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Integrative Roles of Pro-Inflammatory Cytokines on Airway Smooth Muscle Structure and Function in Asthma

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ABSTRACT

Asthma has become more appreciated for its heterogeneity with studies identifying type 2 and non-type 2 phenotypes/endotypes that ultimately lead to airflow obstruction, airway hyperresponsiveness, and remodeling. The pro-inflammatory environment in asthma influences airway smooth muscle (ASM) structure and function. ASM has a vast repertoire of inflammatory receptors that, upon activation, contribute to prominent features in asthma, notably immune cell recruitment and activation, hypercontractility, proliferation, migration, and extracellular matrix protein deposition. These pro-inflammatory responses in ASM can be mediated by both type 2 (e.g., IL-4, IL-13, and TSLP) and non-type 2 (e.g., TNFα, IFNγ, IL-17A, and TGFβ) cytokines, highlighting roles for ASM in type 2 and non-type 2 asthma phenotypes/endotypes. In recent years, there has been considerable advances in understanding how pro-inflammatory cytokines promote ASM dysfunction and impair responsiveness to asthma therapy, corticosteroids and long-acting β2-adrenergic receptor agonists (LABAs). Transcriptomic analyses on human ASM cells and tissues have expanded our knowledge in this area but have also raised new questions regarding ASM and its role in asthma. In this review, we discuss how pro-inflammatory cytokines, corticosteroids, and LABAs affect ASM structure and function, with particular focus on changes in gene expression and transcriptional programs in type 2 and non-type 2 asthma.

1 | Introduction

Airway smooth muscle (ASM) contributes to inflammation, airway hyperresponsiveness (AHR), remodeling, and thickening which leads to airway narrowing, resulting in asthma symptoms, including dyspnea, wheeze, and cough, and acute exacerbation [1–3]. Changes to ASM structure and function is promoted by airway inflammation, which involves immune cell aggregation around peribronchiolar and perivascular regions in close proximity to ASM. Within this pro-inflammatory environment, ASM responds to cytokines and growth factors released by immune cells and the airway epithelium [1–3]. These inflammatory mediators have effects on ASM that promote cytokine and chemokine release, enhanced calcium ion (Ca^{2+}) response and hypercontractility, proliferation, migration, and extracellular matrix (ECM) production. The importance for ASM function in asthma is highlighted by positive correlations between increased asthma severity and worsened lung function, AHR,

and more frequent exacerbations [4, 5]. Studies also show associations between increased ASM layer remodeling and thickening with severe and fatal asthma [6].

Asthma is highly heterogenous with multiple phenotypes and endotypes that are distinguished by airway inflammation, lung function, co-morbidities, and disease severity [7, 8]. While the underlying factors that influence this heterogeneity are not fully understood, it is thought that interactions among the environment (allergens, pathogens, toxins, pollutants), genetics (e.g., atopy), age-onset (childhood vs. adult), and co-morbidities (e.g., obesity, autoimmunity) are influencing factors [8–11]. The type 2 inflammatory response has a central role in asthma pathogenesis and is a primary target for new biologic therapies. However, some individuals with asthma have non-type 2 phenotypes and endotypes with activation of type 1 and/or type 17 pathways, creating a complex inflammatory environment that is difficult to manage with standard therapies [8].

Recent studies, most notably using bulk and single-cell RNA-sequencing, have revealed that distinct pro-inflammatory transcriptional programs are present in immune and airway epithelial cells [12–15]. Despite the diverse immune environments in asthma, changes to ASM function (hypercontractility) and structure (thickening, remodeling) within asthma phenotypes and endotypes are poorly understood. ASM is greatly impacted by cytokines produced during type 2, type 1, and type 17 inflammatory pathways, suggesting ASM dysfunction develops across multiple pathways.

Corticosteroids and long-acting β2 adrenergic receptor agonists (LABAs) are used to manage asthma and their effectiveness in reducing symptoms and minimizing exacerbations are key factors in asthma severity, with insensitivity to treatment defining more severe disease [16, 17]. Studies show that corticosteroids modulate ASM responses to inflammation, but their anti-inflammatory effects can be limited particularly in the presence of type 1 inflammatory cytokines, tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) [18, 19]. Recent studies using microarray and RNA sequencing (RNA-seq) analyses in ASM cells and airway tissues have provided novel insight into the pro-inflammatory signaling and gene expression changes to ASM in asthma. In this review article, we summarize how pro-inflammatory cytokines that distinguish asthma phenotypes and endotypes affect ASM. We also discuss the current understanding of transcriptional programs in ASM that respond to airway inflammation, corticosteroids, and LABAs. Finally, we identify existing knowledge gaps and opportunities to further explore how complex inflammatory environments impact the role of ASM in asthma.

2 | Inflammatory Pathways in Asthma Phenotypes and Endotypes

Distinct asthma phenotypes and endotypes have been identified within asthma patient populations and can be broadly stratified by the presence of type 2 or non-type 2 inflammation based on blood and sputum granulocyte populations among the presence of other inflammatory markers [8, 9]. Type 2 inflammation, which is characterized by increased eosinophils, is the most common inflammatory pathway in asthma and is associated with both mild and moderate–severe asthma [20]. Non-type 2 inflammatory pathways involve type 1 and/or type 17 inflammation, which are associated with increased asthma severity and is characterized by increased neutrophils. Asthma endotypes with mixed granulocytic (eosinophils and neutrophils) infiltration is composed of both type 2 and non-type 2 pathways [8, 9]. An additional asthma endotype, paucigranulocytic inflammation, has airway inflammation in the absence of eosinophils and neutrophils and has been shown to be insensitive to corticosteroids [21].

2.1 | Type 2 Inflammation

The type 2 inflammatory response is central to allergic responses, host defense against parasitic helminths, tissue repair, and fibrosis [22–24]. Type 2 inflammation is induced by allergic responses and is mediated by effector immune cells including T helper 2 (Th2) cells, type 2 innate lymphoid cells (ILC2), B cells, eosinophils, and mast cells [25–27]. Interleukin (IL)-4, IL-13, and thymic stromal lymphopoietin (TSLP) are among the key inflammatory mediators produced in type 2 inflammation that have known effects on ASM structure and function. The importance of type 2-induced signaling pathways are emphasized by the development of monoclonal antibody therapies for severe asthma (anti-IgE, anti-IL-4Rα, anti-IL-5, anti-IL-5R, and anti-TSLP) and their ability to improve lung function and reduce acute exacerbations [28–31]. They can also impact airway structure. For example, benralizumab, a monoclonal antibody against IL-5R, was recently shown to reduce not only eosinophil infiltration but also ASM mass in bronchial biopsy samples from subjects enrolled in a double-blind clinical trial [32, 33].

2.2 | Type 1 Inflammation

The type 1 inflammatory response plays an integral role in host defense against viral and bacterial infection [34]. Increased type 1 inflammation has been observed across multiple pediatric and adult asthma cohorts with increased pathogenic bacterial colonization and microbial dysbiosis [35], obesity [36–39], or other complex co-morbidities such as autoimmune disease [8]. Asthma endotypes with increased type 1 inflammation has been identified and are associated with corticosteroid insensitivity in severe asthma [40, 41]. Type 1 inflammation involves increases in innate and adaptive immune cell populations including macrophages, neutrophils, and Th1 cells. IFNγ, IL-1β, and TNFα are among the key inflammatory mediators produced in type 1 inflammation. Unlike type 2-high asthma, there are currently no effective therapies for managing severe asthma endotypes with increased type 1 inflammation aside from corticosteroids and bronchodilators. Accordingly, there is a continued need to understand how type 1 pathways promote corticosteroid insensitivity, particularly with regard to ASM structure and function.

2.3 | Type 17 Inflammation

The type 17 inflammatory response is important for host defense against bacterial and fungal infections in the lung [42]. Upon activation, Th17 cells produce IL-17A among other inflammatory

cytokines (IL-6, IL-17F, IL-21, IL-22, and IL-23) that recruit and activate neutrophils [43]. In children and adults with severe asthma, increased sputum neutrophils and IL-17A levels are associated with corticosteroid insensitivity in allergic and non-allergic asthma [44–47]. Increased type 17 inflammation in severe asthma is associated with the presence of pathogenic bacteria and fungal sensitization [48–51]. Additionally, type 17 inflammation is also induced upon exposure to diesel exhaust particles or ozone, which augments allergic airway inflammation and AHR to promote corticosteroid insensitivity [52–54]. Despite having an established role in severe asthma endotypes, targeting type 17 inflammation with anti-IL-17RA or -IL-23 monoclonal antibodies were found to be ineffective in patients with severe asthma [55, 56], reinforcing the need to further understand how type 17 inflammation promotes airway inflammation and AHR in asthma.

2.4 | Paucigranulocytic Inflammation

Some individuals with asthma have paucigranulocytic inflammation with an absence of sputum eosinophils and neutrophils, and low levels of type 2 inflammatory biomarkers (e.g., FeNO and eosinophilic cationic protein) [57]. In a pediatric cohort, paucigranulocytic inflammation was identified in 52% children with severe asthma [58]. While paucigranulocytic asthma is more likely to have better lung function compared to other asthma endotypes, their symptoms persist despite corticosteroid treatment [57–59]. The underlying causes of paucigranulocytic asthma remains largely unknown, however, co-morbidities and environmental exposures that promote ASM dysfunction in the absence of eosinophil and neutrophil activation have been implicated [60, 61]. Studies show pro-inflammatory cytokines most associated with type 1, 2, and 17 inflammation are at relatively low levels in paucigranulocytic asthma [21, 62]. While the underlying mechanism remains under investigation, it has been proposed that other inflammatory pathways among airway structural cells such as transforming growth factor (TGFβ) signaling, which has an integral role in regulating inflammation, wound healing, and fibrosis, may be involved [60, 61, 63, 64].

3 | Immune Cell Recruitment and Interactions With Airway Smooth Muscle

In response to inflammation, ASM produces several pro-inflammatory mediators, including chemokines and cytokines, that recruit and activate immune cells around the airway [3]. Bi-directional paracrine signaling between ASM and immune cells can further promote airway inflammation that leads to airway pathology (Figure 1). There are also direct interactions with innate and adaptive immune cells through ASM cell–cell adhesion and co-stimulatory molecules, which contributes to asthma pathogenesis.

