, ***: p<0.001, ****:p<0.0001.

Figure 6: Poly I:C failed to suppress Th2 immunopathology in absence of monocyte-derived macrophages. A-C: Representative flow cytometry plots and quantifications of $Ly6C^{hi}$ macrophages and eosinophils in the lungs of OVA/poly I:C challenged mice with isotype control or anti-Ly6C antibody treatment. Data are mean ± SEM, unpaired Student's t-test. **: p<0.01,

****:p<0.0001. D-F: Representative flow cytometry plots and quantifications of Ly6C+ macrophages and eosinophils in the lungs of WT or CCR2 KO C57BL/6 mice following OVA or OVA/poly I:C challenges. Data are mean ± SEM, 2-way ANOVA with Bonferroni post-test. *:p<0.05, ***: p<0.001. G-H: The expression of IL-5 and IL-13 in lung tissues of WT or CCR2 KO C57BL/6 mice following OVA or OVA/poly I:C challenges as measured by qPCR. Data are mean ± SEM, 2-way ANOVA with Bonferroni post-test. *:p<0.05.

Figure 7: Illustration of the proposed pathway responsible for the dampened Th2 immunopathology in the lung conferred by poly I:C co-administration during allergen challenges. poly I:C induced the recruitment of monocyte-derived macrophages to the lung, which is polarized into M2 phenotype with immunomodulatory functions in response to Th2-high milieu of the lung. Consequently, these macrophages are responsible for inhibiting allergen-induced T cell responses.

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Figure 1

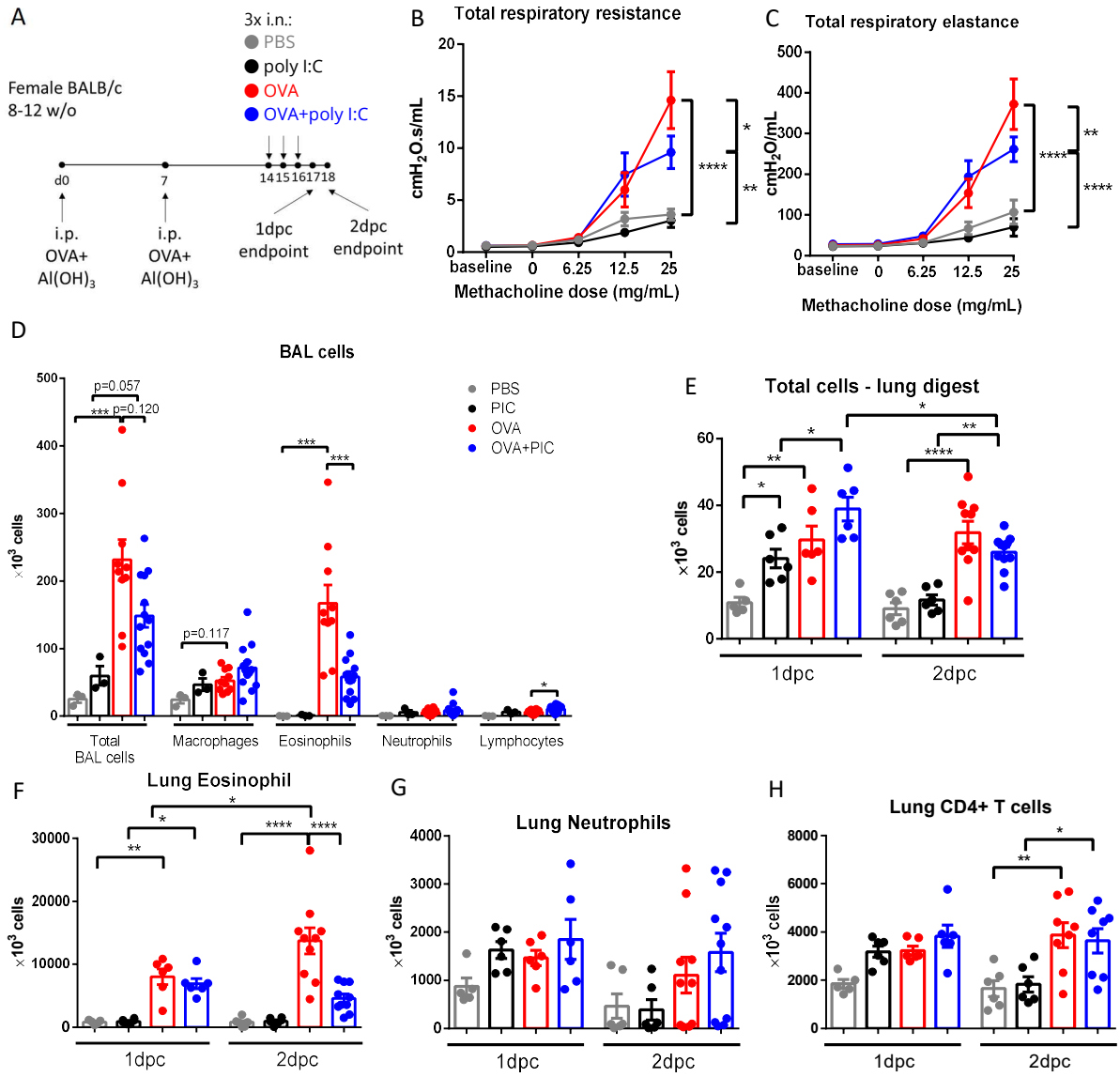
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Figure 2

