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ABSTRACT

Phenotypic changes in airway smooth muscle are integral to the pathophysiological changes that constitute asthma – namely inflammation, airway wall remodelling and *bronchial hyperresponsiveness*. *In vitro* and *in vivo* studies have shown that the proliferative, secretory and contractile functions of airway smooth muscle are dysfunctional in asthma. These functions can be modulated by various mediators whose levels are altered in asthma, derived from inflammatory cells or produced by airway smooth muscle itself. In this review, we describe the emerging roles of the CXC chemokines (GROs, IP-10), Th17-derived cytokines (IL-17, IL-22) and semaphorins, as well as the influence of viral infection on airway smooth muscle function, with a view to identifying new opportunities for therapeutic intervention in asthma.

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1. Overview

Asthma is a complex disease, characterised by inflammation, airway wall remodelling and *airway hyperresponsiveness* (AHR). Although the precise factors underlying either its aetiology, or associated with accelerated disease progression with increasing asthma severity have not been fully defined, the contribution of the altered contractile responses of airway smooth muscle (ASM) that lead to AHR are a key defining feature of asthma. However, ASM has additional potential roles – not only as an effector of airway narrowing, but also as an immunomodulator of inflammation and remodelling [1,2]. It is now apparent that changes in these other ASM functions are also likely to contribute significantly to asthma pathophysiology. ASM can both produce and respond to an array of cytokines, chemokines and growth factors, leading to cell migration and proliferation, production of extracellular matrix (ECM) proteins as well as altered reactivity. This has provided the basis for the

significant body of research which has focussed upon identifying the intrinsic abnormalities which occur in ASM in asthma, as well as the influence of mediators on these diverse ASM functions.

2. Changes in ASM function in asthma

In vitro studies have provided unequivocal evidence that ASM cells derived from asthmatic subjects are intrinsically different, demonstrating an increased rate of proliferation [3] relative to ASM from non-asthmatic subjects, associated with increased mitochondrial biogenesis [4]. ASM from people with asthma has also been shown to produce an altered profile of ECM proteins in culture [5]. This can in turn exert autocrine effects to influence other functions of these cells [6,7]. In support of this, the hypersecretory cytokine production demonstrated in asthmatic ASM [8,9] has been shown to be partially dependent on integrin-mediated interactions with ASM-derived fibronectin [8]. Furthermore, ASM from people with asthma contract at an increased rate when seeded into collagen gels [10], and as isolated single cells [11].

The altered proliferative, secretory and contractile functions of ASM demonstrated *in vitro* are consistent with many of the changes seen in the disease setting. One of the most striking structural changes seen in remodelled airways in asthma is thickening of the ASM layer, accompanied by sub-epithelial fibrosis, goblet cell hyperplasia and angiogenesis [12]. Several mechanisms have been

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proposed for the increase in ASM mass including, but not limited to, increased ASM proliferation and migration [13]. Changes in the profile and levels of cytokines and ECM proteins produced by this increased ASM are also likely to contribute to the persistent airway inflammation and other airway remodelling changes implicated in fixed airflow obstruction [14] and an accelerated loss of lung function [15,16] in asthma. Critically, the increased contraction of ASM invoked by a range of agents in these inflamed, remodelled airways can lead to life-threatening bronchospasm during acute asthma.

The identification of key mediators regulating the contribution of ASM to asthma may provide new opportunities for therapeutic intervention. In this review, we describe the emerging roles of the CXC chemokines CXCL1, CXCL2, and CXCL3 (growth-related oncogenes), CXCL10 (IP-10), Th17-derived cytokines (IL-17, IL-22) and semaphorins, as well as the influence of viral infection, on the regulation of asthma pathophysiology and ASM dysfunction in asthma.

3. Chemokines

Chemokines, also known as chemoattractant cytokines, are essential signalling molecules that have the ability to attract inflammatory cells to the sites of injury [17]. These small proteins (8–10 kDa) are classified into four families based upon the relative position of their N-terminal cysteine residues: CC, CXC, C and CX3C, where X represents any amino acid.