3.1 | Eosinophils

Eosinophils and their activation play a central role in type 2 asthma through their release of inflammatory mediators, proteases, and bronchoconstrictors. IL-4 and IL-13 stimulate human ASM to secrete CC chemokine ligand 11 (CCL11) and CCL24, which are chemokines that recruit eosinophils to the site of inflammation [65, 66]. Inhibitory studies using small interfering RNA (siRNA) knockdown and pharmacological inhibition showed that IL-4- and IL-13-induced CCL11 expression is mediated by their common receptor, IL-4Rα, and regulated by signal transducer and activator of transcription 6 (STAT6), extracellular

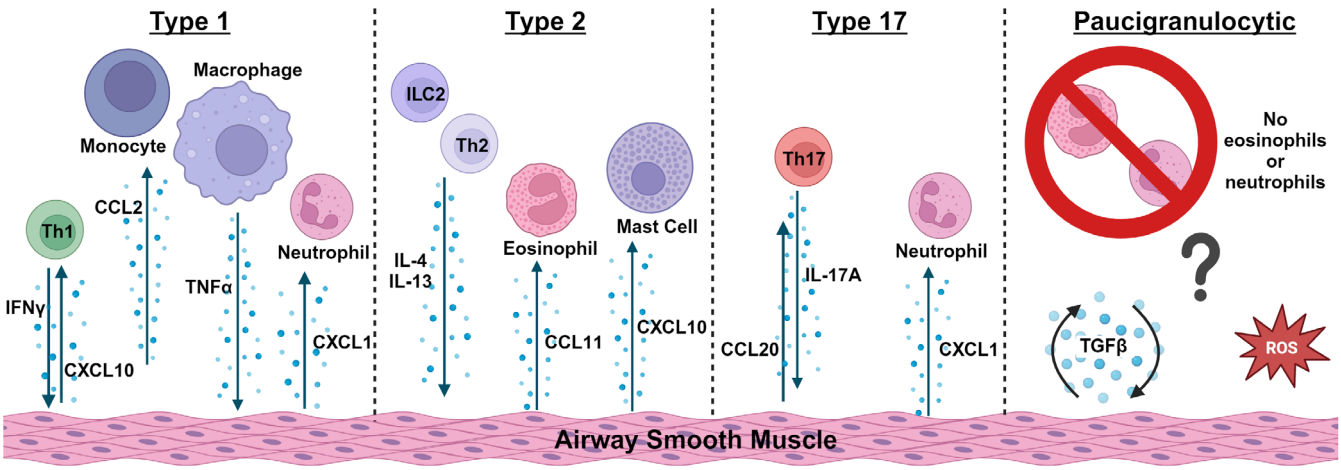

FIGURE 1 | Airway smooth muscle and immune cell interactions in asthma endotypes. Pro-inflammatory cytokines secreted from immune cells that infiltrate the airway stimulate pro-inflammatory cytokine and chemokine release from airway smooth muscle (ASM). In type 2 inflammation, type 2 helper T lymphocytes (Th2) and innate lymphoid cells (ILC2) release interleukin (IL)-4 and IL-13 to promote ASM hypercontractility and pro-inflammatory mediator production. Eosinophils are recruited by ASM through CC motif chemokine ligand 11 (CCL11) release, while mast cells are recruited and activated by C-X-C motif chemokine ligand 10 (CXCL10). In type 1 and type 17 inflammation, ASM releases CXCL1 to recruit neutrophils, while also secreting CXCL10 and CCL20 to recruit Th1 and Th17 cells, respectively. ASM contributes to monocyte infiltration through CCL2. The paucigranulocytic asthma endotype does not have eosinophil and neutrophil infiltration and lacks expression of cytokines and chemokines commonly associated with type 2 asthma. It has been proposed that TGFβ signaling and oxidative stress among airway structural cells are involved. Figure was created using BioRender.com on August 31, 2024.

signal-regulated kinase (ERK), and p38 [67]. The effects of IL-4 and IL-13 on CCL11 are further enhanced by combined treatment with non-type 2 cytokines such as, IL-1β, TGFβ, and TNFα [65, 68, 69]. TSLP also stimulates pro-inflammatory mediator production in human ASM by increasing CCL11, CXCL8, and IL-6 secretion through MAPK and STAT3-mediated pathways [70]. Eosinophils degranulate and release factors to induce AHR and promote ASM proliferation through the production of cysteinyl leukotrienes [71]. Eosinophils also promote TGFβ production, fibronectin deposition, and airway remodeling through interactions [72].

3.2 | Mast Cells

Mast cells also contribute to type 2 inflammation and are a key source of pro-inflammatory mediators and stimuli that promote airway inflammation and AHR in asthma [73]. They are activated by immunoglobulin E (IgE) binding to its receptor, FcεR1, which induces the production of proteases, cytokines, and lipid mediators [73]. Increased mast cells are associated with severe asthma and their localization to sub-airway epithelial cell regions may contribute to increased AHR and ASM layer thickening [74, 75]. ASM release of CXC motif chemokine ligand 10 (CXCL10) can contribute to mast cell recruitment [76], while promoting their survival through cell–cell interactions via stem cell factor (SCF) and cell adhesion molecule 1 (CADM1) [77, 78]. SCF-CADM1 interactions also diminish the ability for LABAs to relax ASM [79]. Conditioned media from mast cells was shown to stimulate ASM production of CXCL8 and fibronectin [80], suggesting mast cell-derived factors can promote inflammatory responses in ASM. Mast cell and ASM interactions also involve TSLP, which is secreted by ASM to activate CXCL8, IL-5, and IL-13 production by mast cells [81, 82].

3.3 | Neutrophils

Neutrophils are increased in asthma endotypes with increased type 1 and type 17 inflammation [45]. Upon their activation, neutrophils produce pro-inflammatory mediators and reactive oxygen species to promote corticosteroid insensitivity and severe asthma pathogenesis [83]. Neutrophils are recruited by chemokines CXCL1, CXCL2, CXCL3, and CXCL8, which are all released by human ASM upon stimulation with TNFα or IL-1β [84, 85]. IL-17A-treated human ASM secrete neutrophil-specific chemokines CXCL1, CXCL2, and CXCL3 [86], while also augmenting IL-1β-induced CXCL8 production [87]. Regulation of CXCL1 expression mediated by TNFα involves nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), ERK, and c-Jun N-terminal kinase (JNK) pathways [85]. In addition to pro-inflammatory cytokines, stimulated neutrophils release exosomes that induce ASM proliferation and contain proteins known to promote airway remodeling such as matrix metalloproteinase-9 (MMP-9) [88].

3.4 | Macrophages

Monocytes and macrophages are also involved in type 1 and 2 inflammation by producing pro-inflammatory mediators, such as IL-1β, TNFα, and IL-13 [89, 90]. Circulating monocytes are recruited to the lung during inflammation, which is primarily through its chemoattractant, CCL2 [91]. Monocyte activation is associated with increased asthma severity [92]. Monocyte recruitment in asthma may be enhanced as evidenced by increased CCL2 expression in asthmatic airway tissues [93] and asthma human ASM cells [94]. Lung resident alveolar and interstitial macrophages may also contribute to asthma pathogenesis and interact with ASM by secreting pro-inflammatory and profibrotic factors, however, this idea has yet to be explored experimentally [89].

3.5 | T Lymphocytes

Helper (Th) T cells release effector cytokines to promote airway inflammation and have a central role in inflammatory pathways involved in asthma pathogenesis [95, 96]. Asthmatic airways show the presence of CD4+ T lymphocytes within ASM bundles, which has been correlated with asthma severity [97]. ASM releases chemokines known to recruit Th cells. Regarding Th2 cell specific chemokines, treatment with IL-4 or IL-13 alone does not induce CCL17 production. However, when IL-4 or IL-13 treatment is combined with TNFα, CCL17 secretion is augmented [98]. In subsequent studies, IL-4/TNFα-induced and IL-13/TNFα-induced CCL17 production was shown to be greatest in human ASM samples with Ile50Val, Ser748Pro, and Gln551Arg polymorphisms in the IL-4Rα gene [98]. In type 1 inflammation, Th1 cells are recruited by chemokines CXCL9, CXCL10, and CXCL11. ASM cells exposed to IL-1β, IFNγ, or TNFα secrete these Th1 specific chemokines in abundance [99, 100]. CCL20 has a key role in the type 17 inflammatory pathway and recruiting Th17 cells. IL-1β, a cytokine involved in Th17 differentiation, stimulates human ASM to release CCL20 [101]. In addition to Th17 cell recruitment, ASM may also contribute to Th17 differentiation through IL-6 production, which can be induced by TNFα or sphingosine 1-phosphate [102, 103]. These studies implicate ASM in type 17 inflammation through neutrophil recruitment, Th17 cell recruitment and differentiation. However, the extent to which ASM is involved in Th17 pathways in asthma remains to be determined.

4 | Airway Smooth Muscle Hypercontractility and Airway Hyperresponsiveness

ASM contraction is a key determinant of lung function in asthma through its regulation of airway tone and lumen diameter size [1, 2]. Airway inflammation promotes ASM hypercontractility and AHR that narrows the airway and obstructs airflow [104]. In asthma, pro-inflammatory cytokines and other mediators prime ASM for hypercontractile responses to affect Ca^{2+} response/dynamics, actin-myosin activation, and contractile force [1, 2]. Contraction is rapidly induced by bronchoconstrictors released by immune cells, airway neurons, or other airway structural cells (e.g., acetylcholine, histamine, leukotrienes, bradykinins, and tachykinins). These bind to their respective G protein coupled receptor (GPCR) and induce downstream signaling that regulates intracellular Ca^{2+} ($[Ca^{2+}]_i$), ion channels, and actin-myosin interactions. $[Ca^{2+}]_i$ plays a critical role in ASM contraction through Ca^{2+}-activated calmodulin that induces myosin

light chain kinase (MLCK) activation. MLCK phosphorylates myosin light chain 20 (MLC20), which enables myosin-actin interactions and conformational changes that favor contraction [105, 106]. ASM relaxation involves myosin light chain phosphatase (MLCP) activity, which de-phosphorylates MLC20 to convert the myosin-actin complex back to steady state [105]. These contraction and relaxation mechanisms are affected within asthma phenotypes and endotypes to promote ASM hypercontractility as detailed in Figure 2. However, the underlying mechanisms are dependent on the effector cytokine(s) and need to be probed in an endotype-specific manner.

4.1 | Intracellular Ca^{2+} Regulation

At steady-state, Ca^{2+} levels are low in the cytosol and predominately stored in the sarcoplasmic reticulum (SR) in ASM. Ca^{2+} release from the SR into the cytosol is mediated by inositol 1,4,5-triphosphate receptor (IP3R) and ryanodine receptor (RyR) upon binding their respective second messengers, inositol 1,4,5-triphosphate (IP3) and cyclic-adenosine diphosphate ribose (cADPR) [105]. IP3 is produced by phospholipase C, while the ADP ribosyl cyclase activity of cluster of differentiation 38 (CD38) produces cADPR [107]. Re-uptake of cytosolic Ca^{2+} into the SR is mediated by the sarcoendoplasmic reticulum calcium ATPase (SERCA) and is an important mechanism for maintaining $[Ca^{2+}]_i$ at homeostatic levels [105].

Pro-inflammatory cytokines, IL-13 and TNFα, are widely known to augment $[Ca^{2+}]_i$ release from the SR in response to different bronchoconstrictors in human ASM cells and promote hypercontractility in mouse and human tracheal/airway tissue [108–116]. These effects are mediated through several different mechanisms, including enhanced IP3-IP3R and RyR activity [108, 117]. IL-13 and TNFα also increase CD38 expression and activity to enhance $[Ca^{2+}]_i$ release through increased cADPR-RyR signaling [118–121]. IL-13 and TNFα increase cytosolic Ca^{2+} oscillation frequency [122, 123], a key factor in ASM hypercontractility and the capacity for relaxation [124, 125]. Using pharmacological inhibitors, enhanced $[Ca^{2+}]_i$ oscillation