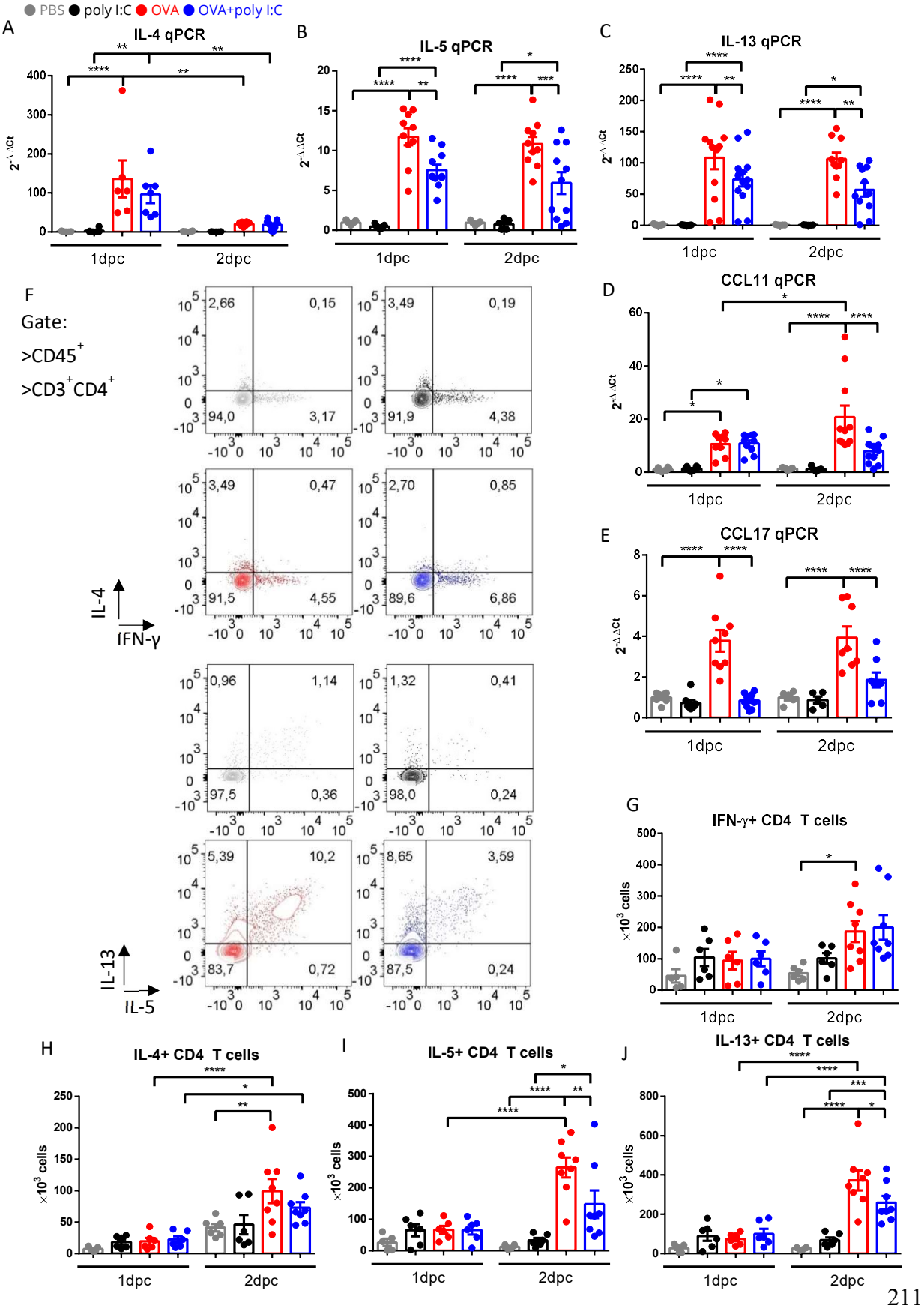
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Figure 3

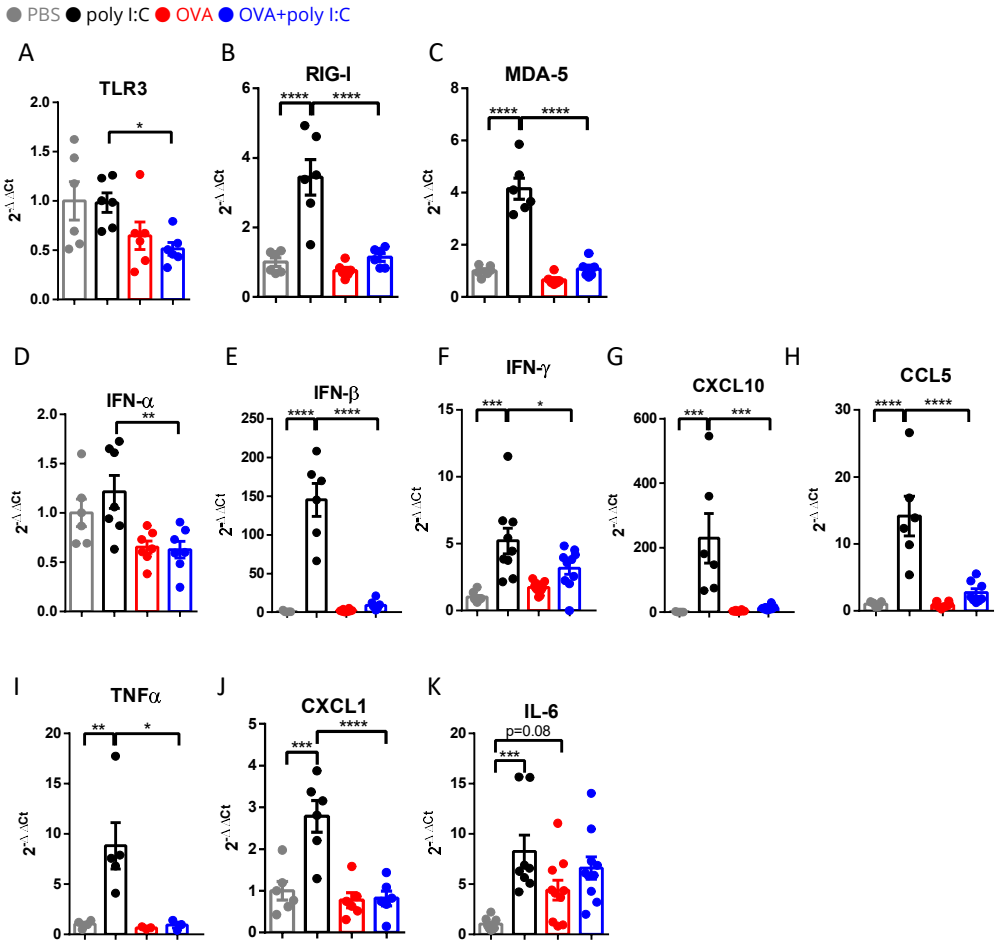
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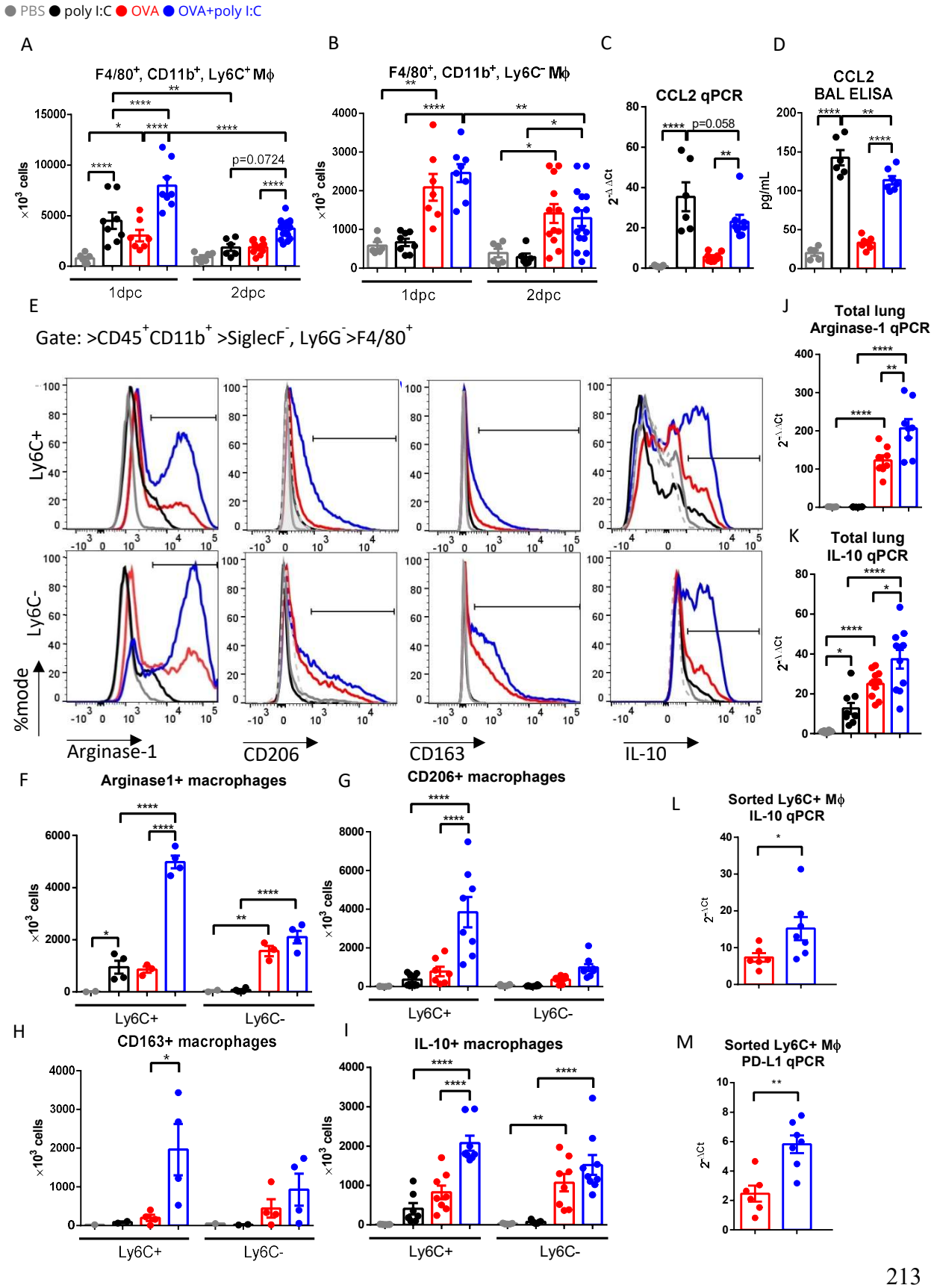
Figure 4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CD163
IL-10
J
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K
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IL-10+ macrophages
M
Sorted Ly6C+ Mϕ PD-L1 qPCR