The CXC group of chemokines can be further sub-divided into an ELR + group, bearing glutamic acid (E), leucine (L) and arginine (R) at the NH₂-terminus, or non-ELR group, lacking the ELR tri-peptide motif [17]. The ELR + family of chemokines comprises CXCL1 (growth-related oncogene [GRO]- α) CXCL2 (GRO- β) and CXCL3 (GRO- γ), together with four other members, namely CXCL5 (epithelium-derived neutrophil-activating peptide 78 [ENA-78]), CXCL6 (granulocyte chemotactic protein 2 [GCP-2]), CXCL7 neutrophil-activating peptide 2 [NAP-2]) and CXCL8 (IL-8) [18].

Chemokines act on receptors that are also classified into CC, CXC, and CX3C groups, whose members are rhodopsin-like, class A, seven-transmembrane-domain, G-protein-coupled receptors (GPCR) [19]. Several studies have demonstrated CXCR2 as the receptor that mediates CXCL1, CXCL2, and CXCL3 signalling [20,21].

CXCL1, CXCL2, and CXCL3 have been long recognized as inflammatory mediators due to their ability to act as neutrophil chemoattractants [22] with roles in several inflammatory diseases such as rheumatoid arthritis [23] and asthma, especially the severe phenotype [24]. Of interest, CXCL1, CXCL2, and CXCL3 have been implicated to play a role in airway remodelling in asthma due to their ability to also induce chemotaxis of endothelial progenitor cells and to increase angiogenesis [25].

CXCL1, CXCL2, and CXCL3 are among the numerous chemokines expressed by ASM [26]. The functionality of these chemokines has been confirmed through their ability to induce neutrophil chemotaxis [27], while our recent findings suggest that they also regulate ASM migration. We have shown that both asthmatic and non-asthmatic ASM preferentially produce CXCL1, CXCL2, and CXCL3 in response to IL-17 stimulation. ASM cell migration in response to the IL-17-induced supernatants containing CXCL1, CXCL2, and CXCL3 could be mimicked by direct administration of a combination of recombinant human CXCL1, CXCL2, and CXCL3 and reduced to basal levels upon blockade of CXCR2 [28].

These studies demonstrate the emerging roles of CXCL1, CXCL2, and CXCL3 as mediators of neutrophilic inflammation and as autocrine regulators of ASM cell migration. The ASM/CXCL1, CXCL2, CXCL3/CXCR2 axis has the potential to regulate airway inflammation

and remodelling, identifying these cytokines and their receptors as potential therapeutic targets in asthma.

An additional ASM-derived CXC chemokine, CXCL10, also known as interferon- γ inducible protein of 10 kDa (IP-10) may also influence the contribution of inflammatory cells and ASM in asthma. CXCL10 is secreted in the lung by a variety of cells including airway epithelial cells [29] and ASM [30] in response to type I and II interferons (IFNs) and lipopolysaccharide (LPS), while its receptor CXCR3 is highly expressed in Th1 lymphocytes, mast cells and eosinophils [31–33]. Higher expression of CXCL10 in asthmatic airways as well as in mouse models of allergic airways disease [30,34,35] suggests an essential role for the CXCL10/CXCR3 axis in the trafficking of these inflammatory cells to contribute to the initiation and progression of allergic asthma [30,33,36].

Of note, ASM of asthmatic patients has been shown to highly express CXCL10 and recruit mast cells via CXCR3 [30], contributing to the localization of mast cells within ASM bundles that is frequently reported in asthmatic airways but rarely observed in the airways of non-asthmatic patients [37–39]. These increased numbers of infiltrated mast cells expressing higher levels of CXCR3 have been correlated with asthma severity [30,33,37,38].

In *ex vivo* and *in vitro* studies, Brightling *et al.* [30] investigated the migration of mast cells toward ASM in response to smooth muscle-derived chemokines and found that CXCR3 is the most abundantly expressed chemokine receptor on human lung mast cells within ASM. They also found that recruitment of mast cells to the lung is predominately mediated by activation of mast cell CXCR3 by ASM-derived CXCL10 and that CXCL10 is released preferentially from Th1-stimulated asthmatic ASM compared with ASM from healthy controls.

The potential bi-directional interactions between mast cells and ASM within the airway wall, either through direct cell–cell contact or the release of specific mediators, are likely to have important consequences for the development of the disordered physiology in asthma. Indeed, mast cells secrete numerous mediators including autacoids, cysteinyl leukotrienes, prostanoids, cytokines and growth factors that can directly affect ASM function and phenotype in asthma [40,41]. For example, histamine, tryptase, platelet-derived growth factor (PDGF) and the cysteinyl leukotriene LTC₄ are mitogenic for ASM [42–44] while other known mast cell products heparin and IL-4 can inhibit ASM proliferation [45,46]. ASM migration occurs in response to PGD₂ and IL-13, while the response to PDGF is further enhanced in the presence of LTE₄ [47,48].