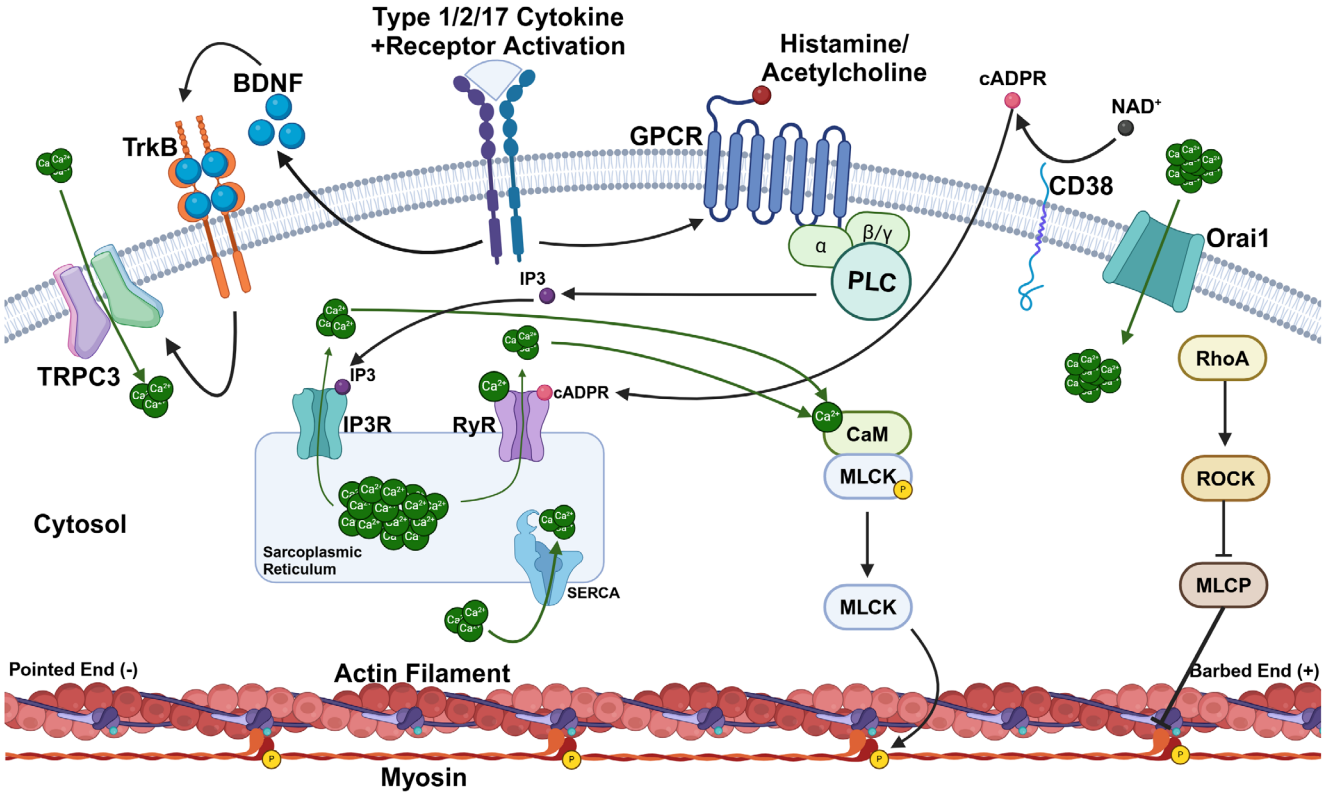

FIGURE 2 | Pro-inflammatory cytokines promote airway smooth muscle hypercontractility and force. The binding of an agonist, such as histamine or acetylcholine, stimulates G-protein coupled receptor (GPCR) signaling to induce airway smooth muscle (ASM) contraction. Pro-inflammatory cytokines augment Ca^{2+} responses through increased Ca^{2+} release from the sarcoplasmic reticulum, via IP3-IP3R and RyR channel activation. Extracellular Ca^{2+} influx involves Orai1 and TRPC3 channels. Pro-inflammatory cytokines can stimulate BDNF release by ASM, which induces autocrine signaling that further promotes extracellular Ca^{2+} influx. Increased intracellular Ca^{2+} activates MLCK to enhance actin-myosin interactions and induce ASM contraction. This mechanism is augmented by the RhoA-ROCK pathway to impair ASM relaxation and sustain contractile force following bronchoconstrictor stimulation. BDNF, brain-derived neurotrophic factor; CaM, calmodulin; cADPR, cyclic adenosine diphosphate ribose; CD38, cluster of differentiation 38; IP3, inositol 1,4,5-trisphosphate; IP3R, inositol 1,4,5-trisphosphate receptor; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; NAD+, nicotinamide adenine dinucleotide; PLC, phospholipase C; RhoA, Ras homolog family member A; ROCK, Rho-associated protein kinase; RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; TrkB, tropomyosin receptor kinase B; TRPC3, transient receptor potential cation channel subfamily C member 3. Figure was created using BioRender.com on August 31, 2024.

frequency by IL-13 was shown to involve IP3R and ryanodine receptor (RyR) [122]. Additionally, Ca^{2+} reuptake into the SR by SERCA is impaired in IL-13 treated human ASM, an effect attributed to Ca^{2+}/Calmodulin Stimulated Protein Kinase II (CaMKII)-mediated SERCA phosphorylation [126, 127]. These studies show that pro-inflammatory cytokines augment $[Ca^{2+}]_i$ responses to promote hypercontractility and inhibit relaxation of ASM.

4.2 | Extracellular Ca^{2+} Influx

In addition to intracellular Ca^{2+} stores, ASM also expresses a variety of voltage-gated Ca^{2+}, transient receptor potential, L- and T-type Ca^{2+}, ion exchange, and store-operated Ca^{2+} entry (SOCE) channels that mediate rapid Ca^{2+} influx from outside the cell [2]. Notably, IL-13 and TNFα increase expression and activity of Na^+/Ca^{2+} exchange (NCX) and Stim1/Orai1 (SOCE) [128–130]. TNFα also induces release of factors that promote ASM hypercontractility via autocrine signaling. For example, ASM exposed to TNFα has increased expression of brain-derived neurotrophic factor (BDNF) and its receptor, tropomyosin-related kinase (TrkB) [114, 131]. BDNF–TrkB signaling in human ASM enhances $[Ca^{2+}]_i$ responses to histamine and acetylcholine [114, 132]. TNFα and BDNF promote Ca^{2+} influx activity by transient receptor potential channel 3 (TRPC3), while blocking BDNF–TrkB signaling in TNFα-exposed ASM blunts Ca^{2+} response [133, 134]. In an allergen mouse model, TrkB conditional knockin mice with disruption in TrkB tyrosine kinase activity and smooth muscle-specific BDNF knockout mice demonstrate that BDNF–TrkB signaling is important for mixed allergen-induced AHR [135]. These studies highlight an autocrine mechanism by which TNFα promotes ASM hypercontractility.

4.3 | Actin-Myosin Regulation

Inflammation leads to changes in the actin-myosin complex that enhances ASM hypercontractility. In its active state, actin polymerizes to form F-actin which allows contraction. TNFα enhances F-actin formation and actin polymerization to augment contractile force in response to acetylcholine [136, 137]. While IL-17A receptor signaling (IL-17RA/RC) stimulates recruitment of Rab35 and DennD1C to promote protein kinase C activation and enhance actin-myosin interactions in human ASM [138]. TGFβ also enhances actin-myosin interactions to augment ASM contraction by increasing transgelin and calponin expression to stabilize actin and regulate myosin motor function [139–141].

Pro-inflammatory cytokines also regulate the myosin-actin complex by increasing activity of Ras homolog family member A (RhoA), a small Rho GTPase that activates its downstream target Rho-associated protein kinase (ROCK). RhoA expression is increased by IL-4 and IL-13 through Stat6 and ERK [142–144]. Multiple studies have shown that IL-17A activates NFκB signaling to increase RhoA expression and activity [145–149]. Regulation upstream of RhoA activation by IL-17A involves Arhgef12, a RhoA guanine nucleotide exchange factor (RhoGEF) identified to mediate AHR in mice treated with IL-17A or challenged with house dust mite (HDM) [150]. RhoA activity and ROCK activation are increased by IL-4, IL-13, TNFα, IL-17A, and TGFβ [142, 143, 145, 149, 151]. ROCK phosphorylates MLCP to inhibit its phosphatase activity, permitting continued MLC phosphorylation and ASM contraction [152, 153]. Treatment with TGFβ increases ASM cell stiffness and enhances $[Ca^{2+}]_i$ responses upon stimulation with carbochol [140]. These hypercontractile effects on ASM are mediated by TGFβR1/Smad3 signaling, which regulates RhoA, ROCK, and phosphorylated MLC [140]. We recently reported that protein phosphatase 2A (PP2A) activation inhibits IL-13 and histamine-induced ASM contraction by reducing MLC phosphorylation at its Thr18/Ser19 residues [154].

Another small Rho GTPase, Rac1, has also been recently shown to be enhanced with pro-inflammatory cytokines and promote AHR [155]. Rac1 regulates phospholipase β2 activity to promote IP3 production and Ca^{2+} release from the SR [155]. A pharmacological inhibitor targeting Rac1 or its guanine exchange factors, NSC23766 and EHT1864, inhibits rat tracheal ring contraction and AHR in HDM-challenged mice [155–157]. The importance for smooth muscle Rac1 in ASM hypercontractility is demonstrated in smooth muscle specific Rac1 knockout mice, who exhibit reduced AHR when challenged with ovalbumin or HDM [155].

5 | Airway Smooth Muscle Thickening and Remodeling

Airflow obstruction in asthma is also influenced by increased airway wall thickness or airway remodeling, an irreversible process with no current treatments [158]. Although asthma has distinct inflammatory pathways, airway thickening and remodeling are common pathology among type 2 and non-type 2 asthma endotypes [1]. Increased ASM layer thickening is associated with worsened lung function and more frequent exacerbations [4, 159]. Thickening within the ASM layer is thought to involve multiple cellular processes including proliferation, migration, and modulation of the ECM surrounding the airway (Figure 3).

5.1 | Proliferation

ASM proliferation is thought to be a key mechanism that contributes to increased ASM mass. Using microscopic polarimetry and optical dissection, ASM hyperplasia is shown to be present in both large and small airways in fatal and non-fatal asthma as compared to non-asthmatic airways [160, 161]. Conversely, some studies have found no differences in proliferating cell nuclear antigen (PCNA) + ASM proliferation patterns in control, nonfatal, and fatal asthmatic airways [162]. Notably, PCNA+ ASM cell numbers in the airway were independent of airway thickness, eosinophil and neutrophil counts, suggesting that ASM proliferation is uncoupled from inflammation [162]. Despite these discrepancies, ASM proliferation continues to be thought of as a key mechanism in ASM layer thickening.

Studies have identified several different growth factors which induce ASM proliferation including platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), insulin-like growth factor, and TGFβ

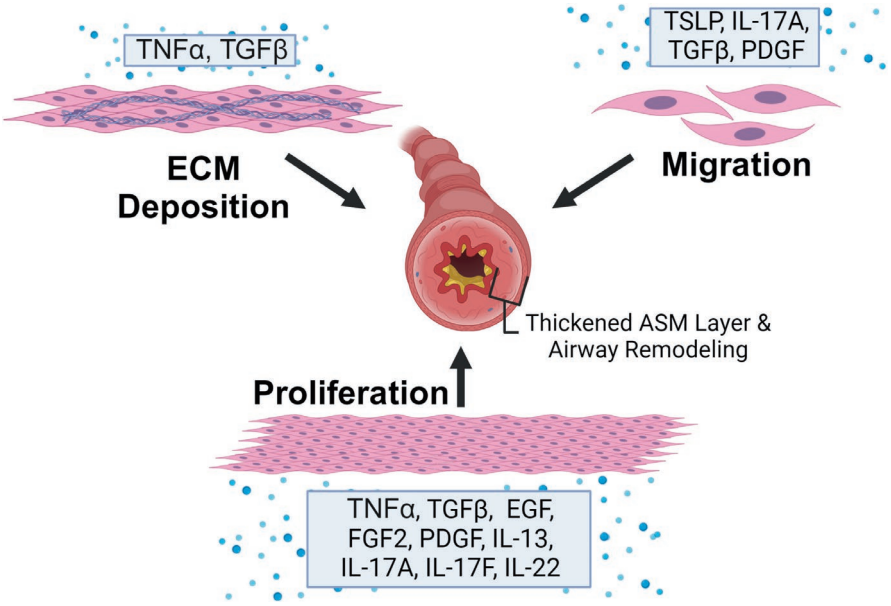

FIGURE 3 | Pro-inflammatory cytokines and growth factors induce airway smooth muscle remodeling. Tumor necrosis factor alpha (TNFα) and transforming growth factor beta (TGFβ) are linked to inducing extracellular matrix (ECM) deposition. Thymic stromal lymphopoietin (TSLP), interleukin (IL)-17A, and platelet-derived growth factor (PDGF) can promote ASM migration. Epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), PDGF, TGFβ, TNFα, IL-17A, and IL-22 have been shown to induce ASM proliferation. Figure was created using BioRender.com on August 31, 2024.

[3, 163–166]. Proliferation can be mediated by different mechanisms mediated by receptor tyrosine kinase, non-receptor tyrosine kinase, and GPCR signaling [167, 168]. These pathways can activate Ras, ERK, and PI3K/Akt pathways that promote cell cycle activity and mitosis [169, 170]. Cell cycle analysis by flow cytometry showed that mitogen exposure enhanced the number of cells in S-phase more profoundly in asthmatic ASM than non-asthmatic human ASM although both cell types were positive for proliferation markers, PCNA and Ki67 [169, 170].