Figure 5

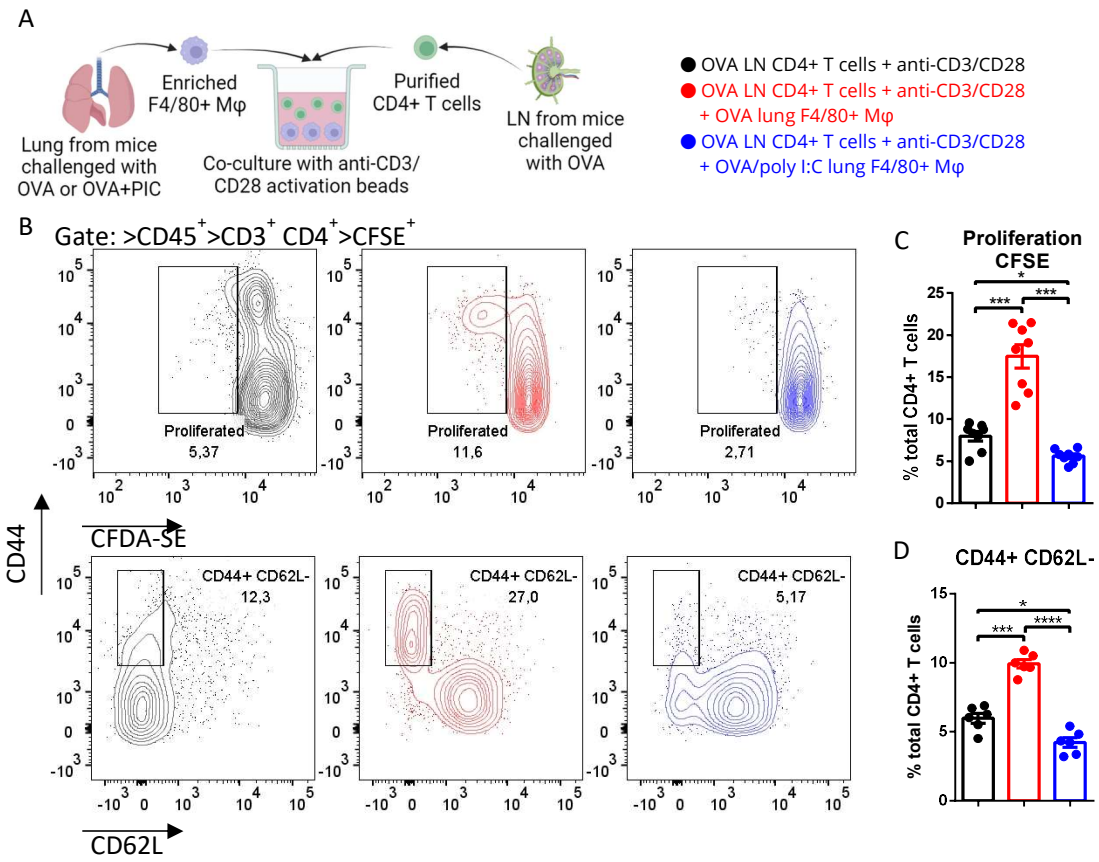

A
Enriched F4/80+ Mφ
Purified CD4+ T cells
Lung from mice challenged with OVA or OVA+PIC
Co-culture with anti-CD3/ CD28 activation beads
LN from mice challenged with OVA
OVA LN CD4+ T cells + anti-CD3/CD28
OVA LN CD4+ T cells + anti-CD3/CD28 + OVA lung F4/80+ Mφ
OVA LN CD4+ T cells + anti-CD3/CD28 + OVA/poly I:C lung F4/80+ Mφ
B
Gate: >CD45+>CD3+ CD4+>CFSE+
Proliferated 5,37
Proliferated 11,6
Proliferated 2,71
CD44
CFDA-SE
CD44+ CD62L- 12,3
CD44+ CD62L- 27,0
CD44+ CD62L- 5,17
CD62L
C
Proliferation CFSE
% total CD4+ T cells
D
CD44+ CD62L-
% total CD4+ T cells