More recently, Xia *et al.* used the Fc ϵ RI α subunit transfected human mast cells (HMC α cells) to examine the modulation of cytokine secretion from ASM cells [49]. These cells are capable of responding to IgE/antigen by releasing an array of mediators [50], and were shown to elicit eotaxin, IL-8 and IL-6 production from ASM [49]. Alternatively, mast cell-derived IL-4 and IL-13 can inhibit ASM cytokine secretion [51].

Mast cell-derived TGF- β could also enhance the ability of ASM to lay down ECM proteins [52] and promote ASM cell differentiation into a more contractile phenotype [53], but could also induce AHR independent of the inflammatory response [54,55], while histamine, LTC₄ and LTD₄ and the prostanoid PGD₂ can all elicit direct ASM contraction [56,57].

The potential effects of ASM-derived CXCL10 on aspects of airway inflammation unrelated to mast cells are less clear. In an *in vitro* study conducted by Loetscher *et al.* [58], CXCL10 was shown to not only facilitate recruitment of Th1 cells via CXCR3, but concomitantly to block the migration of eosinophils and Th2 cells occurring in response to ligands for the CCR3 receptor preferentially expressed on these cells [58]. The observation that this resulted in polarization of effector T cell recruitment led to the

conclusion that CXCL10 may antagonize allergic inflammation. However, an *in vivo* study in a mouse model of allergic asthma using either CXCL10 overexpressing mice or CXCL10 KO mice could not establish an anti-inflammatory role for CXCL10 [35].

In a more recent study, Lin *et al.* [34] have used CXCR3 KO mouse challenged with ovalbumin (OVA) to investigate the role of this receptor in airway inflammation. CXCR3 KO mice showed significantly fewer CD8⁺ and CD4⁺ T cells in BAL fluid along with lower levels of TNF α and IL-4 in lung tissue. They concluded that CXCR3 is crucial for both AHR and airway inflammation, by promoting recruitment of both CD8⁺ and CD4⁺ T cells and initiating the release of proinflammatory mediators following allergen sensitization and challenge.

There is now evidence that targeting interactions of ASM with inflammatory cells by blocking specific chemokine pathways, particularly CXCL10/CXCR3, could attenuate Th1 and Th2 dependent diseases including allergic airway disease [30,59]. Further studies to fully assess the therapeutic potential of antagonists and blocking antibodies targeting CXCL10/CXCR3 for asthma are warranted.

4. Semaphorins

Semaphorins, previously called collapsins, were initially discovered as “axon guidance” cues in neuronal cells [60], but are ubiquitously expressed, including in the immune, cardiovascular and respiratory systems. They are now known to play multifaceted roles in angiogenesis, cell adhesion, survival, proliferation and directional cell migration under physiological and pathological conditions [61]. Semaphorins are therefore of potential interest in the context of asthma since these cellular events can contribute to pathogenesis of airways disease in terms of inflammation, remodelling and hyperresponsiveness [62].

Based on phylogenetic tree analyses, semaphorins have been categorized into eight classes: Classes 1 and 2 are found in invertebrates only, whilst classes 3, 4, 6, and 7 are found in vertebrates only. Class 5 is found in both vertebrates and invertebrates while class V is specific to viruses. All vertebrate secreted semaphorins are grouped in class 3 (Sema3A–3G).

Semaphorins bind to receptors called Plexin (A–D) and Neuropilin (Nrp-1 or 2) to induce axon repulsion in neuronal cells [61]. Their signalling is mainly converged to the cytoskeleton through regulation of actin rearrangement or by modulation of integrin-mediated cell adhesion via small GTPase proteins such as Rho (Ras homologous) and Rac (Rho-related C3 botulinum toxin substrate) molecules [63]. Other signalling pathways such as ERK1/2 MAPK (mitogen-activated protein kinase), Akt/PI3K (phosphatidylinositol 3-kinase), and STATs (signal transducer and activator of transcription) have been also reported to play important roles downstream of the Semaphorin–Plexin/Nrp axis [64]. Interestingly, these molecular events are also involved in regulation of smooth muscle cell function in terms of proliferation and migration [65–67].