In addition to growth factors, pro-inflammatory cytokines that are increased can also induce ASM proliferation. TNF-α and IL-13 increase ASM cell proliferation after 24 h treatment in both asthmatic and non-asthmatic patients compared to vehicle controls. Interestingly, the effect was more pronounced in asthmatic ASM cells [170]. Although, the effects of pro-inflammatory cytokines on ASM proliferation is albeit moderate and comparatively lower than the growth factor effect (such as PDGF) [170]. Th17-associated cytokines such as IL-17A, IL-17F, and IL-22 also promote ASM proliferation and reduce the apoptotic rate of non-asthmatic and asthmatic human ASM cells through ERK and NFκB pathways [171].

5.2 | Migration

Cell migration is a multi-step process involving cell polarization, protrusion, adhesion, contraction, and retraction [172]. For ASM, changes to actin filament dynamics and organization leads to formation of projections at the leading edge and re-arrangement around airways. Increased ASM migration towards the epithelium has been proposed to contribute to airway wall thickening [172–174]. In our recent study using migration and transwell assays, we found that ASM cells exposed to PDGF or TGFβ significantly enhances their migration [175, 176]. This involved enhanced filopodial formation and actin polymerization characterized by increased F/G actin ratio, Arp2/3, and N-Wasp expression in PDGF-exposed ASM cells through the PKA/CREB pathway [175, 176].

Pro-inflammatory cytokines in asthma have also been shown to mediate ASM migration [172]. In type 2 inflammation, TSLP promotes ASM migration in the Boyden chamber assay through increases in actin polymerization and Rac1 GTPase activity [177]. Studies have shown that IL-17A induction of CXCL2 and CXCL3 secretion promotes ASM migration in a chamber assay through chemokine receptors, CXCR1 and CXCR2 [86, 178]. Using pharmacological inhibitors, these effects were shown to involve ERK and p38, which regulate CXCL1, CXCL2, and CXCL3 production [86, 178]. Interestingly, CXCL2- and CXCL3-induced asthmatic ASM migration was mediated by CXCR1-PI3K signaling, suggesting that asthmatic ASM has distinct migration mechanisms [178]. These findings suggest that pro-inflammatory cytokines and chemokines related to type 2 and type 17 inflammation promote airway remodeling by enhancing ASM migration.

5.3 | Extracellular Matrix Protein Deposition

Apart from proliferation and migration, ECM production and deposition play a substantial role in the progression of airway remodeling [179, 180]. ECM in proximity to ASM is more structurally dense and distinct from ECM located outside the airway compartment [181, 182], which highlights direct contribution of ASM-related ECM in regulating airway remodeling. Inflammatory cytokines contribute to changes to ECM composition around the airway and adjacent reticular basement

membrane regions [183, 184]. Asthmatic airways have increased expression of ECM proteins, most notably collagen, fibronectin, and laminins, that are highly disorganized [183, 185, 186]. These pathological changes lead to airway stiffening and influence ASM function [187].

ECM protein production by ASM contributes to airway remodeling in asthma [183]. TGFβ is also widely known to promote collagens and fibronectin in human ASM, which involves the β-catenin/TCF/LEF pathway that promotes transcription of ECM-related genes [188]. TNFα also enhances ECM production and deposition in ASM cells isolated from asthmatic and control individuals [189, 190]. We observed increased levels of collagen I, III, and fibronectin with TNFα exposure in non-asthmatic and asthmatic ASM cells. Subsequently, the effect was more pronounced in asthmatic ASM cells [189]. Furthermore, the expression ratio of ECM regulatory proteins, MMP-2 and MMP-9, and their counter regulatory proteins tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2, are increased after TNFα exposure [189]. In addition, luciferase activity confirmed TNFα effects on MMP and TIMP expression are through NFκB and activator protein-1 (AP-1) activation in both non-asthmatic and asthmatic ASM cells [189]. Several studies further unveiled the mechanisms of TNFα-inducing cytokine-mediated ECM deposition in ASM cells and phosphorylation of ERK, JNK, RhoA, ROCK1/2, and p38 are predominantly suggested mechanisms/pathways through which TNFα regulates ECM deposition in human ASM cells [191].

Integrins are cell surface receptors that mediate cell-ECM interactions. ASM cells express different integrin subtypes, such as α2β1, α5β1, αvβ5, and α7β, which contribute to regulate ECM composition and TGFβ activation [180, 192–194]. A recent study by Ngo U et al., showed IL-13 and IL-17A enhance ASM adhesion to the ECM by activating β1 integrin [147]. They also showed that β1 integrin activation was more robust in ASM from asthmatic patients than controls, indicating the deregulated expression of β1 integrins in disease conditions. Furthermore, mechanisms of IL-13- and IL-17A-mediated β1 integrin activation in ASM involved NF-κB-induced RhoA/Rho kinase and phosphatidylinositol-4-phosphate 5-kinase type-1 gamma (PIP5K1γ)-induced synthesis of phosphatidylinositol 4,5-bisphosphate (PIP2) [147]. Collectively, the researchers identified a novel mechanism by which type 2 and type 17 cytokines mediate β1 integrin activation in ASM cells.

6 | Inflammatory Pathways and Airway Smooth Muscle Transcriptional Programs

Inflammatory pathways involved in asthma phenotypes and endotypes have a profound impact on ASM function and airway pathology. In recent years, the implementation of microarray and RNA-sequencing on human ASM cells and tissues have revealed greater insight into the molecular and cellular changes ASM may undergo in asthma (Table 1). These studies have identified differences in gene expression of untreated asthmatic versus non-asthmatic ASM. The impact of pro-inflammatory cytokines within the asthmatic inflammatory milieu on ASM gene expression is captured by microarray and RNA-seq studies, demonstrating a differential impact on pathways related to ASM dysfunction in asthma (Table 2).

6.1 | Messenger RNA Expression

RNA-seq studies in untreated asthmatic human ASM show evidence for altered gene expression compared to non-asthmatic human ASM. Transcriptome profiling using the cap analysis of gene expression (CAGE), which specifically measures 5′-capped messenger RNA (mRNA), showed 615 differentially expressed genes ($p < 0.005$) in mild–moderate asthmatic human ASM compared to non-asthmatic ASM (GSE63744) [195]. Applying an OncoFinder algorithm for pathway enrichment analysis, asthmatic human ASM were found to have enrichment for several pathways relevant to cell proliferation (Akt, Ras), pro-inflammatory cytokines and growth factors (TNF, TGFβ, IL-10, VEGF, and IL-6), and Ca^{2+} regulation and contractility (cAMP, CREB, and IP3) [195]. A similar study using RNA-seq by Banerjee et al., identified differential gene expression (adjusted p-value < 0.1) in human ASM from individuals with a history of asthma compared non-asthmatic individuals (GSE119578) [196]. Co-expression network analyses on differentially expressed genes identified enriched networks in asthmatic ASM which included TGFβ signaling, hippo signaling, adherens and gap junctions, and ferroptosis. Several upstream transcription factors were predicted to be key regulators including SMADs, zinc finger proteins, and homeobox transcription factors [196]. These studies suggest transcriptional differences in non-asthmatic and asthmatic ASM. Yet it is unclear whether the observed changes in cultured human asthmatic ASM are comparable to airway tissues.

One study utilizing laser capture microdissection and subsequent RNA-seq analysis on the ASM layer from atopic non-asthmatic and asthmatic lung biopsy samples identified 174 differentially expressed genes between atopic asthma and atopic controls (log2 fold change $|>1|$, p-value <0.05) (GSE38003) [197]. Pathway enrichment analysis using Ingenuity Pathway Analysis (IPA) identified that asthmatic samples had enrichment for carbohydrate metabolism and cell–cell signaling [197]. Notably, changes in *RPTOR*, *FAM129A*, *VANGL1*, and *LEPREL1* expression were significantly associated with AHR [197]. *RPTOR* is involved in the mTOR pathway, which has been shown to promote AHR in mice [208]. *FAM129A* (also known as niban apoptosis regulator 1, *NIBAN1*) regulates p53 and apoptosis has been implicated in lung function and corticosteroid sensitivity in asthma [209, 210]. However, the role(s) for *VANGL1* (VANGL Planar Cell Polarity Protein 1) and *LEPREL1* (prolyl 3-hydroxylase-2, *P3H2*) in ASM and asthma are less clear.

In a study among the first to show correlations between collagen gene expression and deposition in asthma, a multiplex NanoString analysis (334 gene panel, 12 housekeeping genes) was performed on airways from 12 asthmatic and 12 non-asthmatic subjects. Asthmatic airways had greater basement membrane thickness than non-asthmatic airways [198]. Among the 334 gene panel, 51 genes (p-value <0.05) were identified to be significantly different in asthmatic airways. Genes related to airway remodeling (*COL1A1*, *COL3A1*, *ITGB6*) were

TABLE 1 | Transcriptomic studies in asthmatic vs. non-asthmatic airway smooth muscle. Microarray and RNA-seq studies show pro-inflammatory pathways that promote inflammation, hypercontractility, and remodeling are enriched in asthmatic airway smooth muscle.

Tissue source	Treatment(s)	Sequencing platform/ method	Key findings	Dataset(s) source	References
Human airway smooth muscle cells	Untreated non-asthma and asthma ASM	Cap Analysis of Gene Expression (CAGE)-seq, 5′-capped mRNA	Increased proliferation, inflammatory response, smooth muscle contraction Akt, SMAD, Ras, MAPK, and JAK/Stat signaling pathways; decreased cell death pathways	GSE63744	[195]
Human airway smooth muscle cells	Untreated non-asthma and asthma ASM	RNA-seq, Illumina HiSeq2000	Increased Hippo and TGF-β signaling, adherens junctions, gap junctions, ferroptosis, lncRNA *KCNQ10T1*	GSE119578	[196]
Human bronchial biopsies	Nonatopic/atopic non-asthma and nonatopic/atopic asthma subjects	RNA-seq, Roche Genome Sequencer FLX (Laser capture microdissection)	Increased carbohydrate metabolism, cell–cell signaling, airway hyperresponsiveness, NFκB, alpha-catenin, cadherins, 14-3-3, and ABL1	GSE38003	[197]
Human airway tissue	Non-asthma and asthma subjects	RNA, NanoString	Increased *COL1A2* and *COL3A1* expression correlated with collagen deposition	Not reported	[198]
Human airway smooth muscle cells	Untreated non-asthma and asthma ASM	Small noncoding RNA-seq, Illumina NextSeq500	Increased PI3K/ Akt, PTEN signaling; Decreased miRNA *let-7*	GSE64744	[199]
Human lung	Non-asthma and asthma subjects	Single-cell RNA-seq, Illumina HiSeq4000	Smooth muscle enriched for *ACTA2*, *MYH11*, *CRISPLD2, SYNPO2*	GSE130148, EGAS00001001755, EGAS00001002649	[200]
Mouse lung	Untreated mice	Single-cell RNA-seq, Illumina HiSeq3000	Distinct gene expression in mouse airway smooth muscle	http://betsholtzlab.org/Publications/SMC/database.html	[201]

TABLE 2 | Transcriptomic studies in cytokine treated airway smooth muscle. Microarray and RNA-seq studies show pro-inflammatory cytokines stimulate transcriptional programs that promote inflammation, hypercontractility, and remodeling in ASM.