Figure 6

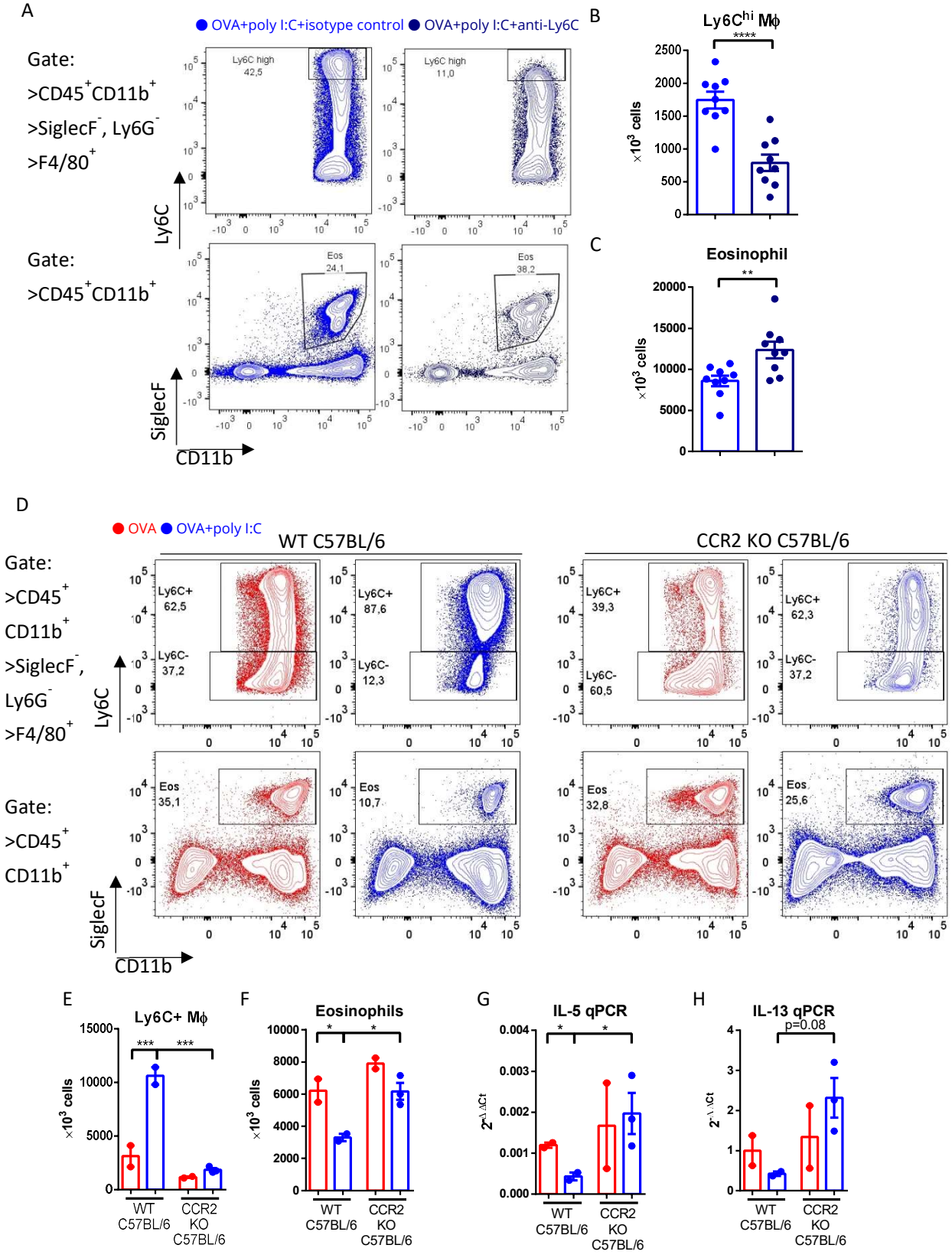
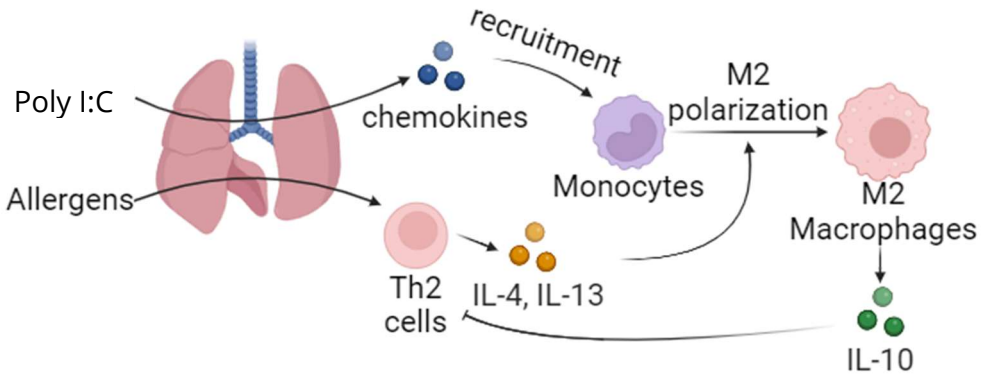

A
OVA+poly I:C+isotype control
OVA+poly I:C+anti-Ly6C
Gate:
>CD45+CD11b+
>SiglecF-, Ly6G-
>F4/80+
Ly6C high 42,5
Ly6C high 11,0
Ly6C
Gate:
>CD45+CD11b+
Eos 24,1
Eos 38,2
SiglecF
CD11b
B
Ly6Chi Mϕ
****
×10³ cells
C
Eosinophil
**
×10³ cells
D
OVA
OVA+poly I:C
WT C57BL/6
CCR2 KO C57BL/6
Gate:
>CD45+
CD11b+
>SiglecF-,
Ly6G-
>F4/80+
Gate:
>CD45+
CD11b+
Ly6C+ 62,5
Ly6C- 37,2
Ly6C+ 87,6
Ly6C- 12,3
Ly6C+ 39,3
Ly6C- 60,5
Ly6C+ 62,3
Ly6C- 37,2
Eos 35,1
Eos 10,7
Eos 32,8
Eos 25,6
Ly6C
SiglecF
CD11b
E
Ly6C+ Mϕ
***
***
×10³ cells
WT C57BL/6
CCR2 KO C57BL/6
F
Eosinophils
*
*
×10³ cells
WT C57BL/6
CCR2 KO C57BL/6
G
IL-5 qPCR
*
*
2^-ΔΔCt
WT C57BL/6
CCR2 KO C57BL/6
H
IL-13 qPCR
p=0.08
2^-ΔΔCt
WT C57BL/6
CCR2 KO C57BL/6