Recent studies indicate that different members of the semaphorin family contribute to lung physiology or pathology. Glycosylphosphatidylinositol (GPI)-anchored semaphorin 7A (Sema7A) is involved in the development of TGF- β induced pulmonary fibrosis through the recruitment of fibrocytes [68] and also plays important roles in myofibroblast hyperplasia, alveolar remodelling, and apoptosis [69]. In addition, different members of class 3 semaphorins have been implicated in pathogenesis of lung cancer, by affecting signalling pathways regulating angiogenesis which is a cardinal process in airway remodelling [70,71]. Interestingly, new studies demonstrate that expression of Sema3A is reduced in a murine model of allergic rhinitis and intranasal administration of

this neuronal chemorepellent alleviates the allergic symptoms in this model [72].

In the context of asthma, it has recently been shown that the expression of immune semaphorins and their receptors is enhanced upon allergen exposure in a mouse model of allergic inflammation [73]. While Sema4D expression was high on immune cells such as T and B lymphocytes, Sema4A was detected in bronchial epithelial cells and ASM, suggesting a potential role for this semaphorin in the regulation of ASM function. Allergen-treated Sema4A(–/–) mice showed a selective increase in eosinophilic airway infiltration accompanied by bronchial epithelial cell hyperplasia relative to wild-type mice [74]. Therefore, Sema4A expression in both lung-resident cell and inflammatory cells regulate inflammatory and remodelling changes. These combined findings pave the way for further investigations of the role of semaphorins in the regulation of ASM function to define their potential as therapeutic targets for asthma.

5. Th17-derived cytokines

Th17 cells are a subset of T cells distinct from Th1/Th2 cells, originally characterized by their production of IL-17A, and subsequently by the production of IL-17F and IL-22 [75–77]. To date six IL-17 family members (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E/IL-25 and IL-17F) and five receptors (IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE) have been identified, which are conserved in rodents and humans [78]. IL-17A and IL-17F display high sequence homology and can be secreted as homodimers, as well as IL-17A/F heterodimers, by both mouse and human cells [79,80]. Other potential sources of IL-17A in the lung include $\gamma\delta$ T cells, natural killer T cells and neutrophils [81,82].

Recent studies suggest that Th17 cells and Th17-associated cytokines are involved in asthma. Polymorphisms on chromosome 17q21, strongly associated with childhood asthma, significantly increased the secretion of IL-17 in cord blood without affecting regulatory T/Th2/Th1 lineages [83], while imbalances in Th1/Th2 and Th17/Treg have been found in patients with allergic asthma [23]. The percentages of Th17 cells and plasma concentrations of IL-17 and IL-22 [84], and the expression of IL-17A and IL-17F in human lung biopsies [85] have also been shown to be increased with asthma severity.

IL-17A may contribute to asthma through its capacity to promote recruitment of inflammatory cells including neutrophils, monocytes and macrophages to the site of airway inflammation [86,87]. In addition, Th17 cells and IL-17 may drive airway remodelling through the accumulation of neutrophils or by stimulating the release of matrix metalloproteases (MMPs) [88] as well as cytokines including IL-6, IL-8, IL-11, GM-CSF and VEGF [89].

Human ASM cells have been shown to express IL-17RA [90,91] and IL-17RC [91]. The receptor for the related cytokine IL-22 consists of two subunits, IL-22R1 and IL-10R2 [92], and we have found that IL-22R1 is present in human ASM cells [91]. IL-17A has been reported to induce the production of cytokines and chemokines from ASM cells. The presence of IL-17A could directly [90,93] or indirectly [94,95] enhance the production of IL-8, via the MAPK (p38 MAPK, JNK and p42/p44 ERK) and NF- κ B pathways. Although IL-17A is not able to directly induce IL-6 production from ASM cells, it could augment TNF- α -induced IL-6 production by enhancing mRNA stability [96]. These studies suggest that IL-17A amplifies the synthetic function of ASM cells, hence possibly amplifying the inflammation in asthma. Interestingly, IL-17A could also induce CCL11 (Eotaxin-1) production from ASM cells through MAPK pathways and STAT-3 [97,98]. Of note, CCL11 suppresses IL-8 production from human endothelial cells [99] and thus negatively regulates neutrophil recruitment to the lung during the acute

inflammation [100]. These findings hint at a potential regulatory role of IL-17A via a feedback mechanism involving CCL11 in establishing homeostasis.