Tissue source	Treatment(s)	Sequencing	Key findings	Dataset(s) source	References
Human airway smooth muscle cells	IL-1β, IL-13, and TGFβ treated non-asthma ASM	RNA Microarray, Affymetrix HG-U95Av2 Array	Differential IL-1β, IL-13, TGFβ effects on gene expression	Not reported	[202]
Human airway smooth muscle cells	IL-13, IL-17A, and IL-13/IL-17A treated non-asthma and asthma ASM	RNA array, Illumina HT12v4 array; DNA methylation, Illumina Omni2.5v8v1A or Human Core arrays	Increased hypercontractility genes are associated with asthma GWAS SNPs	GSE146377	[203]
Human airway smooth muscle cells	TNF, IFN, TNF/IFN, and Fluticasone treated non-asthma pediatric ASM	RNA-seq, Illumina HiSeq4000	TNF/IFN promote corticosteroid insensitivity	GSE179354	[19]
Human airway smooth muscle cells	IL-1β treated non-asthma and asthma ASM	RNA Microarray, Affymetrix GeneChip Human Gene 1.0 ST Array	Increased CCL20 expression contributes to mucus hypersecretion	GSE63383	[101]
Human airway smooth muscle cells	IL-17A treated non-asthma and asthma ASM	RNA Microarray, Affymetrix HuGene v1.0 GeneChip	Increased expression of *CXCL1*, *IL6*, *NFKBIA*, *NFKBIZ*, *MAP3K8*	GSE35643	[204]
Human airway smooth muscle cells	IL-17A and Dexamethasone treated non-asthma and asthma ASM	RNA-seq, Illumina HiSeq	IL-17A and Dex synergize to increase CSF3	GSE135730	[205]
Human airway smooth muscle cells	IL-13 and TGFβ treated non-asthma ASM	RNA-seq, Illumina NovaSeq	IL-13 and TGFβ enhance pro-contractile pathways	GSE220972	[206]
Human airway smooth muscle cells	IL-6 treated non-asthma ASM	RNA-seq, Life Techologies ABI 5500XL W DNA sequencer	Increased IL-6 trans-signaling	Not reported	[207]
Human airway tissue	IL-13 and TGFβ treated non-asthma ASM	RNA-seq, Illumina HiSeq 2000	Increased contractile and type 2 cytokine receptors	Not reported	[109]

significantly increased. These expression changes were positively correlated with histological staining showing increased basement membrane thickness (Masson's trichrome) and airway collagen (picrosirius red) in asthmatic airways [198]. Among the genes significantly decreased in expression were *PTGFR*, *LAMC2*, *CD38*, *PRMT5*, and *RAC1*.

6.2 | Noncoding RNA Expression

In recent years, long non-coding RNA (lncRNA) and microRNA (miRNA) have been identified to have key roles in pro-inflammatory responses in ASM and asthma pathogenesis [211]. LncRNAs and miRNAs regulate gene expression providing additional regulation of cellular responses to inflammation [211]. LncRNAs are transcribed from noncoding DNA regions and are >200 nucleotides in length [212]. They have various functions that include regulating chromatin structure, gene transcription, and miRNA activity [212]. MiRNAs are short, 20–22 nucleotide sequences that regulate mRNA levels through binding to the 3′-untranslated mRNA region, in turn targeting the mRNA for degradation [213]. Recent RNA-seq studies on small noncoding RNAs and miRNAs identified 32 differentially expressed (fold change |> 1.3|, p-value <0.05) lncRNAs and miRNAs in asthmatic human ASM compared to non-asthmatic ASM (GSE64744) [199]. Asthmatic ASM had significant changes in small noncoding RNAs that regulate PI3K/Akt and phosphatase and tensin homolog (PTEN) signaling pathways, which are important in promoting ASM proliferation [199]. Additionally, there was notable downregulation in let-7 expression, which targets pro-inflammatory genes including *IL6*, *IL13*, and *IL10*. This suggests that reduction in let-7 expression leads to elevations in pro-inflammatory signaling in asthmatic ASM. In our recent studies, we identified 15 lncRNAs to be differentially expressed in asthmatic ASM (adjusted p-value <0.1) (GSE119578) [196], which included *KCNQ10T1*, a lncRNA recently implicated in airway remodeling in pediatric asthma [214]. These findings suggest that there are overall transcriptional changes, innate to asthmatic ASM which distinguish their expression profiles from that of non-asthmatic ASM despite the absence of cytokine treatment.

6.3 | ASM Transcriptional Programs in Type 2 Inflammation

While the impact of type 2 inflammatory cytokines on ASM function is well established, less is known about the transcriptional programs induced by these effector cytokines. Among the initial microarray studies, non-asthmatic human ASM showed IL-13 exposure for 4 and 24 h increased a limited number of genes including chemokines CCL11 and CCL2 (fold change |> 2|) [202]. In human ASM cells cultured in a neutralized collagen microtissue, exposure to IL-13 for 7 days significantly changed the expression of 138 genes (log2 fold change |> 1.5|, p-value <0.05) (GSE220972) [206]. Among the top genes increased were *CCL26*, *CCL11*, *PTGDR2*, *IL13RA2*, and *POSTN*, which have established roles in type 2 inflammation [206]. In a separate study by Thompson et al., IL-13 treatment significantly changed expression in 4105 genes in asthmatic and non-asthmatic human ASM (FDR 1%), with *CCL26*, *SOCS1*, *HRH1* (histamine receptor H1), *IL17R1*, and *CCL11* having the greatest increases. Among these differentially expressed genes, 290 were identified to be in the asthma genome-wide association study (GWAS) catalog, suggesting that IL-13 modulates expression of genes that are strongly associated with asthma (GSE146377) [203]. In human airway tissue, IL-13 treatment was shown to increase expression of genes that mediate bronchoconstriction, most notably *HRH1*, *CYSLTR1* (cysteinyl leukotriene receptor 1), and *CHRM3* (muscarinic M3 receptor). Genes encoding receptors for IL-4 (*IL4R*), IL-13 (*IL4R, IL13R1*, and *IL13R1*), and IL-17A (*IL17RA* and *IL17RC*) were also found to be significantly increased [109]. These studies suggest IL-13 induces genes that regulate hypercontractility and eosinophil recruitment.

6.4 | ASM Transcriptional Programs in Type 1 Inflammation

TNFα and IFNγ are among type 1 cytokines most implicated in severe asthma [215, 216]. Using RNA-seq, we identified that TNFα and IFNγ induce distinct gene expression profiles in non-asthmatic human ASM. TNFα significantly increased the expression of 680 genes that promoted ASM inflammation, hypercontractility, and airway remodeling, while 396 genes were significantly decreased (log2 fold change |> 1.5|, FDR <0.05, GSE179354) [19]. For IFNγ, 419 genes were significantly increased, and 124 genes decreased, with pathway enrichment for IFN signaling and host defense genes. In co-expression analysis, we identified separate gene networks enriched for TNFα- and IFNγ-mediated signaling [19]. Within the TNFα network, genes involved in NFκB signaling (*TNFAIP3*, *RELB*, *NFKB2*, and *NFKBIE*) and ASM contractility (*MYH11, CD38*, *BDKRB1*, *ANO9*, and *SLC8A3*) were major network hubs. The IFNγ network included IFN-mediated signaling genes (*ISG15* and *SOCS1*) and cell–cell adhesion (*VCAM1*, *ICAM1*, and *LGALS9*) [19].

IL-1β is an additional type 1 pro-inflammatory cytokine that has been implicated in severe asthma [217]. In a microarray study, non-asthmatic human ASM treated with IL-1β increased several pro-inflammatory cytokines (*CSF3, IL6*), chemokines (*CXCL1*, *CXCL8*), adhesion molecules (*ICAM1*), and signaling pathways (*TNFAIP3*) related to type 1 inflammation (fold change |> 2|) [202]. Using 2 datasets with asthmatic and non-asthmatic human ASM, exposure to IL-1β significantly changed 551 genes (fold change |> 2|, FDR <0.05, GSE63383), 408 up-regulated and 143 down-regulated in dataset A [101]. Meanwhile in the validation dataset B, IL-1β significantly changed 475 genes with 377 up-regulated and 98 down-regulated [101]. Pathway enrichment analysis revealed changes in genes related to IL-1R, NFκB, and IFN signaling pathways. Pro-inflammatory chemokines, *CXCL1*, *CXCL2*, *CXCL8*, *CXCL10*, and *CCL20* were among the genes with the greater increase in expression [101]. Increased CCL20 secretion by IL-1β was subsequently validated and implicated with induction of *MUC5AC* expression and mucus hypersecretion in airway epithelial cells. These studies suggest IL-1β-activated ASM may contribute to a pathological feature commonly associated with type 2 inflammation.

6.5 | ASM Transcriptional Programs in Type 17 Inflammation

The pro-inflammatory effects of IL-17A on ASM is also reflected in changes in gene expression. Microarray analysis observed that non-asthmatic and asthmatic ASM exposed to IL-17A for 2 h significantly changed the expression of 99 genes (fold change $|>1.2|$, p-value <0.05) (GSE35643), with notable changes in NFκB and MAPK pathway genes (*NFKBIA*, *NFKBIZ2, and MAP3K8*), pro-inflammatory chemokines (*CXCL1*, *CXCL2, and CXCL10*), and *IL6* [204]. Similarly, Ouyang et al. observed that human ASM treated with IL-17A significantly changes the expression of genes involved in neutrophil recruitment, maturation, and activation (*CSF2*, *CXCL1*, *CXCL2*, *CXCL3*, *CXCL8*, and *IL6*), and NFκB and MAPK signaling (*NFKBIA* and *NFKBID*) (fold change $|>2|$, FDR <0.05, GSE135730) [205].

IL-6 expression is increased in asthma, particularly in the context of metabolic syndrome and obesity in severe asthma and has been associated with diminished airway function [218, 219]. Along with IL-21, IL-22, IL-1β, and TGFβ, IL-6 serves as a key stimulatory factor in Th17 differentiation and effector function due to STAT3 activation in naïve CD4+ T cells [220, 221]. IL-6-mediated signaling is complex and involves IL-6 receptor (IL-6R) and its co-receptor, glycoprotein 130 (gp130). IL-6 classical-signaling is induced by IL-6 binding to the IL-6R-gp130 complex, while IL-6 trans-signaling is stimulated by the IL-6/soluble IL-6R complex only binding and signaling through gp130 [222]. Both signaling pathways lead to JAK/STAT3 phosphorylation and activation, while only IL-6 trans-signaling increased human ASM proliferation [207].

In RNA-seq studies, human ASM favored IL-6 trans-signaling was observed in non-asthmatic human ASM treated with the IL-6/soluble IL-6R complex. Only 17 genes were significantly increased (log2 fold change $|>0.5|$, FDR <0.05) by IL-6 classical-signaling (IL-6 only) [207]. Conversely, IL-6 trans-signaling significantly changed 205 genes including 38 increasing by >2-fold [207]. Notable genes and enriched pathways were those related to IL-6 trans-signaling were IL-6 signaling (*IL6*, *OSM*, and *SOCS3*), cell proliferation (*IL6*, *NMPT*, *OSMR*, *PLA2G2A*, and *TUBB3*) and type 2 inflammation (*IL13RA1* and *MUC1*). These findings show that ASM may favor IL-6 trans-signaling, which has been implicated in promoting ASM proliferation and airway remodeling [223].

6.6 | ASM Transcriptional Programs in Paucigranulocytic Inflammation

Asthma endotypes with an absence of eosinophils and neutrophils may involve alternative non-type 2 inflammatory pathways, such as TGFβ signaling [60]. In a microarray study, TGFβ was shown to increase expression of growth factors *CTGF*, *IGF*, and *BMP1* in non-asthmatic human ASM (fold change $|>2|$) [202]. Human ASM cells cultured in a neutralized collagen microtissue and treated with TGFβ for 7 days had 3688 differentially expressed genes (log2 fold change $|>1.5|$, p-value <0.05) (GSE220972) [206]. Among the top genes increased by TGFβ were *HAS1*, *IL11*, *MMP10*, *KANK4*, and *COL10A1*. Pathway analysis revealed enrichment for several pathways relevant to ASM hypercontractility and remodeling including epithelial-mesenchymal transition, myogenesis, ECM-receptor interaction, and inflammatory response [206]. Using a kinase inhibitor screen, protein kinase C, Akt, and mTOR signaling were identified as key pathways in TGFβ-mediated contraction [206]. As stated previously, these pathways are known to be involved in ASM hypercontractility, proliferation, and ECM deposition. While TGFβ signaling may be a key driver in paucigranulocytic asthma, it likely has roles in both type 2 and other non-type 2 endotypes [224, 225].