Figure 7


recruitment
Poly I:C
chemokines
M2
polarization
Monocytes
M2
Macrophages
Allergens
Th2
cells
IL-4, IL-13
IL-10

Supplementary materials

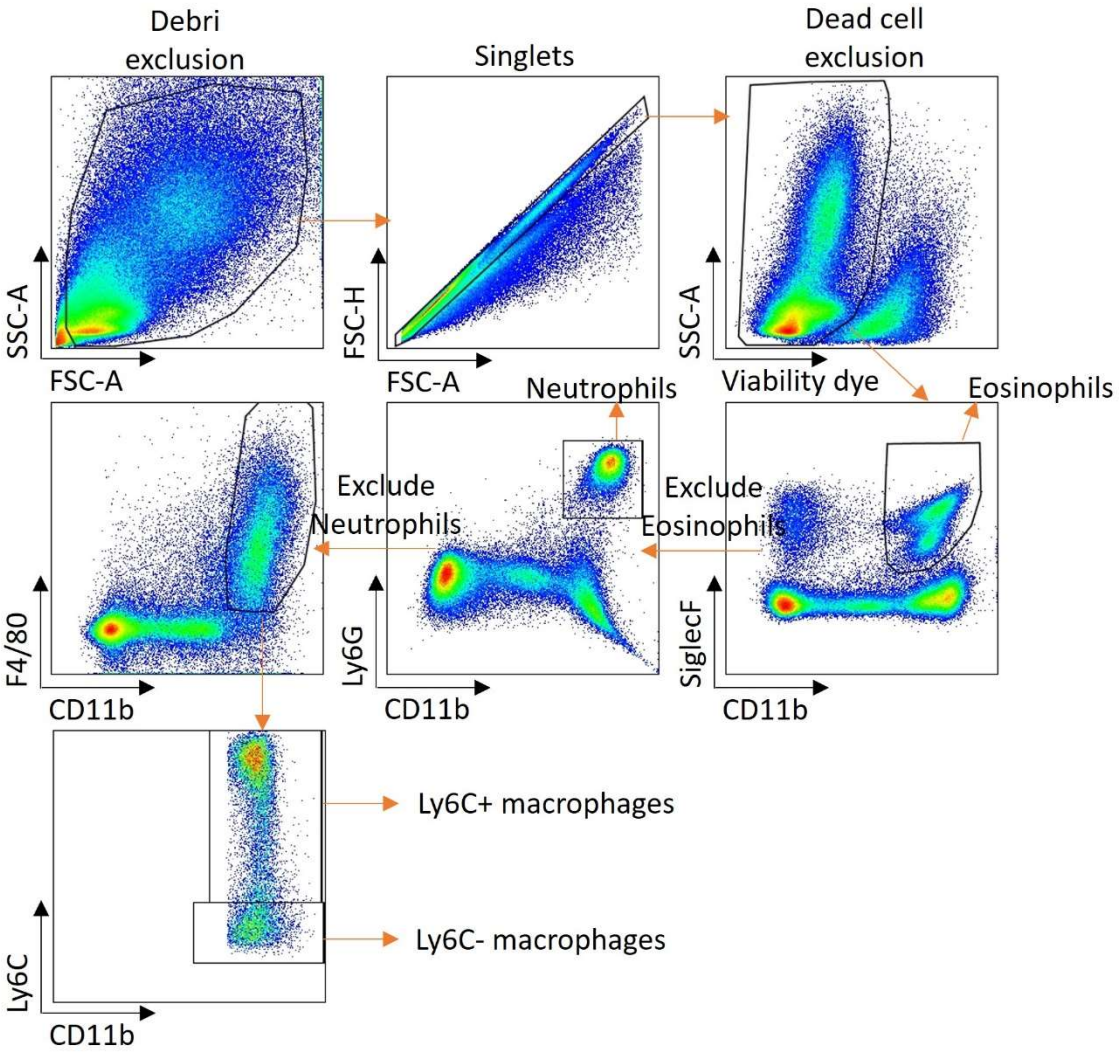
Supplementary Table S1: Antibodies and viability dye used in flow cytometry

Target	Fluorephore	Supplier	Catalog number
CD45	BUV737	BD Biosciences	568344
SiglecF	BV786	BD Biosciences	740956
Ly6G	Alexa Fluor 700	BioLegend	127621
F4/80	PE-eFluor 610	Invitrogen	61-4801-82
Ly6C	APC	Invitrogen	17-5932-82
CD11b	BUV395	BD Biosciences	563553
CD206	BV421	BioLegend	141717
CD163	PE	BioLegend	155307
Arginase-1	PE-Cy7	Invitrogen	25-3697-80
CD3	BV510	BioLegend	100234
CD4	BV421	BioLegend	100443
IFN-γ	Alexa Fluor 700	BioLegend	505824
IL-4	PE	BD Biosciences	554389
IL-5	APC	BioLegend	504305
IL-13	PE-eFluor 610	Invitrogen	61-7133-82
IL-10	PE-Cy7	BioLegend	505025
Viability Dye	eFluor780	Invitrogen	65-0865-18

Supplementary Table S2: Primer sequences for qRT-PCR
GAPDH
Forward: GGTCCTCAGTGTAGCCCAAG
Reverse: AATGTGTCCGTCGTGGATCT
IL-4
Forward: TCAACCCCCAGCTAGTTGTC
Reverse: TCACTCTCTGTGGTGTTCTTCG
IL-5
Forward: ATTGACCGCCAAAAAGAGAAGTG
Reverse: AGCCTCAGCCTTCCATTGC
IL-13
Forward: CATCACACAAGACCAGACTCCC
Reverse: CTTGCGGTTACAGAGGCCA
CCL11
Forward: AGTCGGGAGAGCCTACAGAG
Reverse: AAGTTGGGATGGAGCCTGG
CCL17
Forward: CCACCAATGTAGGCCGAGAG
Reverse: CTGGACAGTCAGAAACACGATG
TLR3
Forward: GCAAAGAAGATAAAGCGAGTTTCAC
Reverse: TCTTTTGGTGCGCGATTGTG
RIG-I
Forward: TTGGATGCCCTGTACCATGC
Reverse: TCCCTTTAGTGTCTCGGATCTGT