Of note, IL-17A was recently shown to mediate severe AHR in mice [101]. In a separate study, Th17 cells were positively correlated with the severity of airway remodelling in a mouse model of allergen-induced airway disease, while adoptive Th17 transfer induced airway remodelling including increased ASM mass that could be abrogated by anti-IL-17A-mAb treatment [102].

These recent studies highlight the evidence of the important role of IL-17 in airway remodelling of asthma. ASM migration is a potential factor contributing to the increased ASM mass in severe asthma [103]. Using an *in vitro* cell migration assay, we have shown that the Th17-associated cytokines IL-17A, IL-17F and IL-22 promote human ASM migration [91]. The effects of IL-17A and IL-17F were mediated through p38 MAPK activation while IL-22 was acting through a separate NF- κ B-dependent signalling pathway. In ongoing studies, we have found that these cytokines also increase proliferation and decrease the apoptosis rate of ASM from both non-asthmatic and asthmatic subjects. Different signalling pathways were involved in the increased proliferation compared with the migratory effect, with ERK1/2 MAPK pathways involved in the response to IL-17A/F and IL-22, while the NF- κ B pathway also contributed to IL-22-induced ASM proliferation.

This emerging evidence demonstrates that Th17 cells and IL-17 family cytokines play important roles in the pathogenesis of asthma including airway remodelling in this disease, further broadening the perspectives for novel asthma treatment.

6. Viruses

Exposure to viruses such as rhinovirus (RV), influenza and respiratory syncytial virus (RSV) can cause lung dysfunction on their own, but can also precipitate severe exacerbations of underlying conditions like asthma. Viral interactions with immune cells, through engagement of pattern recognition receptors such as Toll-like Receptors (TLRs), play an important role in these exacerbations. However, viruses can also directly modulate ASM function since ASM differentially expresses transcripts for most TLR isoforms [104]. While functional TLR2, 3 and 4 are present with TLR3 being the most highly expressed [105], the expression of other TLRs in ASM is likely.

The TLR3 agonist poly(I:C) can be used as a viral exposure mimetic to assess regulation of ASM function. Cooper *et al.* demonstrated that exposure of human lung slices to poly(I:C) resulted in production of numerous cytokines including CCL3 (macrophage inflammatory protein-1alpha [MIP-1alpha] and CCL5 (regulated upon activation normal T-cell expressed, and secreted [RANTES]) [106]. In cultured ASM, stimulation with a combination of poly(I:C) and TNF α or IL-1 β also induced secretion of CCL5 as well as IL-6, IL-8 and CCL11 (eotaxin), with implications for the recruitment of mast cells to ASM *in vivo* [107,108]. In addition, exposure to poly(I:C) alone, or to RV or RSV elicited CXCL10, IL-1 β , IL-6, IL-8, and IL-11 production by ASM [9,107,109,110].

The effects of TLR agonists on ASM reactivity *in vitro* are variable. Although stimulation of either murine or canine airway tissues with LPS, poly(I:C), or both agents enhanced contractility to bradykinin [111,112], poly(I:C) did not influence airway bronchoconstriction to carbachol or the dilation to isoproterenol in human lung slices [106]. Interestingly, it was recently demonstrated that guinea pig ASM expresses TLR7, which recognizes single stranded RNA viruses like influenza, and that incubation of tracheal ring segments with a TLR7 agonist reduced contractile responses to 5-HT [113].

The exact role of TLRs in mediating these changes in inflammatory and contractile responses of ASM to viral exposure is not

clear, and other receptors have also been identified that mediate viral-induced changes in ASM function. In human ASM, Grunstein *et al.* showed that both active and UV-inactivated RV16 stimulated IL-5 and IL-1 β secretion. This inflammatory response was significantly decreased in the presence of an ICAM-1-neutralizing antibody and independent of productive infection of the tissues [114].

In rabbit ASM, Grunstein *et al.* also showed that RV16 exposure enhanced contractile responses to acetylcholine and impaired relaxation responses to isoproterenol [114], and that administration of either ICAM-1- or IL-1 β -neutralizing antibodies prior to RV16 exposure abrogated the enhanced contractile responses [115,116]. This same group demonstrated that chronic RV exposure of ASM induced β_2 adrenoceptor (β_2 AR) hypo-responsiveness via secretion of IL-1 β [117].