6.7 | Single-Cell RNA-Seq

Thus far, ASM gene expression in response to pro-inflammatory cytokines are based mostly on findings from cultured primary human ASM cells. Studies utilizing single-cell RNA-seq to study human asthma have identified significant increases in transcriptional programs known to be involved in type 2, type 1, and type 17 inflammation in bronchial epithelial and immune cells [14, 200, 226]. However, due to sampling primarily using sputum and bronchoalveolar lavage (BAL) samples, insight into potential changes in ASM using single-cell (or nuclei) have thus far been limited. Single-cell RNA-seq on human asthmatic lungs identified a gene signature that distinguishes smooth muscle cells from other lung cell types including epithelial, endothelial, and immune cells [200]. It is important to note that this study did not differentiate airway versus pulmonary vascular smooth muscle cells. Nonetheless, annotated smooth muscle cells showed enrichment for established smooth muscle markers (*ACTA2*, *TAGLN*, and *MYH11*), NADH dehydrogenase 1 alpha subcomplex, 4-like 2 (*NDUFA4L2*), and regulator of G protein signaling (*RGS5*) [200]. While insight into potential differences between asthma and non-asthma was limited in this study, smooth muscle cells did show enrichment for among asthma GWAS genes related to corticosteroid response (*Cysteine-rich secretory protein LCCL domain-containing 2* (*CRISPLD2*)), cyclic GMP signaling (*PRKG1*), and actin cytoskeleton (*ABI3BP*, *SYNPO2*) [200]. Although heterogeneity among human airway and pulmonary vascular smooth muscle in the lung is unknown, single-cell RNA-seq studies in the mouse demonstrate smooth muscle subsets across different tissues and organs [201]. Compared to venous and arterial vascular smooth muscle cells in the lung, mouse ASM had specific expression of *Chrm2*, *Foxf1*, *Gja1*, and *Cldn1* [201]. Understanding the transcriptional profiles of ASM at single-cell resolution is just beginning but could be useful in unlocking the mechanisms that lead to AHR and airway remodeling.

7 | Corticosteroid Sensitivity in Airway Smooth Muscle

Corticosteroid sensitivity is a key determinant of asthma severity and moderate–severe asthma is characterized by persistent airway inflammation despite being on high dose or prolonged corticosteroid therapy [227]. Corticosteroids inhibit inflammation through glucocorticoid receptor (GR) activation [228]. Upon ligand binding, GR translocates from the cytosol to the nucleus and binds to DNA at glucocorticoid response element (GRE) motifs [228]. Pro-inflammatory cytokines impact

corticosteroid sensitivity in ASM by altering GR expression, phosphorylation, nuclear translocation, and DNA binding activity [229–232]. ASM is one of the major structural cells in the lung being targeted by corticosteroids. Consequently, it is important to understand how corticosteroids affect ASM structure and function while also unraveling the mechanisms that affect corticosteroid sensitivity in ASM. Microarray and RNA-seq studies have expanded the understanding for how corticosteroids can inhibit inflammation in ASM and potential mechanisms by which pro-inflammatory cytokines promote corticosteroid insensitivity (Table 3).

7.1 | Corticosteroid-Induced Gene Expression

Recent studies have provided novel insight into understanding the effects of corticosteroids on ASM and how these responses may be altered in asthma. In pediatric non-asthmatic human ASM, we reported that treatment with fluticasone propionate alone for 18 h changed the expression of 506 genes (log2 fold change >|1.5|, FDR <0.05) (GSE179354) [19]. Our pathway enrichment analysis identified changes to several different genes related to inflammation and metabolism. Among the significantly increased anti-inflammatory genes with established roles in inhibiting pro-inflammatory signaling were *DUSP1*, *FKBP5*, *KLF15*, *PER1*, *TSC22D3* [19]. We also found repression of several pro-inflammatory genes including *IL6*, *MMP1*, *PTGS2*, and *TNFSF15* [19]. In adult human ASM, exposure to budesonide for 24 h significantly changed 7835 genes in non-asthmatic ASM and 6957 genes in fatal asthmatic ASM (q-value <0.05) (GSE94335) [234]. Among the top differentially expressed genes (log2 fold change |>3|, q-value <0.05) in non-asthmatic and fatal asthmatic ASM were established target genes *CRISPLD2*, *FKBP5*, *KLF15*, *DUSP1*, *PER1*, and *TSC22D3* that are part of the corticosteroid-induced gene signature [234]. While this gene signature was comparable between pediatric and adult human ASM, the effects of corticosteroids on ASM are different from other cell type lines [234]. For example, *FKBP5* and *TSC22D3* are increased by corticosteroids in human ASM and other macrophage, epithelial, lymphoblastoid, and acute lymphoblastic leukemia cell lines. However, induction of other anti-inflammatory genes, *KLF15*, *PER1*, and *CRISPLD2* was specific for non-asthmatic and fatal asthmatic human ASM [234]. This suggests the anti-inflammatory effects of corticosteroids on ASM may involve specific genes that are distinct from other cell types [234, 242].

Separate from in vitro studies treating cells with corticosteroids, additional sequencing studies observed the transcriptional signatures of cells obtained from patients treated with corticosteroids. A corticosteroid-induced signature was observed using microarray analysis on bronchial brushings and endobronchial biopsies from subjects treated with inhaled budesonide for 6 h. Budesonide changed 96 genes (log2 fold change |>2|, FDR <0.05), with signature genes including *ALOX15B*, *NFKBIA*, and *ZBTB16* (GSE83233) [236]. Pro-inflammatory genes such as *CXCL10*, *CXCR4*, *LIFR*, and *TNFRSF11B* were significantly reduced with budesonide treatment [236]. Gene changes in ASM have also been observed in individuals receiving systemic corticosteroids. RNA-seq studies on the ASM layer from bronchial biopsies from subjects with atopic asthma before and after treatment with oral prednisolone for 14 days found significant changes in 15 genes (p-value <0.05, GSE40996) [209]. Notably, there was an absence of established genes known to be induced in ASM treated with corticosteroids. However, significant changes in *FAM129A* and *SYNPO2* gene expression were associated with AHR [209]. The differential effects on gene expression in these two studies suggest that ASM responses may differ depending on route of delivery (e.g., inhaled versus systemic corticosteroids). Here, inhaled corticosteroids may induce a more robust anti-inflammatory gene expression response in ASM, as opposed to systemic corticosteroids.

Corticosteroids also affect lncRNA expression in ASM. Studies in human non-asthmatic ASM treated with dexamethasone and fetal calf serum for 24 h found changes in expression of several lncRNAs including those known to be miRNA "sponges" and reduce miRNA expression activity, RP11-46A10.4, LINC00883, and BCYRN1 [243]. In a follow up study, microarray analysis in non-asthmatic, non-severe, and severe asthmatic human ASM observed significant expression differences in 4 lncRNAs between severe asthmatic and non-severe asthmatic ASM (p-value <0.05) [235]. The lncRNA, plasmacytoma variant translocation 1 (PVT1), was increased in severe asthmatic human ASM treated with dexamethasone, TGFβ, and fetal calf serum [235]. In contrast, PVT1 expression was unchanged in dexamethasone treated human ASM isolated from non-asthmatic and non-severe asthmatic individuals. Knockdown studies demonstrated that PVT1 selectively promotes proliferation and IL-6 secretion in dexamethasone, TGFβ, and fetal calf serum treated non-asthmatic human ASM [235]. While the implications for these observations remain unclear, these studies show that corticosteroids can regulate lncRNAs that modulate inflammatory responses in ASM.

7.2 | Corticosteroid Sensitivity in Type 2 Inflammation

Type 2-high inflammation is associated with both corticosteroid-sensitive and -insensitive asthma endotypes [8, 244]. While the underlying mechanisms that lead to corticosteroid insensitivity in type 2-high asthma are not entirely clear, recent studies in a severe asthma cohort suggest that older age may be associated with insensitivity to systemic corticosteroids [245]. In an acute ovalbumin challenged allergen mouse model, low dose systemic corticosteroids are effective in reducing AHR, IL-13 levels, and Th2 cell infiltration [246]. Conversely, chronic allergen models, particularly those with multiple allergens, have persistent increases in type 2 inflammation and AHR despite treatment with corticosteroids at greater doses [246, 247]. These findings imply type 2 inflammation can have differential corticosteroid sensitivity, although it is unclear whether this is due to different immune cell effector functions or inflammatory signals ASM receives.

For ASM, there is limited understanding in how Th2 cytokines affect corticosteroid sensitivity. Dexamethasone reduces basal $[Ca^{2+}]_i$ levels and SOCE responses in human ASM exposed to IL-13, suggesting corticosteroids can inhibit Ca^{2+} responses augmented by IL-13 [129]. Contractile force in IL-13 treated human airways is also inhibited by dexamethasone [109]. However,

TABLE 3 | Transcriptomic studies in corticosteroid treated airway smooth muscle. Microarray and RNA-seq studies show changes in gene expression in ASM treated with corticosteroids and pro-inflammatory cytokines.

Tissue source	Treatment(s)	Sequencing	Key findings	Dataset(s) source	References
Human airway smooth muscle cells	Dexamethasone treated non-asthma ASM	RNA-seq, Illumina HiSeq 2000	Dex increases an anti-inflammatory gene, *CRISPLD2*	GSE52778	[233]
Human airway smooth muscle cells	Budesonide treated non-asthma and fatal Asthma ASM	RNA-seq, Illumina HiSeq 2500	Corticosteroid-induced gene signature in human ASM	GSE94335	[234]
Human airway smooth muscle cells	TGFβ and Dexamethasone treated non-asthma, non-severe, and severe asthma ASM	RNA and lncRNA Microarray, Agilent Technologies SurePrint G3 Human GE microarrays	TGFβ Ca^{2+} signaling, airway remodeling, glucocorticoid activity, and reduces lncRNA *PVT1*	Not reported	[235]
Human blood, bronchial brushings, and endobronchial biopsies	Inhaled budesonide treated subjects	RNA Microarray, Affymetrix PrimeView	Increased corticosteroid gene signature that includes *ZBTB16*	GSE83233	[236]
Human endobronchial biopsies	Oral prednisolone atopic asthma subjects	RNA (laser capture microdissection)	Increased *FAM129A* and *SYNPO2* expression is correlated with AHR	GSE40996	[209]
Human airway smooth muscle cells	TGFβ, KLF15 overexpression treated non-asthma ASM	RNA-seq GR and RNA Pol II ChIP-seq	KLF15 inhibits TGFβ-induced hypertrophy	GSE95397 (RNA-seq) GSE95632 (ChIP-seq)	[237]
Human airway smooth muscle cells	Budesonide $G_{\alpha s}$ siRNA knockdown	RNA-seq, Illumina HiSeq 4000	$G_{\alpha s}$-cAMP contributes GR genomic effects	GSE130715	[238]
Human airway smooth muscle cells	EGF, IL-1β, fluticasone, PKA inhibitor treated non-asthma ASM	RNA microarray	PKA and GR regulate pro-mitogenic pathways	GSE13168	[239]
Human airway smooth muscle cells	Dexamethasone treated non-asthma ASM	RNA Microarray, Agilent-014850 Whole Human Genome Microarray	Increased *KLF15* expression	GSE34313	[240]
Mouse lung	Dexamethasone treated wild type and $KLF15^{-/-}$ mice	RNA microarray, Agilent 4x44K ink-jet arrays	KLF15 regulates ~7% corticosteroid-induced gene expression	GSE44695	[241]

IL-13-induced STAT6 phosphorylation is unaffected by dexamethasone [109], suggesting that IL-13-mediated signaling may not be fully inhibited by corticosteroids.