MDA-5
Forward: TTCTGCCTGCTGTCGATGAG
Reverse: AAACTTGTGTCTGATTCTGTGGTC
Pan IFN-α
Forward: CCTGAGAGAGAAGAAACACAGCC
Reverse: TCTGCTCTGACCACYTCCCAG
IFN-β
Forward: AGACTATTGTTGTACGTCTCC
Reverse: CAGTAATAGCTCTTCAAGTGG
IFN-γ
Forward: AGCAAGGCGAAAAAGGATGC
Reverse: TCATTGAATGCTTGGCGCTG
CXCL10
Forward: CCACGTGTTGAGATCATTGCC
Reverse: GAGGCTCTCTGCTGTCCATC
CCL5
Forward: CTCCAATCTTGCAGTCGTGTTTG
Reverse: TGCCCATTTTCCCAGGACC
TNFα
Forward: ACGTGGAACTGGCAGAAGAG
Reverse: GCCATTTGGGAACTTCTCATCC
CXCL1
Forward: CTGCACCCAAACCGAAGTCA
Reverse: CCGTTACTTGGGGACACCTTTTA
IL-6
Forward: AGAAAACAATCTGAAACTTCCAGAGAT
Reverse: GAAGACCAGAGGAAATTTTCAATAGG
CCL2
Forward: GCTACAAGAGGATCACCAGCAG
Reverse:GTCTGGACCCATTCCTTCTTGG
Arginase-1
Forward: GCTCCAAGCCAAAGTCCTTAGAGAT
Reverse: AGGAGCTGTCATTAGGGACATCAAC
IL-10
Forward: CATTTGAATTCCCTGGGTGAGAAG
Reverse: GCCTTGTAGACACCTTGGTCTTGG
PD-L1
Forward: GGCAGGAGAGGAGGACCTTA
Reverse: TTTGCGGTATGGGGCATTGA

Supplementary Figure S1: gating strategy for macrophages, eosinophils and neutrophils



Chapter 5: Discussion, conclusions and future directions

Our investigations started with an emphasis on the pathways governing pathological ASM phenotype alterations, which are important features of asthma. The characterization of transcriptional regulatory mechanisms of ASMC phenotypes in our first study was motivated by the uncertainty around the possible co-existence of proliferation/secretion and contractility. We established the MyoCD-Elk-1 interaction as the molecular basis of the paradigm of the phenotype dichotomy and provided important insights into the role of SRF, based on the stoichiometric relationships among the three transcription factors. The results also raise the question as to potential of SRF as a therapeutic target. Switching the focus towards the aspect of the synthetic phenotype of ASMC and its induction by CD4+ T cells, our second study specifically highlighted the secretion of chemokines responsible for CD4+ T cell recruitment, which has been previously demonstrated as an important extrinsic mediators of ASMC changes in asthma via contact-dependent interaction[1,2]. In this study, we discovered that ASMC mediates *in vitro* CD4+ T cell chemotaxis in a CXCL10/11-CXCR3 axis-dependent manner and emphasized the key role of the Th1 cytokine, IFN-γ in stimulating the expression of these chemokines. This study suggests a pathway by which Th1 immunity may contribute to the pathophysiology of asthma, specifically in mediating smooth muscle alterations. These findings led to our interest in establishing an animal model to study the interaction between Th1 and Th2 immunity in the context of asthma in our third study, as data supporting both protective and pathological roles for the activation of Th1 immunity have been reported. By administering poly I:C, a viral-like PAMP as an inducer of Th1 responses, in an antigen-specific asthma model in mice, we made the unexpected discovery that its effect in inducing interferons and inflammatory cytokines is diminished, and that it recruited a regulatory monocyte-derived

macrophage population that suppresses Th2 immunopathology. This study described a novel tolerogenic pathway that ameliorates asthma pathology, which could be associated with exposures to PAMPs. Overall, our investigations incorporated the research in the aspects of both pathophysiology and immunology of asthma, advancing our understandings in the relationships among ASM biology, Th1 immunity and asthma immunopathology.

Breaking the rules of smooth muscle phenotype dichotomy – SRF upregulation confers dual contractile-proliferative capacity in ASMC

Our study of MyoCD-Elk-1 interactions and their competition for SRF binding provided the molecular basis governing the contractile versus proliferative phenotype dichotomy in ASMCs for the first time. These findings contributed to the understanding of a fundamental molecular mechanism that not only provides insights into asthma-associated ASM changes but is also likely is generalizable to all smooth muscle cell types. Two of the conclusions from this study are particularly noteworthy and provided insight into potential therapeutic interventions.

First of all, as previously described in VSM exclusively, Elk-1's inhibitory effect on MyoCD transcriptional activity has been largely emphasized, as it explains the prevalent loss of contractile properties in VSM following the exposures of growth factors and inflammatory factors in the context of atherosclerosis[3]. However, the investigators of these studies claimed the competitive binding of MyoCD and Elk-1 to SRF without providing sufficient that inversely, an upregulation of MyoCD would suppress Elk-1 function and ultimately proliferation. Our study is the first to confirm such a phenomenon, which completed the description of the contractile-proliferative phenotype paradigm.

More importantly, we proved that in scenarios featuring SRF upregulation where it becomes no longer a limiting factor, ASMCs can demonstrate an augmentation of both contractile and proliferative capacities as a result of unopposed activation of both MyoCD and Elk-1. This is especially pertinent as the observations to date have yet to yield a consensus regarding the direction of ASM phenotype polarization in asthma, with data on both increases in contractility[4-6] and proliferation[1,7] being reported, which raises questions as to whether one specific phenotype is dominant. Our evidence provides a proof-of-concept demonstration that a dual contractile-proliferative ASM phenotype may exist, which could be particularly detrimental to asthma pathophysiology. Furthermore, therapeutic interventions targeting aberrant SRF upregulation, one of which being the inhibitor of the RhoA-ROCK pathway as demonstrated in our study, could be especially effective in attenuating asthma-associated ASM changes.