A study by Billington *et al.* demonstrated that chronic exposure to either IL-1 β or RV16 significantly increased forskolin-induced cAMP production [118], thereby suggesting that a potential feedback mechanism in ASM may reduce the effects of the virus resulting in the hyporesponsiveness to β_2 AR agonists that is normally observed. Another study showed that epithelial infection with RV16 modulated β_2 AR activity in ASM through down-regulation and desensitization of cell surface receptors. It was noted that this effect could not be restored by treatment with either dexamethasone or fluticasone [119], steroids that are known to resensitize β_2 AR in ASM. Others have shown that RSV can productively infect ASM and significantly reduce isoproterenol-induced cAMP production [120] in a similar manner to the modulation of β_2 AR agonist-mediated responsiveness by RV.

In vivo animal models are beginning to unravel the interplay between mediators and the development of AHR. Both AHR and TNF α production in CXCR2-deficient mice following RV1B infection were reduced, thereby suggesting a role for TNF α and IL-8 in neutrophil-dependent AHR following viral exposure [121]. A report in a guinea pig model of RSV infection showed enhanced airway hyper-responsiveness following RSV inoculation, showing infection of the epithelium and stroma [122]. Interestingly, Rho kinase inhibition has been shown to suppress AHR in a model of OVA/RSV-

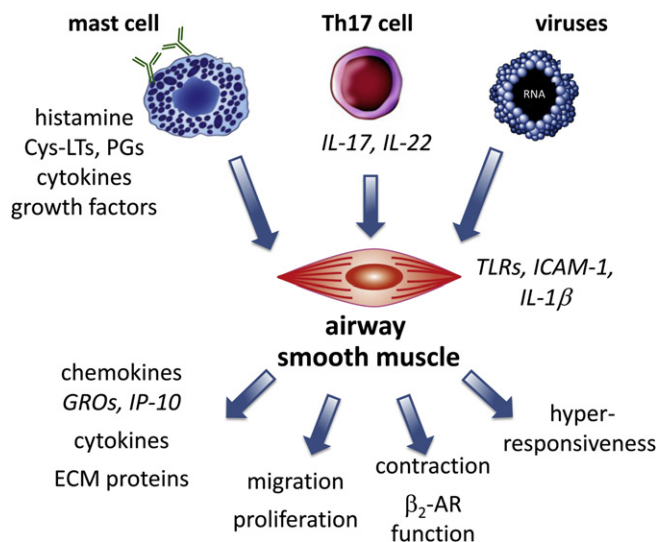


Fig. 1. Airway smooth muscle dysfunction in asthma. Airway smooth muscle (ASM) function can be altered by mediators derived from mast cells, Th17 lymphocytes and ASM itself as well as in response to viral infection. ASM can then contribute to airway inflammation (through cytokine and chemokine production) and airway remodelling (through ASM migration, proliferation and extracellular matrix (ECM) production). Contraction of the increased ASM mass in inflamed, remodelled airways is associated with airway hyperresponsiveness (AHR) in asthma.

induced AHR [123], suggesting that modulation of downstream signalling pathways by mediators produced during allergic airway disease/viral infection may be key to alleviating enhanced AHR due to viral exposure.

These combined data suggest that viral exposure of ASM elicits the release of inflammatory cytokines, and generally sensitizes the airways to contractile agonists while decreasing bronchodilator responsiveness. This poses a problem for treating virus-induced exacerbations of asthma, as the mainstays for therapy for these events are steroids and β_2 AR agonists. Defining the receptors engaged by these viruses, as well as the signalling pathways activated downstream of these receptors, may provide alternative therapeutic targets to treat the effects of viral exposure on ASM function in people with asthma.

7. Concluding remarks

It has been clearly demonstrated that changes in ASM function contribute to asthma pathophysiology. The plethora of data from animal models of allergic airways disease and in people with asthma have implicated numerous causative factors and mechanisms underlying ASM dysfunction in the disease setting. This review has described emerging mediators derived from ASM itself as well as from other sources such as mast cells and Th17 cells, and the many ways in which the response of ASM to these mediators and to viral infection could induce abnormal ASM behaviour (see Fig. 1). It is hoped that this will facilitate the development of novel approaches targeting ASM dysfunction to regulate aspects of airway inflammation and remodelling as well as AHR that may be resistant to current asthma therapy.

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