Interestingly, IL-13 has been implicated in regulating endogenous glucocorticoid metabolism by increasing 11β-hydroxysteroid dehydrogenase (11β-HSD) expression and enzymatic activity to boost conversion of cortisone to cortisol levels in the lung [248]. Increased 11β-HSD activity in human airways treated with IL-13 reduces hypercontractility and improves airway relaxation [249]. IL-13 was also shown to augment GR phosphorylation and DNA binding activity in human ASM [250]. Thus, IL-13 may play a role in local glucocorticoid metabolism and affect corticosteroid sensitivity. The effect of other type 2-associated cytokines, such as IL-4 and TSLP, on glucocorticoid metabolism in the airway and corticosteroid sensitivity remains to be investigated.

7.3 | Corticosteroid Sensitivity in Type 1 Inflammation

Cytokines associated with type 1 inflammation (TNFα, IFNγ, and IL-1β) are associated with corticosteroid insensitivity in severe asthma. For inflammatory mediator production, fluticasone propionate or dexamethasone exposure inhibits TNFα-induced CCL5, CXCL8, and CXCL10 production in human ASM [18, 84, 251]. Similarly, another corticosteroid, ciclesonide, inhibits CCL2 production in human ASM treated with TNFα or IL-1β [90]. These studies show that the individual effects of TNFα or IL-1β on chemokine production in human ASM are sensitive to corticosteroids.

Among the asthma endotypes with type 1 inflammation often involves increases in IFNγ-producing Th cells and IFNγ levels [215]. Combined exposure to TNFα and IFNγ (TNFα/IFNγ) is an established model to induce corticosteroid insensitivity in human ASM [18, 230]. Studies aimed to understand mechanisms of corticosteroid insensitivity in this in vitro model have identified multiple underlying mechanisms including increased expression of the GRβ (dominant negative isoform) and reducing GR phosphorylation at Ser211 which is important for GR DNA binding activity [230, 252]. In regard to inflammatory signaling pathways, TNFα augments IFNγ-induced expression and activity of interferon response factor 1 (IRF1) which persists in human ASM treated with fluticasone propionate [253]. We showed that combined exposure to TNFα and IFNγ substantially increases NFκB p65 expression and Stat1 phosphorylation [18]. The importance of IRF1 and Stat1 in TNFα/IFNγ-induced effects is highlighted by siRNA studies demonstrating significantly reduced CD38 and CCL5 expression in ASM cells transfected with IRF1 and STAT1 siRNA, respectively.

RNA-seq analysis showed that TNFα/IFNγ has a synergistic effect on non-asthmatic human ASM gene expression by significantly increasing 1951 genes and decreasing 2116 genes (log2 fold change $|>1.5|$, FDR <0.05) (GSE179354) [19]. These changes in gene expression were largely unaffected by the addition of fluticasone propionate. In TNFα/IFNγ exposed ASM, we observed enrichment for several notable pathways including cytokine-cytokine receptor, IFN, MAPK, PI3K-Akt, and calcium signaling pathways [19]. These notable findings implicate TNFα/IFNγ in inducing corticosteroid insensitivity by promoting persistent pro-inflammatory signaling, immune cell recruitment and interactions, heightened Ca^{2+} responses and hypercontractility.

Interestingly, our analysis showed that despite substantial corticosteroid insensitivity by TNFα/IFNγ treatment, the corticosteroid-induced gene expression profile was largely unchanged. This would suggest that GR activity is maintained at some level in this in vitro model. Expression of notable GR target genes (*FKBP5*, *PER1*, and *ZBTB16*) and key anti-inflammatory genes (*DUSP1*, *NFKBIA*, and *TNFAIP3*) remained increased in human ASM treated with TNFα/IFNγ and fluticasone propionate [18, 19]. These observations lead us to speculate that synergy among distinct pro-inflammatory pathways can promote corticosteroid insensitivity. Here, interactions between TNFα- and IFNγ-mediated signaling and their synergistic pro-inflammatory effects may provide an example for how type 1 inflammation mediates corticosteroid insensitivity in ASM.

7.4 | Corticosteroid Sensitivity in Type 17 Inflammation

The type 17 inflammatory pathway in asthma is also associated with corticosteroid insensitivity, which may involve limited corticosteroid sensitivity in ASM exposed to IL-17A. RNA-seq in human ASM studies show that IL-17A-induced gene expression of pro-inflammatory cytokines and chemokines involved in neutrophil recruitment, maturation, and activation (*CSF2*, *CXCL1*, *CXCL2*, *CXCL3*, *CXCL8*, and *IL6*) was sensitive to dexamethasone (log fold change $|>2|$, FDR <0.05, GSE135730) [205]. Interestingly, several inflammatory genes induced by IL-17A were insensitive to corticosteroids. Most notably, colony stimulating factor (CSF3), a cytokine that promotes neutrophil maturation and activation, was synergistically increased in ASM treated with both IL-17A and dexamethasone [205]. GR binding sites adjacent to NFκB binding motifs were identified within the human CSF3 gene promoter region and found to regulate CSF3 mRNA levels [205]. In an HDM and complete Freund's adjuvant (HDM/CFA) mouse model, neutralization of CSF3 with a monoclonal antibody significantly reduced BAL immune cell infiltration and improved corticosteroid sensitivity [205]. These findings provide an example for how corticosteroids can interact with pro-inflammatory cytokines to further promote inflammation. This may be an example of the limitations corticosteroids can have, particularly within the context of severe asthma where neutrophil infiltration is known to promote corticosteroid insensitivity in severe asthma.

While IL-6 is associated with severe asthma [218], in vitro studies show that IL-6 expression in ASM is sensitive to corticosteroids. Studies by Che et al., reported that sphingosine 1-phosphate (S1P)-induced IL-6 secretion is inhibited by dexamethasone through mitogen-activated protein kinase phosphatase-1 (MKP-1), a negative regulator of MAPK signaling and known anti-inflammatory GR target gene [103, 254]. Dexamethasone rapidly induces MKP-1 expression to inhibit p38 activity and increase tristetraprolin (TTP) activity to blunt IL-6 mRNA stability and protein synthesis [255, 256]. While

corticosteroids can regulate IL-6 production, it remains unclear whether corticosteroids can inhibit the pro-remodeling effects of IL-6 trans-signaling on ASM.

7.5 | Corticosteroid Sensitivity in Paucigranulocytic Inflammation

With TGFβ implicated in paucigranulocytic inflammation and severe asthma [257], it is important to understand how TGFβ affects corticosteroid sensitivity in ASM. While TGFβ is known to promote ASM hypercontractility, hypertrophy, and ECM protein production, the impact of corticosteroids on these effects is not well understood. Treatment with fluticasone propionate and dexamethasone can inhibit TGFβ-induced αSMA expression, MLCK activation, and actin filament formation in non-asthmatic human ASM in vitro [258]. This study showed that corticosteroids increased the αSMA protein turnover rate to inhibit TGFβ-induced contraction [258]. In regard to ECM protein deposition, fibronectin expression remained increased in non-asthmatic and asthmatic human ASM cells treated with TGFβ and budesonide [259]. In a similar study, budesonide failed to inhibit the pro-remodeling effects of endothelin-1 (ET-1) on human ASM contraction and collagen deposition [260]. These findings suggest limitations to corticosteroids in inhibiting mechanisms that promote AHR and airway remodeling, particularly when they are driven by TGFβ or ET-1.

7.6 | Notable Corticosteroid-Induced Anti-inflammatory Mediators

Corticosteroids and GR inhibit inflammation through multiple mechanisms that largely involve genomic regulation [228], although non-genomic mechanisms are also recognized [261]. GR inhibits pro-inflammatory signaling pathways by direct gene repression and promoting the expression of anti-inflammatory mediators [228]. Within this concept, the effects on the pro-inflammatory NFκB and AP-1 signaling pathways are the most widely studied, however, understanding of underlying mechanisms continues to evolve [262]. In human ASM treated with dexamethasone, GR was found to bind to more than 6000 DNA regions using chromatin immunoprecipitation sequencing (ChIP-seq) (FDR <0.05) (GSE95632) [237]. Notable GR binding sites were in close proximity or within known GR target genes (*FKBP5*, *DDIT4*) and those demonstrated to contribute to anti-inflammatory actions by corticosteroids (*CRISPLD2, KLF15, and IRS2*) [237]. Studies have shown the functional roles for anti-inflammatory genes in human ASM, supporting their importance in corticosteroid sensitivity in ASM.

CRISPLD2 was identified as a GR target gene and demonstrated to have anti-inflammatory function in human ASM (FDR <0.05) (GSE52778) [233]. CRISPLD2 mRNA was identified be increased in human ASM stimulated with dexamethasone or the pro-inflammatory cytokine, IL-1β. In CRISPLD2 siRNA knockdown studies, CRISPLD2 was shown to contribute to reductions in IL-1β-induced IL-6 mRNA expression in ASM treated with dexamethasone, suggesting a role in the anti-inflammatory actions of corticosteroids. Genomic analyses identified single nucleotide polymorphisms (SNPs) in the CRISPLD2 gene locus were nominally associated with bronchodilator response, implicating CRISPLD2 in the response to LABA treatment [233].

Among the anti-inflammatory genes induced by corticosteroids are negative regulators of the NFκB pathway, a key pathway in type 1 inflammation. TNFα-induced protein 3 (TNFAIP3), also known as A20, is a deubiquitin ligase that targets polyubiquitinated signaling proteins upstream of NFκB (e.g., TRAF6) and has important roles in regulating lung inflammation in airway diseases [263]. TNFAIP3 gene expression is rapidly induced upon TNF receptor (TNFR)- and TLR-mediated NFκB activation and serves as a critical negative feedback mechanism for the temporal regulation of NFκB [264]. In developing human ASM, TNFAIP3 protein expression is increased with 1 h exposure to TNFα and TNFAIP3 siRNA knockdown augments TNFα-induced CCL5 and CXCL10 expression [18]. Luciferase activity assays and chromatin immunoprecipitation (ChIP) studies in human ASM and human bronchial epithelial cells (BEAS-2B) show increased NFκB and GR binding and activity within the TNFAIP3 intron 2 region in cells treated with TNFα and dexamethasone [265, 266]. Similarly, we observed increased transcriptional activity at NFκB and GR TNFAIP3 intron 2 binding sites in developing human ASM [18]. NFκB and GR cooperativity is thought to be synergistic in enhancing TNFAIP3 expression and a key negative feedback mechanism to NFκB-mediated signaling [262]. Interestingly, LABAs enhance corticosteroid-induced TNFAIP3 expression in BEAS-2B cells [267]. These findings suggest TNFAIP3 has an important role in the anti-inflammatory effects of corticosteroids on NFκB signaling and may be a key mechanism in combined corticosteroid/LABA therapies for asthma.

Kruppel-like factor 15 (KLF15) is a zinc finger transcription factor that regulates gene transcription with 2 GR binding sites [240, 241, 268]. KLF15$^{-/-}$ mice treated with dexamethasone had significant changes to approximately 7% of GR regulated genes in the lung [241], suggesting that KLF15 is important for mediating the effects of corticosteroids. In the context of inflammation, KLF15 overexpression in human ASM inhibits TGFβ-induced F-actin expression and hypertrophy. Using RNA-seq and ChIP-seq (log2 fold change $|>2|$, adjusted p-value <0.05) (GSE95397, GSE95632), phospholipase C delta 1 (PLCD1) was identified as a KLF15 target with KLF15 occupancy at the PLCD1 promoter [237]. PLCD1 overexpression inhibited TGFβ-induced ASM hypertrophy, however, the underlying mechanism is unknown [237]. PLCD1 has been shown to regulate cofilin expression and actin polymerization in breast cancer cell lines and murine neurons [269, 270], which is key in ASM hypercontractility and hypertrophy. Transcriptional regulation between GR-KLF15 is implicated as an important feed-forward mechanism for GR-mediated suppression of inflammation [271].