Lessons learned from the investigations of transcriptional regulation of contractile protein genes - smooth muscle-related biomechanical engineering as the next steps

In terms of the limitations of this study, we recognize that the contractile property of ASMC was mainly defined by gene expression, and it lacks advanced functional characterization of contraction through assessment of ASM mechanics. Unlike the experiments with tissues, there are more difficulties associated with the measurement of contraction in cultured ASMCs, with the loss of contractility due to prolonged culturing being a main obstacle. The investigations of single cell contraction using traction force microscopy and by measuring cell shortening have encountered similar difficulties[8]. Using ASMC-embedded in a collagen gel and a high concentration of KCl that acts as a contractile agonist, we aimed to mainly assess the maximum

force generation by the cells. We chose KCl rather than more physiologically relevant agonists because the cells responded the best to KCl in comparison to histamine and methacholine. A suboptimal amount of force generation was induced by the latter two agonists despite MyoCD overexpression (data not shown). We hypothesize that KCl produced a superior contractile response as the induction of intracellular calcium signals is receptor-independent, and that the histamine and acetylcholine receptor signaling might also be impacted by culturing, resulting in the poor responsiveness. Regardless, we believe that the current approach was sufficient for the purposes of this study, as it has demonstrated that the increased force generation is due to the upregulation of contractile protein, regardless of the source of the activation signal. Efforts are currently made by various groups of researchers to develop a reliable method to measure cultured ASMC contraction. The impacts of MyoCD overexpression on other aspects of contraction, such as shortening velocity, force maintenance, calcium handling and impairment in relaxation are all of interest, as the changes in these parameters have been reported in asthmatic ASMC[9-11]. These topics can be easily investigated once an optimized system of single cell contraction measurement is established.

Furthermore, this study is solely based on a cultured cell model without a simulation of the actual disease states. Therefore, for future directions, the experiments should also be adapted to models of tissues and whole organisms. These new models will also allow us to better explore the therapeutic potential of inhibiting the RhoA-ROCK-SRF axis in preventing or reversing ASM changes in the actual disease context.

In addition, the comparisons between naïve ASM tissues and MyoCD-transfected ASMCs also allowed us to evaluate the degree of contractile protein gene expression lost due to culturing that is attributable to MyoCD-mediated gene regulation. Our results indicated that

MyoCD expression was indeed downregulated in cultured cells compared to intact tissues, but regardless of an overexpression of MyoCD that exceeded the level of that in naïve tissue, the abundance of contractile proteins was far from being fully restored. Indeed, factors in the cell culture that causes drastic alterations to the environment of the cells, such as the stiffness of the growth surface, ECM compositions, sustained pro-growth stimuli in the culturing medium have all been reported to impact smooth muscle contractile properties, presumably through the influences unrelated to MyoCD-mediated gene transcription[12-15]. The effective preservation of contractile properties has always been a difficulty for *in vitro* research of smooth muscle physiology. These observations provided insights of interest to cell and tissue engineering and facilitate the search of an optimal culturing condition.

ASM secretory phenotype – the outstanding roles of IFN-γ-induced chemokine expression

A study of VSM suggests that MyoCD and NF-κB also demonstrated a mutually inhibitory relationship via direct protein-protein interaction, resulting in a contractile-secretory phenotype dichotomy[16]. We investigated whether such a phenomenon exists in ASM and observed that MyoCD upregulation did not have the expected impact on TNFα-induced NF-κB activation and gene transcription. Such discrepancies in observations could be due to the differences in the cell type, but they once again highlighted the gap of knowledge in our understanding of phenotype regulation in ASM.

Instead, we focused on the exploration of CD4+ T cell-reactive chemokine secretion by ASM. As the accumulating evidence on CD4+ T cell-ASM interaction indicates that it plays a direct role in mediating asthma-associated ASM changes[1,2], the inhibition of T cell infiltration into ASM poses potential therapeutic value. Using the Boyden chamber cell migration assay,

we discovered that the CXCL10/11-CXCR3 axis, the Th1-associated, IFN-inducible chemokine signaling pathway is primarily involved in ASM-mediated T cell recruitment. Besides these chemokines, ASMCs are also known to prominently express CCL3 and CCL5. Our study confirmed that these chemokines are unlikely to be involved, as the small molecule antagonists of their cognate receptors, CCR3 and CCR5, did not inhibit ASMC-mediated T cell migration. While ASMCs constitutively secreted CXCL10 and CXCL11 which were sufficient to induce T cell chemotaxis, we also found that T cells once migrated to ASMC are a major contributor to IFN-γ, which drives further upregulation of CXCR3 ligands.

Next steps for the investigations of ASM-derived chemotactic factors

Alone with IFN-γ, CXCR3 ligands are shown to be upregulated in the context of asthma, as demonstrated in both patients and animal models[17,18]. An elevated CXCL10 level is also particularly linked to severe, corticosteroid-insensitive asthma[17,18]. Furthermore, certain CXCR3 polymorphisms have been associated with an increased risk of asthma[19]. This clinical and experimental evidence consolidated the involvement of the CXCL10-CXCR3 axis in asthma pathophysiology. An observation that is closely related to this study is that the CXCL10-CXCR3 axis is responsible for mast cell recruitment to ASM tissues, another cell type prominently observed to infiltrate the smooth muscle bundles[20]. Along with our study, these findings suggested a mechanism through which the chemokine signaling axis contributes to the disease.

A limitation of this study is the lack of the representation of the actual inflammatory environment in disease. Both ASMCs and T cells were derived from non-asthmatic volunteers. Particularly, the *in vitro* anti-CD3/CD28 activation of the T cells significantly ramped up the

level of cytokine production, and even possibly resulted in a Th1 bias. Future effort should focus on performing a similar study with cells derived from asthmatic patients or incorporating the stimulation of either cell types in the assay with relevant immune mediators, particularly Th2 cytokines. One noteworthy observation we made is that compared to the prominent induction of the CXCR3 ligand by IFN-γ, the induction of Th2-associated chemokines, including CCL11, CCL17 and CCL22 by IL-4 and IL-13 was proven to be difficult, as the magnitude of induction was relatively low and required co-stimulation by TNFα (data not shown), which is consistent with existing evidence[21]. Therefore, it is possible that chemokine secretion by ASMC is inherently biased towards Th1-related factors.

We demonstrated the expression of CXCL10 and 11 by asthmatic ASM in tissue sections, but when we compared ASMCs derived from healthy and asthmatic donors for their CXCR3 ligand secretion capacity in response to recombinant or T cell-derived IFN-γ, we found no notable difference. This suggests that there is no alteration in asthmatic ASMCs leading to changes in innate responsiveness to cytokines, and that elevated chemokine secretion by ASM *in vivo* is solely a result of the pro-inflammatory milieu associated with asthma. Indeed, an increase in expression of CXCL10 has been shown in asthmatic ASM by other studies[20]. Whether the products of mast cells and T cells located within the ASM tissue contribute to the upregulation of chemokine expression remains to be examined.

The future directions of this investigation involve re-establishing the rat model of asthma evoked by OVA sensitization and challenges to confirm whether CXCL10/11-CXCR3 axis is indeed responsible for *in vivo* ASM-mediated T cell recruitment[2]. Though the mouse model is the most commonly used, this species does not feature prominent changes of ASM, or T cell infiltration into ASM. Indeed, the mouse airways are only several cell layer thick and

lack a bronchial circulation. In contrast, a single sensitization and three OVA challenges within the course of three weeks were shown to be sufficient in the Brown-Norway rats to evoke a doubling of ASM mass and also goblet cell differentiation[2]. In addition, novel, more efficient methods for detecting T cells localized within the smooth muscle should be established, as immunohistochemical assessment is inefficient in measuring relatively rare events such as in this case.