7.7 | Corticosteroid and β2-Adrenergic Receptor Agonists Interactions

Combination anti-inflammatory and bronchodilator therapies with inhaled corticosteroid and LABA are used to manage asthma. LABAs relax ASM by binding the β2-adrenergic receptor (β2-AR) to stimulate GPCR $G_{\alpha s}$ signaling, leading to rapid cyclic adenosine monophosphate (cAMP) production and protein kinase

A (PKA) activation [272]. Subsequently, PKA activity promotes relaxation through multiple mechanisms including inhibition of Ca^{2+} regulatory protein and channel activity, and MLCK activation [272]. Despite its proven ability to reduce ASM contractility, LABAs have some limitations that can lead to poor asthma control with development of desensitization [273] and ineffectiveness in severe asthma [274, 275]. Studies have demonstrated that LABAs and corticosteroids can reciprocally enhance β2-AR and GR signaling to augment their respective bronchodilation and anti-inflammatory effects [239, 276, 277]. Combined exposure to corticosteroids and LABA inhibits IL-8 production [84], while also reducing PDGF-induced proliferation and collagen deposition in bovine tracheal smooth muscle strips [278]. Interestingly, these effects were achieved in tracheal tissue treated with relatively low-dose fluticasone propionate (3 pM) and fenoterol, suggesting LABA can improve corticosteroid sensitivity [278]. LABAs, such as formoterol, synergize with budesonide to increase the expression of key anti-inflammatory mediators, TNFAIP3 and MKP-1, in human ASM (Figure 4) [267, 279].

Pro-inflammatory cytokines, such as TGFβ, have been shown to interfere with LABA-induced β2-AR signaling and impair cAMP production. Human ASM exposed to TGFβ lose the ability for a β2-AR agonist, isoproterenol, to reduce cell stiffness and induce relaxation [280]. These effects were attributed to a diminished ability for isoproterenol to stimulate cAMP levels through Smad2/3-mediated increases in phosphodiesterase 4D expression [280]. These findings suggest that TGFβ can promote ASM hypercontractility while also impairing key relaxation mechanisms that enhance bronchodilation in asthma. This effect is reversed in human ASM treated with dexamethasone, which reduces phosphodiesterase 4D expression and enables ample cAMP production for relaxation [281]. Corticosteroids can also acutely stimulate increases in cAMP levels in ASM that involve non-genomic GR-$G_{\alpha s}$ signaling, via membrane-bound GR, which was recently shown to account for approximately 1/3 of the genomic effects of corticosteroids [238, 261]. Using RNA-seq and bioinformatic analyses of human ASM transfected with $G_{\alpha s}$ siRNA, 48 genes were found to be dependent on non-genomic GR signaling, including commonly known GR target genes, *PER1* and *ZBTB16* (GSE130715) [238]. These studies highlight interactions between LABAs and corticosteroids are important for modulating pro-inflammatory and hypercontractile responses in ASM and asthma.

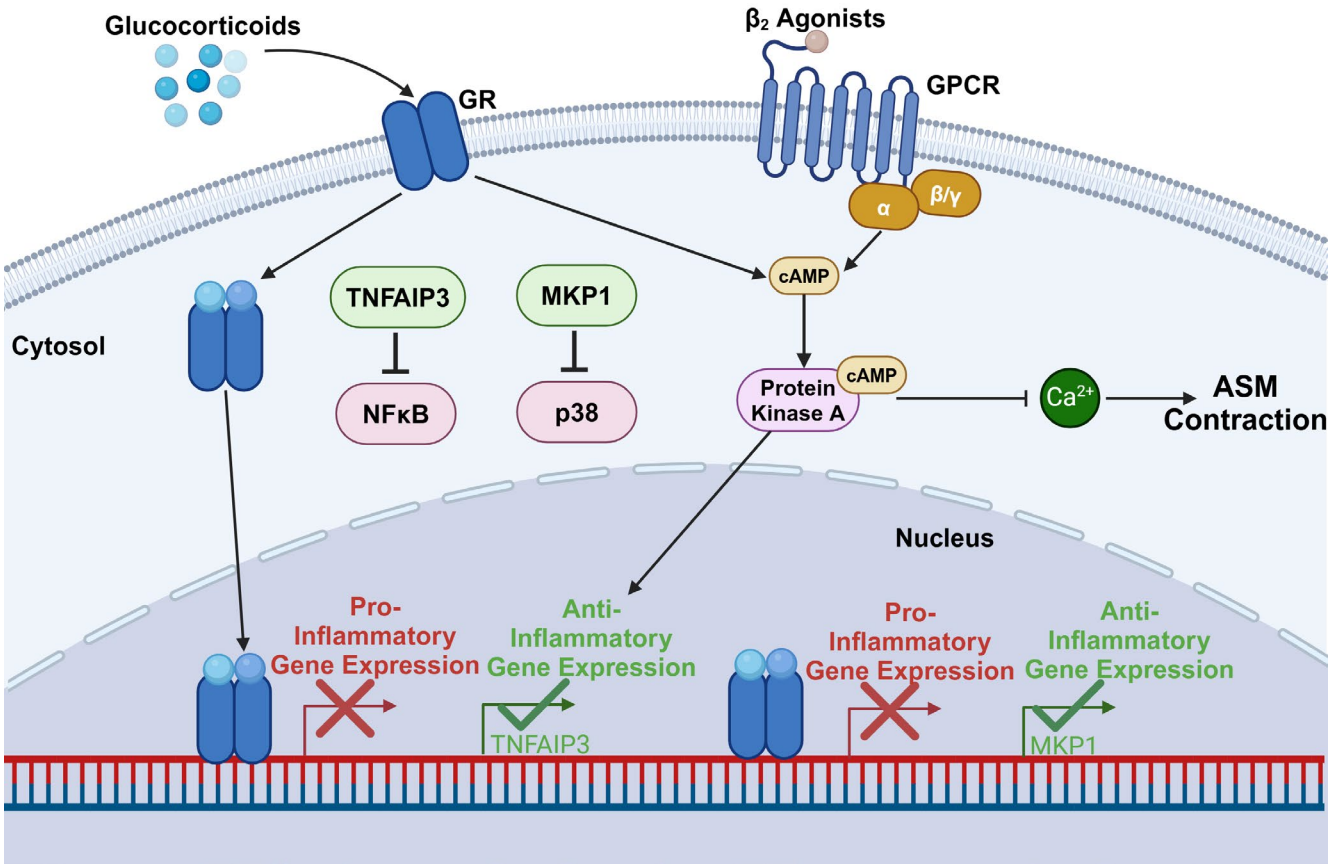

FIGURE 4 | Corticosteroids and long-acting β2-adrenergic receptor agonists synergize to promote anti-inflammatory responses and bronchodilation in airway smooth muscle. Corticosteroids bind to the glucocorticoid receptor (GR) in the cytosol and translocate to the nucleus where it will directly bind DNA and regulate gene expression to inhibit pro-inflammatory responses. Long-acting β2- adrenergic receptor agonists (LABAs) bind the GPCR β2-adrenergic receptor to activate the cyclic adenosine monophosphate (cAMP)-protein kinase A pathway, which promotes ASM relaxation. In non-genomic GR signaling, corticosteroids can also stimulate cAMP production. Combined exposure to corticosteroids and LABAs can enhance the expression of anti-inflammatory genes, such as tumor necrosis factor-induced protein 3 (TNFAIP3) and mitogen-activated protein kinase phosphatase 1 (MKP-1). Figure was created using BioRender.com on August 31, 2024.

8 | Conclusions and Future Directions

In this review, we have summarized the current understanding for how pro-inflammatory cytokines involved in type 2 and non-type 2 asthma endotypes promote inflammatory responses, hypercontractility, and remodeling in ASM. ASM receive inflammatory signals from infiltrating innate and adaptive immune cells and airway structural cells that produce an abundance of cytokines to promote AHR and ASM thickening/remodeling. The emergence of phenotyping and endotyping studies in asthma have identified different immune profiles and suggests heterogeneity among asthma populations [8]. Currently, cellular and molecular differences among asthma endotypes have primarily focused on immune and airway epithelial cells collected from sputum, BAL, nasal and bronchial epithelial brushings [13, 15, 282]. Given that ASM can respond dynamically to multiple pro-inflammatory pathways and changes in mechanical forces in asthma, it is likely that ASM also has heterogeneity. Moving forward this will be an important question to address in future research.

Studies using transcriptomic methods reveal unique transcriptional programs (messenger and noncoding RNAs) related to individual (or combined) pro-inflammatory cytokine exposures and corticosteroid sensitivity. Differences in asthmatic ASM is a consistent finding across microarray and RNA-seq studies comparing gene expression profiles in asthmatic and non-asthmatic human ASM cells and tissues. Although it is likely premature to declare a unique transcriptional program for ASM in asthma, bioinformatic analyses suggests enrichment for genes related to ASM inflammation, hypercontractility, and remodeling. Across these studies multiple pro-inflammatory signaling pathways are consistently implicated in promoting inflammation in asthmatic ASM including JAK/STAT, PI3K/Akt, NFκB, and MAPK signaling. Induction of these pathways in asthmatic ASM are likely dependent upon the cytokine(s) they are exposed to. Here, one would speculate that ASM changes may be distinct based on exposure to type 2 and/or non-type 2 cytokines. However, given the current understanding of asthma endotypes and heterogeneity, there is likely overlap across these pathways. For example, type 2/type 1 and type 2/type 17 endotypes are recognized in children and adults with asthma [40, 215, 283]. Studies show that interactions between type 2/type 1 and type 2/type 17 cytokines can augment inflammatory responses in ASM and diminish corticosteroid sensitivity [215, 283]. Further, other cytokines such as TGFβ, may play an important role in ASM dysfunction regardless of asthma endotype. This is certainly important with therapeutic implications for identifying more specialized therapies that can be effective for heterogenous populations.

Inflammatory, functional, and pathological changes in ASM are managed by corticosteroids and LABAs. Studies in recent years have advanced the understanding in how these important therapies used in combination synergize to alleviate asthma symptoms. While they have different canonical mechanisms (GPCR vs. nuclear receptor signaling), studies in human ASM show that they do interact to enhance and/or maintain cAMP levels and increase important anti-inflammatory mediators that negatively regulate pro-inflammatory signaling pathways. With the emergence of novel biased $G_{\alpha s}$ β2-AR agonists aimed to optimize LABA therapy [284, 285], it will be interesting to know whether emerging LABAs can maintain their synergy with corticosteroids.

Advancing the understanding of how inflammation adversely affects ASM will likely provide opportunities to identify novel mechanisms and improve asthma management and/or disease trajectory. This is particularly important for slowing or reversing airway thickening and remodeling, which remains a hindrance to improving lung function in asthma. Additionally, there have been limited advancements in treatments for corticosteroid-insensitive non-type 2 asthma endotypes. Perhaps impactful progress can be achieved by leveraging cutting-edge technologies in -omics (e.g., RNA, proteins, lipids, and metabolites) platforms, molecular biosensors, computational tools, and bioinformatic analyses that are becoming more established in asthma research. To maximize impact, integration of additional sequencing methods, bioinformatic analysis tools, and pipelines for single-cell RNA and spatial transcriptomic sequencing, assay for transposase-accessible chromatin sequencing (ATAC-seq) for chromatin accessibility, precision run-on sequencing (Pro-seq) for nascent RNA to existing datasets can help further define ASM transcriptional programs in asthma endotypes. In addition, complementing these with mechanistic and functional validation studies in multiple approaches using novel animal and human model systems, including multifunctional spheroids and organoids, is needed for the advancement of the field. For ASM biology and its role in asthma pathogenesis, these methods can be utilized and integrated to address existing knowledge gaps and complex questions related to inflammation and its impact on airway structure and function.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The sequencing data sets from the authors supporting the findings discussed in this article are publicly available in the Gene Expression Omnibus (GEO) under accession numbers GSE119578 and GSE179354 and corresponding references. The data that support the findings of this study are available from the corresponding author upon reasonable request. Availability for additional studies discussed is outlined in Tables 1–3 with accession numbers and corresponding references.

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