Lastly, other observations in this study specifically related to the behavior of CXCR3 offered pertinent insights in the receptor biology. The upregulation of CXCR3 in all T cells following anti-CD3/CD28 activation suggests that the T cell receptor activation signal is a direct contributing factor to its expression, regardless of their subset destination. However, given that anti-CD3/CD28 is a strong activation signal that differs from the physiological stimuli, it is important to investigate whether described changes in CXCR3 expression occur following normal T cell receptor signaling *in vivo*. In addition, CXCR3 internalization upon exposure to its ligands, potentially via the arrestin-mediated mechanism, which has also been reported in several other studies[22,23], hinted to the existence of a negative feedback mechanism protecting T cells from excessive chemokine receptor activation in response to a high ligand concentration. Its biological implications merit further investigation.

<u>An unexpected effect of viral-related PAMP on asthma-associated Th2 immunopathology</u>

Our investigations on the involvement of IFN-γ and CXCR3 ligands in mediating T cell migration to ASM led to our interests in the roles of Th1 immunity in asthma. This topic is also particularly relevant as emerging evidence suggests that severe asthma displays elevated Th1 signatures[24,25]. In addition, viral infections, which commonly induce Th1 responses, are the

leading cause of asthma exacerbations[26]. In order to study the general interaction between Th1 immunity and asthma immunopathology, we decided to co-administer poly I:C, a viral-like PAMP that induces interferons and pro-inflammatory cytokines during allergen challenges in a mouse atopic asthma model.

Contrary to our initial hypothesis that poly I:C would contribute to additional immunopathology in allergen challenge mice, it dampened AHR, eosinophilia and Th2 cells and cytokines. Furthermore, when we verified the expression of poly I:C-inducible interferons and inflammatory factors, they were also suppressed in the co-administered group, indicating the reduction in Th2 immunopathology was not due to the antagonist effect of poly I:C-induced Th1 immunity. Instead, we discovered an enrichment of monocyte-derived macrophages with a prominent M2 phenotype and elevated levels of IL-10 secretion in the lungs following OVA-poly I:C co-administration, which we demonstrated to possess immunosuppressive function *in vitro*. Finally, we provided the proof that the monocyte-derived macrophages were indeed involved in suppressing Th2 immunopathology, as the depletion of this population with anti-Ly6C antibody or in the CCR2 knockout mouse strain impaired the protective effect offered by poly I:C co-administration.

Given the context of our investigation, many tend to make comparisons between our findings and studies of viral infections in asthma, which often feature worsened inflammation and exacerbation[26]. The key difference is that stimulation of antiviral pathways by poly I:C is self-limiting and only induces a transient inflammatory response that completely resolves by day two following the challenge, as reflected by the level of cytokine expression (data not shown). In contrast, viral infections are prolonged processes that involve the propagation of the pathogen and complex host-pathogen interactions[27]. A balanced immune response that

efficiently eliminates pathogen without excessive tissue-damaging cytokine and cellular responses is required for a successful resolution. Therefore, impairments in pathogen tolerance or resistance are often the underlying causes of severe diseases[28]. These issues are not associated with the mild and transient immune activation induced by poly I:C exposures. In fact, our data support the notion that a deficient antiviral response in asthma is the underlying cause of worsened outcomes and exacerbations following infection, as the induction of type 1 interferon, which is essential for the control of many common respiratory viral infections, is impaired in the allergen-challenged mice, which is consistent with the findings in asthmatic patients[29,30].

Rather than being a model of asthma exacerbation, our studies revealed a PAMP-induced immunoregulatory mechanism that could potentially be a part of the normal resolution of inflammatory processes. Similar observations can be found in the literature, in which poly I:C and bacterial PAMP CpG were known to induce regulatory macrophages that dampen immune responses, including asthma-associated inflammation[31,32]. Our data add to the accumulating evidence that pathogen exposure might be essential for establishing immunoregulatory pathways that curb atopic disorders, a hypothesis backed by epidemiological observations[33].

<u>Future directions – a better description of the mechanism by which the modulatory macrophage alleviates asthma immunopathology</u>

Though we have demonstrated the immunosuppressive roles of the poly I:C-indued monocyte-derived macrophages and highlighted the prominent expression of IL-10 by these cells, we have yet to definitively prove that this cytokine is indeed the major factor responsible for the

regulatory function. In fact, other M2-associated molecules expressed by this population, including arginase-1 and PD-L1, could also play a role in suppressing T cell activation and subsequent immune responses[34,35]. Further research is required to decipher the exact molecular pathway involved.

Another intriguing finding in our study is that the modulatory macrophage population seemed to originate from an immunological milieu uniquely induced by the combination of poly I:C and OVA. Our current explanation is that poly I:C acted as a recruiting signal of the monocytes, which differentiated into the M2 phenotype in response to the Th2-high environment in the lung. However, whether other factors, such as the signaling of the PAMP receptors, or enrichment of monocytes in the bone marrow via myelopoiesis, contribute to the generation of this population remains to be investigated. The immunosuppressive myeloid population is extensively studied in cancer, and has been assigned distinct nomenclature, such as tumor-associated macrophages and myeloid-derived suppressor cells[36,37]. The macrophage population described in our study and these cancer-associated myeloid cells might be two sides of the same coin. A deep immunophenotyping of such a population would likely provide important insights into the origin and function of this population and subsequently facilitate the development of a relevant therapeutic interventions.

Conclusions and final remarks

Overall, our studies of cultured ASMCs yielded comprehensive characterizations of the molecular mechanisms responsible for smooth muscle cell phenotype determination, which are especially pertinent in asthma. The key findings, including that an increase in SRF activity allows simultaneous activation of MyoCD and Elk-1, permitting a dual contractile-proliferative

phenotype, and that IFN-γ is the key cytokine to stimulate CD4+ T cell-recruiting signals, established the important biological basis of pathological ASM changes. The future investigations should focus on examining these pathways in animal models or the actual disease context in order to demonstrate their relevance. Furthermore, our exploration of the interaction between Th1 immunity and atopic asthma in a mouse model revealed an unexpected immunomodulatory pathway mediated by monocyte-derived macrophages and implicated the potential roles of pathogen-related signal in shaping a regulatory pathway for the protection against atopic disorders. The more detailed cellular and molecular underpinning of this pathway, and the actual relevance of these findings in human disease requires further investigation.

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